CHARACTERIZATION OF DETERMINANTS OF AIRWAY SMOOTH MUSCLE CELL HETEROGENEITY

BY

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A Thesis

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in Partial Fulfilment of the Requirements

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS x

LIST OF FIGURES xiv

LIST OF TABLES xix

LIST OF ABBREVIATIONS xx

ABSTRACT xxi

1. INTRODUCTION 23

2. LITERATURE REVIEW 29

2.1 CHARACTERISTICS OF DIFFERENTIATED SMOOTH MUSCLE CELLS: SMOOTH MUSCLE CELL PHENOTYPE 30

2.1.1 Smooth Muscle Cells are Multifunctional 30

2.1.2 Differentiated SMCs Exhibit Fetal and Mature States 31

2.1.3 Phenotypic Characteristics of Smooth Muscle Cells 35

2.1.3.1 Mature SMCs 36

2.1.3.2 Synthetic SMCs 37

2.1.4 Molecular Markers of SMC Phenotype 38

2.1.4.1 Myosin 41

2.1.4.2 Actin 43

2.1.4.3 Thin Filament Associated Proteins 43

2.1.4.4 Cytoskeletal Proteins 46

2.1.4.5 Extracellular Matrix Proteins 49

2.1.4.6 Cell Surface Markers 50

2.1.4.7 Transcription Factors 51

2.2 SMOOTH MUSCLE HETEROGENEITY 52

2.2.1 Diversity Between Different Smooth Muscles 52

2.2.2 Diversity of Myocyte Populations in Smooth Muscle 54

2.2.3 Developmental and Molecular Aspects of Smooth Muscle Heterogeneity .58

2.2.4 Smooth Muscle Cell Heterogeneity and the Disease State 63

2.3 SMOOTH MUSCLE AND HYPERRESPONSIVE AIRWAYS DISEASE 68

2.3.1 Airways Remodelling - Smooth Muscle Hyperplasia and Hypertrophy . . . 68

2.3.1.1 Paradigm for the Contribution of Airway Smooth Muscle Cells to Airways Remodelling 71

2.3.2 Regulation of Airway Smooth Muscle Cell Proliferation 76

2.3.2.1 Growth factors, cytokines and other molecules 76

2.3.2.2 Mechanism of Heparin-Mediated Inhibition of Smooth Muscle Cell Proliferation 79

2.3.3 Alterations in Airway Smooth Muscle Properties in Asthma 81

2.3.4 The Airway Smooth Muscle Cell in Culture 86

2.3.4.1 Cell Isolation and Seeding 87

2.3.4.2 Phenotypic Changes of Airway Smooth Muscle Cells in Primary Culture 88

2.3.4.3 Ultrastructure of Cultured Airway Smooth Muscle Cells 91

2.3.4.3.1 Organelles 91

2.3.4.3.2 Cell-to-cell junctions 92

2.3.4.3.3 Cytocontractile structures 93

2.4 GENERAL PERSPECTIVES - AIRWAY SMOOTH MUSCLE CELLS 95

3. MATERIALS AND METHODS 96

3.1 MATERIALS 97

3.2 CELL CULTURE 98

3.2.1 Canine Airway SMC Primary Culture 98

3.2.2 MCH6 Fibroblast Cell Line 101

3.2.3 Effect of Various Agents on Smooth Muscle Cell Cultures 102

3.3 ANALYSIS OF CELLULAR CONSTITUENTS OF SMOOTH MUSCLE CELLS 102

3.3.1 Freshly Isolated and Cultured Airway Smooth Muscle Cells 102

3.3.2 Pulmonary Arterial Smooth Muscle Cells 103

3.3.3 Protein Assay 104

3.3.4 Protein Electrophoresis: SDS-PAGE 104

3.3.5 Western Blot Analysis of Protein Homogenates 105

3.3.6 Laser Densitometry 107

3.3.7 Fluorescent Immunohistochemistry 108

3.3.7.1 Collagen Coating of Glass Coverslips 108

3.3.7.2 Fluorescent Immunostaining of Cells 110

3.3.8 Immunoperoxidase Staining - Cytospin Analysis 112

3.3.9 Measurement of Mitogen Activated Protein Kinase Activity 116

3.3.10 Analysis of Tyrosine Phosphorylation 119

3.3.11 Northern Blot Analysis 120

3.4 FLOW CYTOMETRY 122

3.4.1 Analysis of Intracellular Proteins and DNA Content 122

3.4.1.1 Preparation of Single-SMC Suspensions from Intact Tissue . . 122

3.4.1.2 Preparation of Single-SMC Suspensions from Primary Cultures 123

3.4.1.3 Fluorescent Staining of SMCs for Intracellular Proteins and DNA 124

3.4.1.4 Analysis of Cellular Constituents by Fluorescence Activated Cell Sorting (FACS) 125

3.4.2 Cell Cycle Analysis 126

3.4.2.1 Cell Preparation 126

3.4.2.2 FACS Analysis of Cell Cycle Distribution - PI Staining 127

3.4.2.3 Cell Preparation - Bromodeoxyuride (BrdU) Incorporation . . 128

3.4.2.4 FACS Analysis of Cell Cycle Distribution - PI Staining / BrdU Incorporation 130

3.4.3 Cell Sorting 131

3.5 STATISTICAL ANALYSES 133

4. MARKERS OF AIRWAY SMOOTH MUSCLE CELL PHENOTYPE 134

4.1 BACKGROUND 135

4.2. SPECIFIC AIMS 138

4.3 EXPERIMENTAL DESIGN 138

4.4 RESULTS 139

4.4.1 Growth Characteristics and Morphology 139

4.4.2 Temporal Expression of Marker Proteins 142

4.4.3 Marker Proteins in Freshly Dispersed and Cultured Airway SMCs 143

4.4.4 Marker Protein Expression in MCH6 Fibroblasts 145

4.4.5 Marker Proteins in Freshly Dissociated Tracheal and Arterial SMCs . . . 145

4.5 DISCUSSION 168

4.6 SUMMARY 177

5. HETEROGENEITY IN THE RESPONSE OF AIRWAY SMOOTH MUSCLE CELLS TO HEPARIN-MEDIATED INHIBITION OF PROLIFERATION 179

5.1 BACKGROUND 180

5.2 SPECIFIC AIMS 183

5.3 EXPERIMENTAL DESIGN 183

5.4 RESULTS 183

5.4.1 Effects of Heparin on Growth Characteristics and Phenotypic Modulation 185

5.4.2 Effects of Heparin on Airway SMC Cell Cycle Progression 187

5.4.3 Effects of Heparin on Signalling Pathways 190

5.5 DISCUSSION 212

5.6 SUMMARY 219

6. PHENOTYPIC HETEROGENEITY OF MYOCYTES FROM AIRWAY SMOOTH MUSCLE 223

6.1 BACKGROUND 224

6.2 SPECIFIC AIMS 226

6.3 EXPERIMENTAL DESIGN 226

6.4 RESULTS 227

6.4.1 Airway Myocyte Ploidy 227

6.4.2 Smooth Muscle MHC in Airway Myocytes 228

6.4.3 Smooth Muscle α-Actin in Airway Myocytes 229

6.4.4 Calponin and *h*-Caldesmon in Tracheal SMCs 230

6.5 DISCUSSION 245

6.6 SUMMARY 250

7. CHARACTERIZATION OF ISOLATED AIRWAY SMOOTH MUSCLE CELL SUBPOPULATIONS 252

7.1 BACKGROUND 253

7.2 SPECIFIC AIMS 255

7.3 EXPERIMENTAL DESIGN 256

7.4 RESULTS 257

7.4.1 Isolated Tracheal SMC Morphology: Flow Cytometry Light Scatter Pattern and Cell Sorting 257

7.4.2 Cytospin - Immunocytochemistry of Acutely Dissociated Tracheal Myocytes 259

7.4.3 Characteristics of Type A and Type B Tracheal Cells in Primary Culture 260

7.5 DISCUSSION 282

7.6 SUMMARY 287

8. CONCLUSIONS AND SIGNIFICANCE 289

9. FUTURE PROSPECTS 294

10. BIBLIOGRAPHY 298

DEDICATION

To my Dad -

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What gifts I gained from you I shall never truly be able to measure.

That which marks this journey and vests these pages,

dedication, love, integrity, perseverance and hard work,

are your legacy.

Would it that I might be able to put into words my pride if I could just hear you say

that I haven't wasted all these years "just warming the seat".

Miss you Dad -- Love, Andrew

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LIST OF FIGURES

1. Page 31. Schematic representation of the myogenic program which determines the phenotypic state of SMCs.

2. Page 65. Changes which occur in smooth muscle cell phenotype during vascular wall remodelling associated with progression of an atherosclerotic lesion.

3. Page 73. Changes in smooth muscle cell phenotype which could occur in the course of remodelling of bronchial airway walls in chronic asthma.

4. Page 147. Growth characteristics of cultured canine tracheal smooth muscle cells.

5. Page 149. Indirect immunofluorescense staining, using FITC-conjugated secondary antibodies, of myosin in cultured canine tracheal SMC.

6. Page 151. Indirect immunofluorescent staining, using FITC-conjugated secondary anti-bodies, of smooth muscle-α-actin and *h*-caldesmon in canine tracheal myocytes in primary culture.

7. Page 153. Demonstration of dense bodies / focal adehsion plaques and intermediate filaments in cultured canine tracheal SMC using indirect immunohistochemical techniques.

8. Page 155. Coomassie blue stained 10% SDS-PAGE gels showing the profiles of total protein in crude homogenates of acutely dissociated tracheal and pulmonary arterial SMCs.

9. Page 157. Temporal changes in abundance of specific smooth muscle proteins in cultured tracheal smooth muscle cells as assessed by Western blotting and laser densitometry.

10. Page 159. Comparison, using Western blot analysis and scanning laser densitometry, of the abundance of specific smooth muscle proteins in freshly isolated tracheal smooth muscle cells with those in primary cultures of tracheal smooth muscle cells 7 days after seeding.

11. Page 161. Northern blot analysis of steady state sm-MHC and actin mRNA contents in freshly isolated tracheal smooth muscle cells and in primary cultured tracheal smooth muscle cells 7 days after seeding.

12. Page 163. Comparison of the presence of specific protein markers in total homogenates of primary cultured tracheal smooth muscle cells (5 days after seeding) and in homogenates from cultured human foreskin fibroblasts, cell line MCH6, by Western blot analysis.

13. Page 165. Western blot analysis of the abundance of specific smooth muscle proteins in freshly isolated tracheal SMCs and pulmonary arterial SMCs.

14. Page 191. Effects of heparin on the growth characteristics of canine tracheal SMCs in primary culture.

15. Page 193. Effects of heparin on the temporal expression of phenotypic marker proteins in primary airway SMC cultures.

16. Page 195. Cell cycle analysis of primary cultured tracheal SMC proliferation by flow cytometry.

17. Page 197. Histogram depicting the fraction of arrested SMCs which entered the cell cycle from G_0/G_1 after serum-stimulation in the presence and absence of heparin.

18. Page 199. Flow cytometric analysis of the concentraction-response relationship of tracheal SMCs to heparin-induced inhibition of cell cycle entry.

19. Page 201. Bi-variate FACS analysis of the effects of heparin on the cell cycle distribution of serum-stimulated airway SMCs.

20. Page 203. Distribution of airway srSMCs which entered the cell cycle in the presence and absence of heparin.

21. Page 205. Autoradiograms obtained using the in gel assay for MAP kinase activity in cultured tracheal myocytes in the presence and absence of heparin.

22. Page 207. Effects of heparin on temporal MAP kinase activation in cultured canine tracheal myocytes after serum-stimulation.

23. Page 209. Detection of tyrosine phosphorylation using an immunoblot assay in canine tracheal myocytes grown in the presence and absence of heparin.

24. Page 220. Hypothesized distribution of airway SMC subpopulations based on differences in response to serum stimulation and heparin-mediated

inhibition of proliferation.

25. Page 230. FACS analysis of ploidy in canine tracheal and bronchial SMCs.

26. Page 232. Bivariate FACS analysis of smMHC content and ploidy of canine tracheal and bronchial SMCs.

27. Page 234. FACS analysis of the distribution of indirect-immunofluorescence of smMHC in freshly dispersed canine tracheal and bronchial SMCs.

28. Page 236. Bivariate FACS analysis of sm-α-actin content and ploidy of freshly dissociated canine tracheal SMCs.

29. Page 238. FACS analysis of the distribution of indirect-immunofluorescence of sm-α-actin in freshly dissociated canine tracheal and bronchial SMCs.

30. Page 240. Bivariate FACS analysis of calponin content and ploidy of dissociated canine tracheal SMCs.

31. Page 242. Bivariate FACS analysis of *h*-caldesmon content and ploidy of freshly dispresed canine tracheal SMCs.

32. Page 263. Phase contrast micrograph of enzymatically dispersed canine tracheal SMCs.

33. Page 265. Flow cytometry light scatter pattern obtained for freshly dissociated tracheal SMCs.

34. Page 267. Cytospin of acutely dissociated tracheal SMCs stained for sm-α-actin using peroxidase-anti-peroxidase.

35. Page 269. Immunofluorescence staining for smMHC in cytospins of acutely dissociated canine tracheal SMCs.

36. Page 271. Cytospin of acutely dissociated tracheal SMCs stained for desmin using peroxidase-anti-peroxidase.

37. Page 273. Immunofluorescence staining for vimentin in cytospins of acutely dissociated canine tracheal SMCs.

38. Page 275. Growth characteristics of sorted Type A and Type B tracheal myocytes in primary culture.

39. Page 277. Morphological diversity of sorted Type A and Type B canine tracheal myocytes in culture.

40. Page 279. Fluorescent immunocytochemical staining for sm-α-actin, smMHC and DNA of Type A and Type B airway SMCs in primary culture.

LIST OF TABLES

1. Page 38. Markers of the phenotypic state of vascular SMC in vitro and in vivo (after Sartore et al., 1994).
2. Page 77. Asthma-associated factors and the smooth muscle cell growth response that they are known to effect.
3. Page 108. Primary antibodies used in immunoblotting to study marker protein content in isolated and cultured SMCs.
4. Page 112. Primary antibodies used in immunocytochemical analyses of cultured canine tracheal SMCs.
5. Page 146. Abundance of marker proteins determined by Western blot analysis.

LIST OF ABBREVIATIONS

BrdU - bromodeoxyuridine

Cy3 - indocarbocyanine

EDTA - ethylenediaminetetraacetic acid

FACS - Fluorescence Activated Cell Sorting

FITC - fluorescein isothiocyanate

kDa - kilodalton

MLCK - myosin light chain kinase

nm - nonmuscle

nmMHC nonmuscle myosin heavy chain

PAGE - polyacrylamide gel electrophoresis

PDGF - platelet derived growth factor

PI - propidium iodide

PKC - protein kinase C

SDS - sodium dodecyl sulphate

siSMC - serum insensitive smooth muscle cell

sm - smooth muscle

SMC - smooth muscle cell

SMCs - smooth muscle cells

smMHC - smooth muscle myosin heavy chain

srSMC - serum responsive smooth muscle cell

ABSTRACT

Airway smooth muscle contraction plays an indisputable role in exacerbations of bronchial asthma, a disease in which morbidity and mortality are increasing. Changes in airway smooth muscle function are associated with hyperresponsiveness and remodelling of the airways, including airway smooth muscle cell (SMC) proliferation. Airway myocytes are thought to be similar, but recent data indicate that arterial SMC constitute phenotypically disparate subpopulations of myocytes. Primary cultures of canine airway SMC were established to identify phenotype-specific protein markers. Mature airway SMC were characterized by abundant contractile protein content, whereas proliferative, cultured cells expressed non-muscle isoforms of cytostructural proteins. Adult tracheal SMC were distinct from less contractile, pulmonary arterial SMC, on the basis of marker protein content. Analysis of cell cycle progression by flow cytometry revealed airway SMC cultures to be composed of several distinct subpopulations differing in responsiveness to mitogenic and antiproliferative agents. Distinct subgroups of acutely dissociated canine airway myocytes were discriminated on the basis of ploidy and contractile protein content using immunocytochemistry and flow cytometry. The fraction of SMC possessing high levels of contractile proteins was greatest in muscle from large airways indicating that inter-airways heterogeneity in contractile properties may be due to differences in the distribution of functionally divergent airway myocytes. Flow cytometry was used to sort two populations of canine tracheal SMC, called Type A and Type B, that differed morphologically and in marker protein content. Primary cultures of each population were established. Type A myocytes were small, grew well in culture and

expressed an immature phenotype. Type B cells were large, fusiform cells that were resistant to cell culture and presented a terminally differentiated, contractile phenotype. Collectively, these findings demonstrate that airway smooth muscle is composed of distinct, heterogeneous subpopulations of SMC that differ in contractile and proliferative potential. The distribution of myocyte subpopulations may be important in determining the normal physiology and pathophysiology in different areas of the lung. Future studies aimed at elucidation of the developmental and molecular mechanisms that determine SMC phenotype should provide valuable insights that may be used therapeutically for human asthma.

1. INTRODUCTION

Smooth muscle comprises the medial layers in the walls of most hollow organs. These tissues have traditionally been studied for their ability to regulate lumen diameter via contraction. Smooth muscle cells (SMCs) are now known to play a primary effector role in pathogenesis of fibroproliferative disorders of the vascular wall associated with atherosclerosis and post-angioplasty restenosis (Ross, 1993). Proliferation and hypertrophy of SMCs are also critical determinants of arterial remodelling in hypertension (Owens, 1989), airways remodelling in asthma (Dunnill et al., 1969), urinary bladder hypertrophy associated with urethral stenosis (Uvelius et al., 1989), and intestinal narrowing and stricturing seen in chronic inflammatory bowel disease (Snape and Kao, 1988). It has become clear that SMCs possess the capacity for multifunctional behaviour, including contraction, proliferation and deposition of excess extracellular matrix, which may contribute to the etiology of atherosclerotic and hypertensive vascular disease. There is also compelling evidence that myocytes in mature smooth muscle exist as subsets of phenotypically heterogeneous populations which may differ in their contribution to muscle properties (Sartore et al., 1994).

It appears that SMCs are unique in comparison to skeletal and cardiac muscle cells -- mature SMCs do not appear to be terminally differentiated and they retain the capacity for further replication (Katoh and Periasamy, 1996). Indeed, the smooth myocyte has proven to be phenotypically plastic by virtue of its capacity to respond to environmental cues and insults. Cell-to-cell, cell-matrix, and cell-ligand interactions contribute to maintenance of the normal, differentiated phenotype and determine phenotypic state during development and in disease (Owens, 1995). Specific sets of

transcription factors are thought to direct phenotype-specific gene expression. This knowledge has lead to investigations aimed at elucidation of the complex regulatory mechanisms governing transcription of smooth muscle specific genes. These studies may provide insight into the mechanisms of smooth muscle cell (SMC) activation and recruitment to a pathophysiologic phenotype in atherogenesis. It is important to note at this point, that the information summarized heretofore has been obtained *exclusively from studies on smooth muscle cells of vascular origin.*

Two recent, independent studies (Li et al., 1996b; Moessler et al., 1996), utilizing transgenic mice harbouring a smooth muscle specific gene promoter, have revealed that "distinct transcriptional regulatory programs control muscle gene expression in vascular and visceral SMCs" (Li et al., 1996). This finding clearly demonstrates a molecular basis for discriminating between different smooth muscles and between individual SMCs. Indeed, it has been known for many years that striking differences exist in the contractile and pharmacological properties of smooth muscle from different organs, and smooth muscle from different regions of the same organ (Vanhoutte, 1978; Fleisch, 1980). Ultrastructural and morphological studies have also confirmed that myocytes from different smooth muscles differ in the abundance of contractile apparatus and protein synthesizing organelles (Uehara et al., 1990). Further evidence that heterogeneity is a typical feature of smooth muscle has been obtained from elegant, developmental studies, which demonstrated that the commitment of progenitor cells from different embryological origins is a fundamental mechanism leading to the segregation of different smooth muscle beds in the adult (Le Lièvre and LeDouarin, 1975). It is now believed

that a subset of differentiated arterial SMCs situated near the arterial lumen, which retain the capacity to rapidly revert to an immature state, may subserve restenotic and atherogenic lesion formation in endothelium-injured blood vessels (Holifield et al., 1996). The knowledge obtained from studies using vascular SMCs provide important direction to investigations aimed at clarifying the contribution of myocyte populations in pathogenesis of diseases in nonvascular smooth muscle organs. However, in light of the fundamentally distinct nature of different smooth muscles, clearly, analysis of the phenotypic properties of nonvascular smooth muscle must be undertaken on an individual basis prior to reliance upon paradigms developed from vascular smooth muscle research.

Epidemiological trends suggest a substantial increase in morbidity and mortality from asthma since 1970 (Sears, 1996). In fact, anti-asthma drug sales, hospital admissions and emergency room visits are presently at an all time high, which in conjunction with the results of epidemiological studies, indicate that disease prevalence may be well in excess of 10% in North America (Sears, 1996). It has been suggested that the remodelling which occurs in chronic asthmatic airways parallels the pathogenic features of focal atherosclerotic lesion formation (Halayko and Stephens, 1994). The pathogenic and pathophysiologic role of airway smooth muscle in asthma appears to be indisputable, yet remains unclear. Acute exacerbations of asthma manifest from reversible bronchial smooth muscle spasm. The disease is considered to be a chronic inflammatory affliction in which structural alterations of the airways, including, adventitial and sub-epithelial fibrosis, and thickening of the medial smooth muscle layer

due to myocyte hyperplasia and hypertrophy, develop over time (Ebina et al., 1993).

Classical studies have characterized *in vitro* contractile responses of airway smooth muscle from animal models in an attempt to ascertain whether abnormal contraction might be the basis of airways hyperresponsiveness, a diagnostic symptom of asthma. Unfortunately availability of human material has hampered progress, however, some pharmacological, biophysical and biochemical data have been obtained from animal models and they suggest smooth muscle properties may be altered (Stephens et al., 1993; Fan et al., 1997). The potential for contribution of airway SMCs to adventitial and sub-epithelial fibrosis has not been investigated, in spite of observations that myofibroblast-like cells accumulate subepithelially in asthmatic airways (Brewster et al., 1990).

The use of cell biology techniques to study the airway SMC has lagged far behind studies concerning vascular SMCs. Indeed, the first reports describing the characteristics of airway smooth muscle in primary cell culture only appeared in 1981 (Avner et al., 1981), more than 60 years after the first vascular SMC cultures were used for study (Lewis and Lewis, 1917; reviewed by Chamley-Campbell et al., 1979). Hence, it is not surprising that, in comparison to vascular circles, little is known regarding the fundamental phenotypic characteristics of airway myocytes and the factors that regulate phenotypic expression *in vitro* and *in vivo*. The majority of studies to date have used cultured airway myocytes to measure the mitogenic strength of a variety of cytokines and growth factors and, to describe second messenger signalling pathways that regulate proliferation and contraction (Hall and Kotlikoff, 1995). Heterogeneity in pharmacologic

and contractile properties among different airways has been described (Fleisch, 1980; Ma et al., 1996). However, the possibility that the airways media might be composed of functionally and phenotypically diverse groups of SMCs has not been investigated.

The information summarized here has led to the derivation of the a general hypothesis under which studies reported in this thesis were carried out:

GENERAL HYPOTHESIS -- Airway smooth muscle is composed of divergent subsets of myocytes which differ in phenotype and functional properties.

Within the context of the stated General Hypothesis a general objective was set:

GENERAL OBJECTIVE -- To characterize biochemical and functional properties of airway smooth muscle cell populations in their native state and in culture.

It is hoped that the information obtained in these studies will provide impetus for new directions and approaches to be used in research programs aimed at elucidating the pathogenesis of human asthma.

2. LITERATURE REVIEW

2.1 CHARACTERISTICS OF DIFFERENTIATED SMOOTH MUSCLE CELLS: SMOOTH MUSCLE CELL PHENOTYPE

2.1.1 Smooth Muscle Cells are Multifunctional

Fully differentiated, mature smooth muscle cells (SMCs) differ fundamentally from striated muscle cells in their ability to respond to environmental stress by modulating their phenotype from that of a mitotically quiescent, contractile cell to one which is mitotically active, recapitulates a fetal gene expression profile, and secretes extracellular matrix, cytokines and growth factors (Owens, 1995). The capacity of the mature smooth muscle cell (SMC) for phenotypic plasticity allows a given myocyte to express a broad range of different phenotypes in response to different physiologic and pathophysiologic factors (Schwartz et al., 1986; Campbell and Campbell, 1987; Ross, 1993; Owens, 1995). During development, vascular repair and in vascular disease the SMC fulfills multiple functions exclusive of contraction which is the function normally associated with the mature myocyte (Wissler, 1968; Owens, 1995). Indeed, in combination with a spatially and temporally diverse developmental and differentiation repertoire, phenotypic plasticity yields divergent SMC populations between different organs and between different regions within the same organ (Katoh and Periasamy, 1996; Li et al., 1996). At present, assessment of the differentiation state of a SMC depends upon several criteria, including it's anatomic location, cellular and sub-cellular morphology, functional characteristics of the cell and expression of genes encoding SMC-specific marker proteins (Campbell et al. 1979; Sartore et al 1984; Owens, 1995).

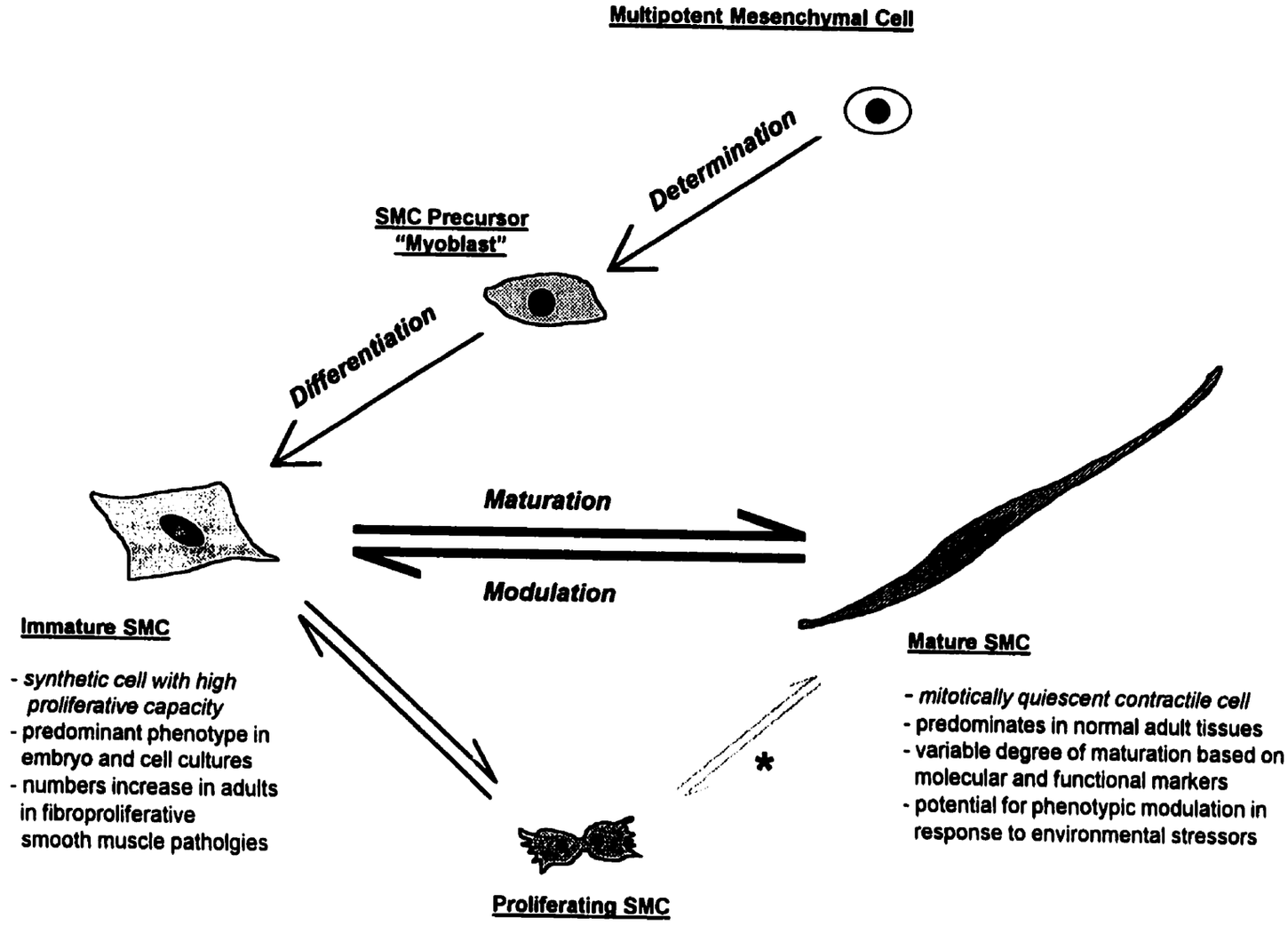
2.1.2 Differentiated SMCs Exhibit Fetal and Mature States

Individual SMCs demonstrate striking dissimilarities in morphology, contractile and cytoskeletal protein content, electrophysiological properties, proliferative responsiveness and ability to synthesize extracellular matrix components (Frid et al., 1994; Wohrley et al., 1995; Archer et al., 1996; Caleb et al., 1996; Meer and Eddinger 1996). Differentiated SMCs demonstrate a range of phenotypes which fall between two extreme states, which are classically referred to as the "contractile" and the "synthetic" phenotype (Chamley-Campbell et al., 1979; Campbell and Campbell, 1987). The contractile phenotype is associated with fully mature SMCs whereas the synthetic phenotype, although still considered to be that of differentiated SMCs, appears to be common to SMCs in developing fetal tissues and pathophysiologic conditions (Owens and Thompson, 1981; Zanellato et al., 1990; Giuriato et al., 1992; Frid et al., 1993; Sartore et al., 1994).

A schematic representation of the ontogeny of differentiated SMCs and the potential for phenotypic plasticity in the mature SMC is depicted in Figure 1. The scheme presented depicts the elements common to presently accepted paradigms for vascular SMC development and maturation (eg. McHugh, 1995; Katoh and Periasamy, 1996). Salient details of the complex molecular and cellular events which regulate commitment

FIGURE 1. Schematic representation of the myogenic program which determines the phenotypic state of SMCs.

Multipotent cells are derived from local mesenchyme for most developing organs, though regions of the aorta, pulmonary and carotid arteries arise from neural crest ectomesenchyme. Mesenchymal determination and differentiation is thought to occur in response to local paracrine, cell-to-cell and extracellular matrix interactions. Immature and adult SMCs are analogous with the so-called "synthetic" and "contractile" phenotypes and they represent end points of a maturation continuum in which differentiated SMCs normally exist. Modulation occurs in culture and *in vivo* as a response to environmental cues. * - Proliferation of mature SMCs may occur, however, the frequency is extremely low.


Multipotent Mesenchymal Cell
Determination
SMC Precursor
"Myoblast"
Differentiation
Maturation
Modulation
Immature SMC
- synthetic cell with high proliferative capacity
- predominant phenotype in embryo and cell cultures
- numbers increase in adults in fibroproliferative smooth muscle patholgies
Mature SMC
- mitotically quiescent contractile cell
- predominates in normal adult tissues
- variable degree of maturation based on molecular and functional markers
- potential for phenotypic modulation in response to environmental stressors
*
Proliferating SMC

and differentiation of SMCs are given in later sections. For the purposes of presenting an overview of the determinants of SMC heterogeneity, it is necessary to clearly discriminate between differentiation, maturation and modulation of SMCs.

Differentiation is the event in which embryonic cells, which have been committed to a particular lineage, *i.e.* undergone determination (Figure 1), begin to demonstrate fundamental biochemical, morphological and/or physiological characteristics that are particular for a specific mature cell type. *Maturation* is a late stage of differentiation during which SMCs that have differentiated to an irreversible fetal phenotype develop the predominant characteristics of adult SMCs in fully developed organisms. In the case of SMCs this means that myocytes demonstrating a fetal, synthetic/proliferative phenotype acquire contractile characteristics common to myocytes of the adult phenotype (Figure 1). A feature which distinguishes mature, contractile SMCs from other differentiated, contractile muscle cell types is the capacity of the former for reversible *modulation* to an immature phenotype including reiteration of fetal gene expression programs (Figure 1). Strictly speaking, phenotypic modulation does not deliver a cell to the multipotent, undifferentiated state, and is not considered to be dedifferentiation (Chamley-Campbell et al., 1979), though the latter term is often used incorrectly to describe modulation. Morphological and biochemical changes accompanying phenotypic modulation have been best characterized in primary cultures where contractile SMCs undergo rapid, reversible modulation after seeding (Chamley-Campbell et al., 1979; Shanahan et al., 1993; Halayko et al., 1996a). Similar changes in subsets of mature vascular SMCs accompany the migration and proliferation of myocytes during injury repair and

atherogenesis *in vivo* (Ross, 1993). In the context set out heretofore, heterogeneity of SMCs in the adult is the product of two factors: 1) an irreversible developmental schedule which determines the commitment and differentiation of precursor cells to the smooth muscle lineage, and, 2) the dynamic interplay between modulation and maturation in response to humoral, hormonal and physical environmental cues during development and in the adult.

2.1.3 Phenotypic Characteristics of Smooth Muscle Cells

Several characteristics, including, cellular morphology and function, anatomic location and the abundance of SMC-specific proteins and their isoforms, are recognized as markers for discriminating SMC of different phenotype. The primary role of the mature SMC is contraction, hence, the vast majority of SMCs in the adult are thought to exist, more or less, in a contractile state (Chamley-Campbell et al., 1979). However, early histological observations revealed that SMC were the only cell type present in the media of large, elastic arteries suggesting that these differentiated cells must also play a role in the deposition and maintenance of collagen, elastin and other extracellular matrix components (Pease and Paule, 1960; Wissler, 1968). The contractile and synthetic phenotypic states have been described to represent idealized extremes indicative of the multifunctional nature of SMCs (Campbell et al., 1979).

Most SMCs likely exist in a native, intermediate state which falls within an existent phenotypic continuum particular to specific organs and species (Campbell et al., 1988). Some of extracellular matrix surrounding SMCs is likely synthesized by the

myocytes themselves indicating that they are capable of sustaining both a contractile and synthetic phenotype (Jones et al., 1979). In vivo, myocytes of the visceral smooth muscles, including the vas deferens, and small, muscular arteries, express the physiologic extreme in contractile cell phenotype (eg. Campbell and Campbell, 1987). Interestingly, only a small fraction of matrix material is evident surrounding the SMCs in these muscles and much of it is thought to be derived from resident fibroblasts (Gabella, 1984). Extremes in synthetic phenotype are seen in response to pathological stimuli and during development (eg. Sartore et al., 1994). It is also well established in primary culture, that native, contractile SMCs from a variety of organs, rapidly modulate to a mitotically active, synthetic phenotype in the presence of serum (Chamley-Campbell et al., 1979; Shanahan et al., 1993; Halayko et al 1996a).

2.1.3.1 Mature SMCs

Mature SMCs generally approximate a contractile state characterized by the presence of a high volume fraction of myofilaments and the expression of numerous smooth muscle-specific genes encoding contractile proteins and proteins which regulate contraction (Campbell and Campbell, 1987; Shanahan et al., 1993; Halayko et al., 1996a). The contractile apparatus of mature SMCs is the dominant ultrastructural component (Chamley-Campbell et al., 1979). Thin myofilaments, composed of actin and other actin-binding proteins such as calponin, caldesmon and tropomyosin, and thick myofilaments, composed of smooth muscle-specific myosin, can occupy 80-90% of total cytoplasmic volume in contractile SMCs (Gabella, 1984). Myofilament abundance

appears to be correlated with the content of myosin, actin and myofilament-associated proteins (Somlyo et al., 1973; Cohen and Murphy, 1978; Halayko et al., 1996a). The presence of a large number of membrane-associated and cytoplasmic dense bodies, which act as anchor points for thin and intermediate filaments, is another feature characterizing mature SMCs (Gabella, 1990; Chou et al., 1992). Intermediate filaments in mature SMCs are composed of desmin and vimentin whereas in immature SMCs the filaments are almost exclusively composed of vimentin (Kocher et al., 1985). Linear arrays of flask-shaped invaginations of the sarcolemma, called caveoli, are seen between membrane dense bodies -- the caveoli increase in number during SMC maturation and are a distinctive feature of the mature SMC (Devine et al., 1971). Organelles associated with protein synthesis (eg. Golgi apparatus, rough endoplasmic reticulum, free ribosomes) are located near the nuclear poles but comprise only a small fraction of total cell volume (Campbell and Campbell, 1987). Fully mature SMCs have been shown to be proliferative *in vivo* (Cobb and Bennett, 1970; Imai et al., 1979) but these observations are infrequent, indicating that replication of contractile myocytes proceeds at low rates.

2.1.3.2 Synthetic SMCs

Synthetic SMCs have an abundance of organelles for protein processing and synthesis, including rough endoplasmic reticulum and Golgi apparatus, and a limited number of myofilaments (Campbell and Campbell, 1987). SMCs maintaining this phenotype replicate frequently in comparison to contractile SMCs, they synthesize and secrete extracellular matrix proteins and proteases abundantly, and express genes for

growth factors, cytokines and their requisite receptors (Ross, 1993; Shanahan et al., 1993). Cells possessing these characteristics are commonly seen during morphogenesis and during arterial remodelling associated with atherosclerotic lesion formation (Schwartz et al., 1986). Mature smooth muscle cells spontaneously modulate their phenotype in primary culture and their responsiveness to various spasmogenic and mitogenic stimuli differs significantly from those of freshly isolated, uncultured cells (Hall and Kotlikoff, 1995). The phenomenon of phenotypic modulation in culture has been exploited for vascular myocytes, with relevance to atherosclerosis and hypertension, to identify protein markers of SMC phenotype which correlate with the physiological function of vessel wall smooth muscle cells (Shanahan et al., 1993; Sartore et al., 1994).

2.1.4 Molecular Markers of SMC Phenotype

Numerous *in vivo* and *in vitro* studies have attempted to identify molecular markers for vascular SMC phenotypic state. Table 1 summarizes findings obtained from such investigations (Sartore et al, 1994). Loss of contractility by proliferative vascular SMCs in sub-confluent primary culture is a manifestation of changes in contractile and cytoskeletal protein content. Proliferating cells exhibit a reduction in smooth muscle α and γ-actins (Shanahan et al., 1993), smooth muscle myosin heavy chain (smMHC) isoforms (Rovner et al., 1986), the smooth muscle regulatory 20kDa myosin light chain isoform (Monical et al., 1993), thin filament associated regulatory proteins calponin (Shanahan et al., 1993) and *h*-caldesmon (Ueki et al., 1987), the sarcoplasmic reticulum membrane associated protein, phospholamban (Shanahan et al., 1993), and the

TABLE 1. Markers of the phenotypic state of vascular SMC in vitro and in vivo (after Sartore et al., 1994).

Markers	Fetal or Phenotype Modulated Cultured SMC	Adult or Confluent Cultured SMC	References
Smooth muscle α-actin	+/-	+++	Kocher & Gabbiani, 1986; Blank et al., 1988
β-actin	+++	+/-	Blank et al. 1988; Eddinger & Murphy, 1991
Smooth muscle γ-actin	+/-	+++	Shanahan et al. 1993; McHugh, 1995
l-Caldesmon	+++	+/-	Ueki et al. 1987
h-Caldesmon	+/-	+++	Ueki et al. 1987; Frid et al. 1992
Calponin	+/-	+++	Frid et al. 1992; Shanahan et al. 1993
SM22α	+/-	+++	Shanahan et al., 1993
smMHC (all isoforms)	+/-	+++	Rovner et al., 1986; Babij et al., 1993; Woodcock-Mithcell et al., 1993
Non muscle myosin heavy chain	+++	+/-	Rovner et al., 1986; Zanellato et al., 1990; Kuro-o et al., 1991
α-Tropomyosin	+/-	+++	Kocher et al., 1985
Phospholamban	+/-	+++	Shanahan et al., 1993
Desmin	+/-	+++	Kocher et al., 1985
Vimentin	+++	+++	Kocher et al., 1985
Meta-vinculin	+/-	+++	Belkin et al., 1988; Wohrley et el., 1995
Fibronectin (A and B isoforms)	+++	+/-	Glukhova et al., 1990
Tenascin	+++	+/-	Hedin et al., 1991
Tropoelastin	+++	+/-	Majesky et al., 1992
Versican	+++	+/-	Lark et al., 1988; Lemire et al., 1996
Osteopontin	+++	+/-	Giachelli et al., 1991; Shanahan et al., 1993
α_1-Procollagen	+++	+/-	Majesky et al., 1992

cytoskeletal proteins, desmin (Kocher et al., 1985) and meta-vinculin (Belkin et al., 1988). Furthermore, phenotypically modulated, cultured cells are known to re-express foetal and non-muscle isoforms of several proteins including, β-isoactin (Fatigati and Murphy, 1984), non-muscle myosin heavy chains (Rovner et al., 1986), the non-muscle regulatory myosin light chain isoform (Monical et al., 1993), *l*-caldesmon (Ueki et al., 1987), the intermediate filament associated protein, vimentin (Kocher at al, 1985) and several secreted proteins including collagen and elastin (Majesky et al., 1992). Cultured SMCs retain the capacity to re-express marker proteins which become down regulated upon seeding in primary culture (Campbell and Campbell, 1987; Halayko et al., 1996a). This capacity to re-express contractile proteins and to concomitantly down regulate expression of proteins associated with the fetal state, is cell density and growth-state dependent and occurs in contact inhibited and growth-arrested, post-confluent SMC cultures (Campbell and Campbell, 1987; Blank et al., 1988; Halayko et al., 1996a).

A number of adult smooth muscle-specific protein markers have been identified, including α-sm actin (Gabbiani et al. 1981), γ-sm-actin (Sawtell and Lessard 1989), smMHC (Nagai et al. 1989), calponin (Gimona et al. 1992), *h*-caldesmon (Frid et al. 1992), SM22α (Gimona et al 1992), and, most recently, a cytoskeletal protein called smoothelin (van der Loop et al. 1996). Some of these markers are differentially expressed during development and between muscles with different contractile and secretory properties in the adult (Cohen and Murphy, 1978; Fatigati and Murphy 1984; Halayko et al. 1996a). These findings suggest that the relative abundance of these particular markers may be the most useful molecular indices of the contractile and/or

secretory function of a SMC. Identification and cloning of SMC phenotype-specific markers has important implications for the design of molecular approaches to study the mechanisms regulating smooth muscle differentiation and maintenance of phenotypic state.

2.1.4.1 Myosin

Myosin is a heterohexameric motor protein composed of one pair each of myosin heavy chains, regulatory light chains and essential light chains; different isoforms of each of these subunits in sarcomeric, and nonsarcomeric smooth muscle and nonmuscle systems have been identified (Rovner et al., 1986; Swynghedauw, 1986; Helper et al., 1988; Babij and Periasamy, 1989; Gaylinn et al., 1989; Mohammed and Sparrow, 1989; Nagai et al., 1989; Kelley et al., 1993). Several isoforms of smMHC, including SM1A, SM1B, SM2A and SM2B, are generated via alternate mRNA splicing of a single gene (Nagai et al. 1989; Kelley et al., 1993; White et al. 1993; Katoh and Periasamy, 1996). The SM1A and SM2A isoforms appear to be expressed somewhat ubiquitously in adult smooth muscle. The SM2 isoforms also appears to be a reliable marker of fully mature, contractile cells as it only accumulates in SMCs during later developmental stages and it is the first smMHC isoform to disappear when cultured cells modulate to the fetal phenotypic state (Nagai et al 1989; Woodcock-Mitchell et al., 1993). Currently, smMHC appears to be the most dependable definitive marker of the SMC lineage as no evidence of its expression in any other cell type exists (Katoh and Periasamy 1996). This is in contrast with a number of other putative markers for the smooth muscle lineage,

including α-actin, SM22α and calponin, which are transiently expressed in some non-SMC types during development and in culture (Sawtell and Lessard, 1989; Darby et al., 1990; Kapanci et al., 1990; Leslie et al., 1990; Birukov et al., 1991; Li et al., 1996b).

Interestingly, the SM1B isoform, which has a faster ATPase than other isoforms, due to the presence of a seven amino-acid insert in the ATP-binding region of the myosin head (White et al. 1993), is present mainly in visceral smooth muscle and is in low abundance in vascular and uterine muscles. Biophysical studies have demonstrated that visceral smooth muscles have faster shortening velocities that arterial smooth muscle (Antonissen et al., 1979; Kong et al., 1981; Malmqvist and Arner, 1991; Stephens et al., 1993). Differences in the contractile properties between smooth muscles have also been linked to the distribution of the 17kDa essential light chain (Helper et al., 1988; Malmqvist and Arner, 1991; Morano et al, 1993). Non-muscle isoforms of myosin heavy chain and the 20kDa regulatory light chain are expressed in adult SMC although the abundance of these isoforms in fetal and cultured SMCs is much greater (Gaylinn et al., 1989; Kuro-o et al 1991; Halayko et al. 1996a). Indeed, it appears that the fetal phenotype can be clearly distinguished from the mature phenotype on the basis of SM1A, SM2 and non muscle (nm) MHC expression patterns (Katoh and Periasamy 1996).

Using reverse transcriptase-polymerase chain reactions (RT-PCR) on single isolated rabbit arterial SMCs, Meer and Eddinger (1996) have recently shown that the ratio of SM2-to-SM1 varied widely in cells from the same vessel. This indicates that a broad range of SMCs exhibiting heterogeneous contractile protein content can exist in smooth muscles. This finding implies that SMC with different contractile capacities may

exist and that the distribution of these myocyte subtypes might be a key determinant of the contractile properties of a given smooth muscle.

2.1.4.2 Actin

The earliest marker of differentiation is the thin filament protein, sm-α-actin, which is also the most abundant protein in mature SMCs (Owens 1995; McHugh 1995). Several other actin isoforms are also expressed in smooth muscle including cytoplasmic-β, cytoplasmic-γ,and smooth muscle (sm)-γ (McHugh 1995). The cytoplasmic isoforms are present in a broad range of cell types whereas the sm-γ and sm-α isoforms are abundant in contractile smooth muscle. Adult smooth muscles which possess a relatively higher abundance of sm-isoactins appear to generate more force than smooth muscle with lower sm-isoactin content (Fatigati and Murphy, 1984; Halayko et al 1996a). Expression of the sm-isoactins decreases significantly after phenotypic modulation of adult SMCs in primary culture (Shanahan et al. 1993). Neither of the sm-isoactins can be considered an unequivocal marker of the smooth muscle lineage because transient expression of both has been reported in several non-smooth muscle cells during development (McHugh 1995; Owens 1995).

2.1.4.3 Thin Filament Associated Proteins

Tropomyosin, a fibrous protein, exists in muscle and nonmuscle cells in a number of isoformic variations which are derived via alternate splicing from multiple genes (Gunning et al., 1990). It associates with actin filaments and is an important regulator of

actin-myosin interactions in skeletal muscles, however, its role in determining the contractile properties of smooth muscle is less well defined (Fatigati and Murphy, 1984; Marston and Smith, 1985). Tropomyosin is thought to be important in controlling acto-myosin interactions and has an co-operative activating effect on actomyosin ATPase activity through its ability to stabilize thin filament structure (Phillips and Chacko, 1996). An α-tropomyosin isoform that is unique to smooth muscle has been described (Wieczorek et al., 1988). Immunohistochemical analyses indicate that expression of the protein is developmentally regulated and it is exclusive to SMCs in the adult suggesting that its presence, in large abundance, is an index of maturation (Kocher et al., 1985; Muthuchamy et al., 1993). However, Shanahan et al. (1993) reported no difference in α-tropomyosin transcript abundance between proliferating, cultured vascular SMCs and freshly dispersed myocytes.

Calponin is a putative regulatory protein of smooth muscle contraction based on its Ca^{2+}-sensitive ability to regulate actin-activated myosin ATP hydrolysis (Winder and Walsh, 1990). Several isoelectric, pI 5-10, and molecular weight variants, Mr 28-34 kDa, of calponin, derived from three genes, are expressed in a sequential manner during development in human, murine and avian smooth muscles (Draeger et al., 1991; Strasser, et al., 1993; Applegate et al., 1994; Trabelsi-Terzidis, 1995; Takahashi et al., 1996). The last isoforms to be expressed, h_1- and l- isoforms, appear to be exclusive to SMCs in the adult and are thought to be reliable markers of the late maturation process of contractile myocytes (Gimona, et al., 1990; Draeger et al., 1991; Frid et al., 1992; Duband et al., 1993). An h_2-calponin isoform has been shown to be expressed in both SMCs and

nonmuscle cells (Strasser et al., 1993). Interestingly, the h_1- and l- isoforms are the first to disappear in culture as SMC modulate from the mature state (Draeger et al., 1991). The role of isoform diversity of smooth muscle-calponin in the regulation of contraction of mature SMC is unclear, however, different isoforms may also play an auxiliary role in inhibition of cell proliferation (Takahashi et al., 1993 and 1995).

SM22 possesses homologous sequence motifs with calponin and displays a development-dependent isoform expression pattern (Gimona et al., 1992; Duband et al., 1993; Nishida et al., 1993). SM22α, the most abundant isoform present in adult smooth muscle, appears to be present only in SMCs (Lees-Miller, et al. 1987; Duband et al., 1993), however, it has been shown to be transiently expressed in both skeletal and cardiac muscle during development (Li et al., 1996a and 1996b). The functional role of SM22 is unknown. In contrast to h_1- and l-calponin, SM22α does not appear to be dramatically down regulated in proliferative SMCs in culture (Gimona et al. 1992; Solway et al., 1995), although one study (Shanahan et al., 1993) demonstrated a gradual decrease in SM22α expression during long term culture of vascular myocytes. Collectively, both calponin and SM22α are thought to belong to a group of late differentiation markers which may be useful in demarcating myocytes resembling the contractile state (Duband et al., 1993).

Caldesmon is an actin-, myosin-, tropomyosin- and calmodulin-binding protein thought to be involved in regulation of contraction in nonsarcomeric cell types via an ability to modulate actomyosin ATPase activity (Sobue et al., 1981; Ngai and Walsh, 1984; Graceffa, 1987; Ikebe and Reardon, 1988; Sobue and Sellers, 1991). Two

isoforms, *l*- and *h*-caldesmon, which arise via alternative splicing of a single gene, differing in molecular mass, 70 and 150 kDa respectively, have been identified (Sobue et al, 1981; Bretscher and Lynch, 1985; Hayashi et al., 1991; Humphrey et al., 1992). The *h*-caldesmon isoform is predominant and appears to be exclusive to SMCs in adult tissues (Ueki et al, 1987). *l*-caldesmon is widely distributed in nonmuscle tissues and cultured cells, in immature SMCs, and is present in low amounts in some adult smooth muscles (Ueki et al, 1987; Halayko et al., 1996a). The expression of caldesmon isoforms is developmentally regulated with accumulation of the *h*-isoform during latter stages of embryogenesis when immature SMCs mature to a contractile phenotype (Ueki et al., 1987; Frid et al., 1992). In concordance with these findings are the observations that *h*-caldesmon decreases in abundance while *l*-caldesmon increases in abundance in cultured SMCs as they modulate from the mature state after plating (Ueki et al., 1987; Halayko et al., 1996a). Collectively, these results support the identification of *h*-caldesmon as a marker for differentiated SMCs which have matured to a contractile state.

2.1.4.4 Cytoskeletal Proteins

Smoothelin is a novel, 59 kDa, cytoskeletal protein recently cloned and characterized from a human smooth muscle cDNA library (van der Loop et al 1996). The protein is expressed in smooth muscle from a broad array of species but its presence appears to be limited exclusively to mature, contractile SMCs. Transcription of smoothelin mRNA was also permanently and rapidly downregulated in phenotypically modulated SMCs in culture indicating that the abundance of the protein may be a marker

of the maturational state of smooth muscle.

Intermediate filaments constitute an important intracellular structural entity in cells and may serve as a lattice which organizes and anchors the contractile apparatus in mature SMCs (Lazarides, 1980; Small et al., 1992; Small, 1995). Desmin, vimentin and cytokeratin are the structural proteins which comprise intermediate filaments in immature and mature SMCs (Lazarides, 1980; Osborn et al., 1981; Jahn et al, 1987). The expression of these proteins is developmentally regulated in SMCs -- cytokeratin is chiefly seen only during embryogenesis and atherogenesis, desmin is predominant in adults whereas vimentin appears to be expressed throughout development and adulthood (Gabbiani et al. 1981; Kocher et al., 1985; Bader et al., 1988; Jahn and Franke, 1989; Nanaev et al, 1991). The accumulation of desmin intermediate filaments is a common feature of the myogenic programs for skeletal, cardiac and smooth muscle (Kocher et al 1985; Li et al., 1989). Individual SMCs have been shown to be heterogeneous with respect to desmin:vimentin content (Gabbiani et al., 1981; Kocher et al, 1985; Rubbia and Gabbiani, 1989). Using immunohistochemical techniques, Kocher et al. (1985) observed that 87% of medial cells in the fetal rat aorta contained only vimentin and 13% contained both desmin and vimentin whereas 12 weeks after birth only 51% of aortic SMCs were solely vimentin positive, 48% were positive for both desmin and vimentin, and 1% were solely desmin positive. Interestingly, desmin is lost as a cellular protein constituent upon phenotypic modulation of SMCs in primary culture (Rubbia and Gabbiani, 1989; Halayko et al., 1996a). In the adult, the relative abundance of vimentin and desmin between smooth muscles is heterogeneous; desmin is the major intermediate

filament protein in visceral smooth muscle and small muscular arteries whereas vimentin filaments predominate in large elastic arteries (Gabbiani et al., 1981; Nanaev et al., 1991). Hence, smooth muscles with superior contractile capacity in the adult, appear to be desmin-enriched although a direct causal relationship is not clear a this time.

Metavinculin, 150k Da, and vinculin, 130 kDa, are cytoskeletal-associated proteins derived via alternate splicing of a single gene that are co-expressed in smooth muscle and are believed to be involved in actin-membrane linkage (Gieger, et al., 1980; Glukhova et al., 1986; Byrne et al., 1992;). Two variants of metavinculin, namely α- and β-, exist and four isoelectric vinculin isoforms, α-, α'-, β-, and γ-, have been identified (Belkin et al, 1988; Koteliansky et al., 1992). Smooth muscle and cardiac muscle-specific expression of α- and β-metavinculin and γ-vinculin appears to occur (Belkin et al., 1988). Metavinculin expression appears to be developmentally regulated and begins to accumulate quite late in embryogenesis (Frid et al., 1994). Metavinculin was observed to be heterogeneously expressed along with *h*-caldesmon, and the SM2 isoform of smMHC in morphologically distinct, adult, bovine pulmonary arterial smooth muscle cells suggesting that it may be a good marker for fully mature vascular SMCs (Frid et al., 1994). Furthermore, Wohrley et al. (1995) reported that in response to hypoxic stress in neonatal bovine calves, metavinculin-negative pulmonary arterial SMCs proliferated preferentially and contributed to arterial wall remodelling. These data suggest that metavinculin may be a unique marker capable of distinguishing SMC populations in vivo with the capacity for phenotypic modulation to a proliferative state in response to environmental insult.

2.1.4.5 Extracellular Matrix Proteins

There is steady-state expression of secreted proteins in adult medial vascular SMCs (Ross and Klebanoff, 1971; Cizmeci-Smith et al., 1993; Yao et al., 1994; Lemire et al., 1996). Numerous reports exist describing the upregulation and initiation of transcription of genes encoding secreted proteins in SMCs in response to injury, in culture and in atherosclerotic lesions (Giachelli et al., 1991; Nikkari et al., 1994; Wolf et al., 1994; Belknap et al., 1996). Modulation of some medial SMCs to a synthetic/proliferative phenotype is a critical feature of atherogenesis and the secretion of excess matrix material is though to be a requisite part of the fibrotic repair process (Ross, 1993; Simons et al., 1993). Using a fetal rat arterial injury model, Belknap et al. (1996) recently demonstrated that high level transcription of tropoelastin mRNA was evident only in post-replicative, quiescent SMCs. This finding suggests that synthetic and proliferative SMCs represent distinct, differentiated states. Deposition of extracellular matrix proteins constitutes a primary role of vascular SMCs during morphogenesis of the vasculature (Owens, 1995). Examples of secreted matrix proteins which are synthesized by SMCs include elastin (Majesky et al., 1992; Belknap et al., 1996), collagens (Majesky et al., 1992), fibronectins (Glukhova et al., 1990), laminin (Glukhova et al., 1993), osteopontin (Giachelli et al., 1991; Shanahan et al., 1993) and a variety of proteoglycans. The latter group includes, syndecans (Czimeci-Smith, et al., 1993), versican (chodroitin sulfate proteoglycan) (Schonherr et al., 1991), perlican (heparin sulfate proteoglycan) (Wight, 1989), decorin (Schonherr, et al., 1993) and biglycan (dermatin sulfate proteoglycan) (Schonherr, 1993). SMCs also express numerous membrane receptors for

extracellular matrix proteins (Hedin et al., 1989; Clyman et al., 1990; Glukhova et al., 1993; Halayko et al., 1996a) which increase in content with phenotypic modulation *in vivo* and *in vitro* (Glukhova et al., 1993; Halayko et al., 1996a). The nature of the extracellular matrix is an important determinant of SMC phenotype, hence, by virtue of the ability to secrete matrix proteins and to interact with them via membrane receptors, SMCs possess a unique feedback mechanism for self-determination of phenotypic expression.

2.1.4.6 Cell Surface Markers

A paucity of reports describing cell surface receptors exist. A single, definitive report (Lazaar et al., 1994) concerning airway SMCs confirms that cellular adhesion molecules may be of interest in defining phenotypic status. Human bronchial SMCs appear to constitutively express CD44, also called homing cellular adhesion molecule (HCAM), and the levels of the protein become increased in cultured myocytes (Lazaar et al., 1994). Similar observations have also been reported for canine airway SMCs (Halayko et al., 1996a). In addition, both vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) become expressed in cultured human bronchial myocytes (Lazaar et al., 1994). Interestingly, exogenous application of the inflammatory mediator, tumour necrosis factor α (TNFα), potentiates expression of these adhesion molecules in cultured cells. These data suggest that changes in myocyte phenotype induced by culture or by exposure to inflammatory mediators are associated with changes in cell adhesion molecules. Therefore, SMCs may be important in

regulating migration of inflammatory cells, such as T-lymphocytes, which adhere to SMCs *in vivo* and in culture (Lazaar et al., 1994).

2.1.4.6 Transcription Factors

Recently several protein transcription factors including, HoxB7 and HoxC9 (Miano et al., 1996), Gax (Gorski et al., 1993) and smLIM (Jain et al., 1996), which all contain homeodomains, have been implicated to be expressed in a phenotype specific fashion. The homeodomain is a specific DNA-binding motif found in the members of the homeobox gene family (Cardoso, 1995). The genes were first described in *Drosophila* (Lewis, 1978) and the protein products are involved in determining correct spatial body plan (Scott et al., 1989; Cardoso, 1995). Furthermore, some homoebox proteins are thought to direct cell-specific transcription of some genes in a variety of cell types (e.g. Bodner et al., 1988; Ingraham et al., 1988; Komuro and Izumo, 1993).

cDNA clones of five homeobox genes, including *HoxA5, HoxA11, HoxB1, HoxB7, and HoxC9,* were obtained from human fetal aortic SMC cultures (Miano et al., 1996). HoxB7 and HoxC9 were present only in fetal SMCs, therefore, they appeared to be translated in a SMC phenotype-dependent manner suggesting they may play some role in specifying the fetal SMC phenotype. *Gax*, a homeobox gene expressed in adult cardiovascular tissues, becomes concomitantly downregulated with smooth muscle contractile proteins in serum-grown SMCs in primary culture (Gorski et al., 1993). One of the specialized members of the homeobox transcription factor family are the LIM proteins (DeLuca, 1991) which are known to play important roles in cellular

differentiation (Jain et al., 1996). A recently cloned nuclear LIM-only protein, called smooth muscle LIM (smLIM), was shown to be preferentially expressed in arterial smooth muscle compared to venous and visceral smooth muscles (Jain et al., 1996). Interestingly, as was the case for *Gax*, expression of smLIM is down regulated in proliferative SMCs in culture and after balloon catheter injury in rat carotid arteries.

2.2 Smooth Muscle Heterogeneity

2.2.1 Diversity Between Different Smooth Muscles

Broad diversity exists in the pharmacological, contractile and electrophysiological properties of mature smooth muscles from different tissues and from different sites within the same tissue. Differences in innervation and cell-to-cell coupling via gap junctions have resulted in classification of different muscles as being either phasic (eg. gastrointestinal), tonic (eg. arterial), or intermediate (eg. airway) in nature (Somlyo and Somlyo 1994). Comparative electron microscopic analyses of smooth muscles from various sources have shown that the volume fraction of myofilaments is variable (Campbell and Campbell 1987). The SMCs from large elastic arteries contain lower numbers of myofilaments than myocytes from muscles which possess low amounts of extracellular matrix, eg. vas deferens. We have compared the profile of proteins expressed in SMC from the trachealis and the pulmonary artery and found that the former, which generated more force and shortened faster compared to the latter, was enriched in myofilament-associated proteins including, sm-α-actin, *h*-caldesmon,

calponin, smMHC, myosin light chain kinase and β-tropomyosin (Halayko et al. 1996a).

Molecular and biochemical factors known to underlie heterogeneity between smooth muscles include the differential expression of proteins comprising receptors, ion channels or the contractile apparatus and those regulating signal transduction and contraction in smooth muscle (Somlyo and Somlyo 1994; Daemen and DeMey 1995; Halayko et al. 1996a). For example, a distinction can be made between arterial smooth muscle and airway smooth muscle in the composition of adrenergic receptors. In general, α-receptors predominate in most arteries whereas β-receptors predominate in the airways and as a result of this disparity, intervention with adrenergic agonists results in contraction of the vessels and relaxation of the airways. Regional differences in the contractile response of arterial beds to α- and β- adrenergic agonists are also well documented (Vanhoutte, 1978; Kolbeck and Spier, 1987). Similarly, regional disparity in responsiveness to other contractile agonists have been noted for airway smooth muscle from different sized airways (Chitano et al., 1993 and 1996; Sigurdsson et al., 1996).

Adding to the breadth of heterogeneity that occurs normally between smooth muscles, are the changes in smooth muscle properties that develop during the pathogenesis of some diseases. Structural changes at specific sites in hollow organs, due to the proliferation of SMCs and their secretion of extracellular matrix components, are common in the pathogenesis of arteriosclerosis, hypertension, asthma and inflammatory bowel diseases. Sites of atherosclerotic lesions, where remodelling of the arterial muscle layers has occurred, are known to be more prone to vasospasm in response to a variety of stimuli (Egashira et al., 1989; Seto et al., 1993). Decreases in colonic contractility with

decreased smooth muscle membrane resistance occur in patients with ulcerative colitis (Snape and Kao, 1988). There are also reports of increased contractile responses or increased sensitivity to contractile agonists of airway smooth muscle from animal models of asthma (Stephens et al., 1993) and from patients suffering from asthma (Björk et al., 1992; Black, 1996). Fully understanding inter-smooth muscle heterogeneity and the factors that control its manifestation are critical for the development of therapeutic interventions aimed at precise locations in specific smooth muscle beds, e.g., potent anti-asthma drugs which relax smooth muscle in small, conducting airways of the lung.

2.2.2 Diversity of Myocyte Populations in Smooth Muscle

The properties of whole tissues have typically been attributed to their requisite myocytes with the general belief that the SMCs in a particular tissue are relatively homogeneous in form, function and biochemical properties. However, it is now known that the medial layers of the vasculature are, in fact, composed of phenotypically heterogeneous populations of multi-functional mesenchymal cells (Schwartz et al., 1986). There is a paucity of data regarding myocyte heterogeneity in non-vascular smooth muscle, however, recent reports show that investigators have begun studying the occurrence of SMC heterogeneity in the airways (Halayko et al., 1994 and 1996b), urinary bladder (Lau and Chacko, 1996) and gastrointestinal tract (Liddell et al., 1993). With the advent of technology to study the molecular mechanisms underlying development, differentiation, and the pathophysiology of diseases such as atherosclerosis and hypertension, has come the realization that distinct cellular phenotypes may be

involved with smooth muscle organ morphogenesis and with proliferative and fibrotic responses of SMCs as seen in diseased arteries (Schwartz et al., 1986; Ross, 1993; Sartore et al., 1994; McHugh, 1995; Owens, 1995). The features of specific phenotypic subtypes of SMCs and their pertinence to development, maintenance of normal function, and to pathogenesis of fibroproliferative diseases involving smooth muscle are areas of considerable research interest in current cell biology.

Based on differences in morphology, ultrastructure, and expression of marker proteins, adult arterial vessels are thought to be composed of at least two broad subpopulations of SMCs. The majority of cells are of an adult phenotype and are medially located, relatively large and fusiform or spindle shaped, and readily express smMHC and sm-α-actin (Frid et al., 1994; Ehler et al., 1995; Mironov et al., 1995; Holifield et al., 1996). The intimal, intralamellar, and adventitial layers of arteries contain round or stellate, epitheloid-like SMCs of a fetal or immature phenotype which stain positively for sm-α-actin and nmMHC but stain negatively or only weakly for smMHC isoforms (Frid et al., 1992; Sartore et al., 1994; Holifield et al., 1996; Mironov et al., 1995). Neylon et al. (1994) reported that the resting membrane potential of medial, spindle-shaped SMCs is less negative than that of the immature cell type. The latter are also sensitive to agonists that activate charybdotoxin-sensitive Ca^{2+}-activated K^+ channels, which may be important in regulation of cell proliferation. Of note are morphological and immunohistochemical observations that SMCs present in neointimal thickenings of atherosclerotic lesions are of an immature, epitheloid-like phenotype, suggesting that these cells may originate from fetal SMCs or mature SMCs capable of

phenotypic modulation, near the lumen of normal blood vessels (Benditt and Benditt, 1973; Owens, 1995; Holifield et al., 1996).

Several clonal cell culture populations, thought to be representative of mature and immature arterial SMCs, have been established (Blaes et al., 1991; Fugita et al., 1993; Weissberg et al., 1993; Lemire et al., 1994; Holifield et al., 1996). Clonal cell cultures thought to represent SMCs of a mature state have been established by sequential arterial digestion and by limiting dilution techniques (Weissberg et al., 1993; Holifield et al., 1996). When isolated by enzymatic dispersal from arterial samples these cells appeared in a fusiform shape commonly attributed to freshly isolated SMCs (Holifield et al., 1996). These cells were viable in culture but were poorly adherent to plastic and collagen-coated matrices, the abundance of smMHC and sm-α-actin did not decrease with time in culture, and they were nonreplicative in the presence of a variety of mitogens (Weissberg et al., 1993; Holifield et al., 1996). These cells were the dominant medial cell type and it has been postulated that they may represent a terminally differentiated, vascular SMC population (Holifield et al., 1996).

Several clonal cell cultures of putative myoblastic or immature vascular SMCs have been established and described. Schwartz and co-workers (Walker et al., 1986; Giachelli et al., 1991; Majesky et al., 1992; Lemire et al., 1994 and 1996) have described a π-phenotype rat aortic SMC line which is thought to be analogous to vascular SMCs derived from the aorta of rat pups and adult neointimas that form after induced-endothelial injury. Cultured π-phenotype cells are of an epitheloid morphology, secrete platelet derived growth factor B chain (PDGF-B), replicate readily in an autocrine-driven

fashion and secrete high levels of osteopontin and elastin. Similarly, Bochaton-Piallat et al. (1996) recently reported the characterization of two highly proliferative clonal rat aortic SMC populations. One was epitheloid and the other small-spindle shaped in morphology -- both types were obtained in higher numbers from rat aortas 15 days after inducing endothelial injury using a balloon catheter. Cook et al. (1994) also characterized cultures obtained from embryonic rat aortae and identified an embryonic SMC line that possessed the capacity for self-driven replication. Holifield et al. (1996) isolated a population of spheroid (10-20 μm diameter) cells which predominated in adventitial and intimal layers of canine carotid arteries. These cells adhered readily to all culture matrices, appearing small and spindle-shaped or epitheloid-like, and proliferated vigorously in the presence of low serum concentrations. In culture the cells expressed sm-α- actin, SM22α and nmMHC but were devoid of smMHC suggesting that they were of an immature phenotypic state (Holifield et al., 1996).

Heterogeneous populations of SMCs have also been characterized on the basis of other criteria. Goldberg et al. (1984) established stable diploid and tetraploid clonal rat aortic SMC populations in culture which differed in replicative rate and cell size. Populations of cultured SMCs have also been established which differ in sensitivity to specific growth factor-induced replication (Majack et al., 1996) or in heparin-mediated inhibition of proliferation (Letourneur et al., 1995; Bârzu et al., 1996). Weissberg et al. (1993) have cloned three different groups of arterial SMCs which differ in morphology and growth characteristics. Frid et al. (1994) have described up to four, site-specific heterogeneous SMC populations in the fetal, neonatal and adult pulmonary arterial wall.

The different populations were discriminated on the basis of immunobiochemical staining for contractile proteins and meta-vinculin, cell morphology and elastic lamellae arrangement. Two muscle cell types have also been identified in cultures of SMCs derived from adult and neonatal rabbit urinary bladder (Lau and Chacko, 1996). These cells were distinguished on the basis of differences in cell size, shape and degree of staining for various contractile smooth muscle markers.

2.2.3 Developmental and Molecular Aspects of Smooth Muscle Heterogeneity

The precise mechanisms leading to the commitment of precursor cells to the smooth muscle lineage and to the divergence of normal smooth muscle into heterogeneous groups are not known. Spatiotemporal differences in the concentration and identity of growth factors (Minoo and King, 1994; Owens, 1995), tissue-specific transcription factors (Cardoso, 1995; Katoh and Periasamy, 1996; Li et al., 1996; Miano et al., 1996; Jain et al., 1996) and local interactions between mesenchyme and epithelial or endothelial cells (Minoo and King, 1994; Hungerford et al., 1996) are thought to be important in determining the recruitment of precursor cells and the development of diversity between smooth muscle in different organs and at different sites in the same organ.

During development smooth muscles appear to be segregated on the basis of differences in the source and site of progenitor cells (Le Lièvre and Le Douarin, 1975; Li et al., 1996; Topouzis and Majesky, 1996). In general, developmental paradigms suggest that SMC in most organs are recruited from local embryonic mesenchyme in response to

chemoattractant peptides and growth factors secreted from organized, growing tubes composed of either immature endothelial or epithelial cells (McGowan, 1992; Minoo and King, 1994; Katoh and Periasamy, 1996; Hungerford et al., 1996). It is also established, however, that the SMCs of the proximal regions of the aorta and pulmonary and carotid arteries are derived from ectomesenchymal cells of the neural crest (Le Lièvre and Le Douarin, 1975). The SMCs of more distal portions of these arteries appear to be a mixture of mesenchyme- and ectomesenchyme-derived cells. Hence, SMCs in different organs are derived from at least two distinct embryonic origins, mesoderm and the neural crest. Topouzis and Majesky (1996) recently compared the properties, in culture, of chick aortic SMC of ectodemal origin with those of mesodermal origin. The two SMC lineages express nearly identical levels of numerous phenotype marker proteins, however, cells of ectodermal origin displayed a greater capacity for growth in serum free conditions and proliferated in response to transforming growth factor-β (TGF-β) exposure, whereas mesodermally derived SMCs were growth inhibited by TGF-β. These data suggest that vascular SMCs respond in a lineage-dependent fashion to growth factors and morphogens which are known to be important in development and progression of vascular disease in the adult.

Organ development and pattern formation is regulated through a complex assortment of local paracrine, cell-to-cell, and cell-matrix interactions which determine the precise pattern of expression of cell-specific transcription factors (McGowan, 1992; Minoo and King, 1994; Cardoso, 1995; Hungerford et al., 1996). The most striking demonstration of the molecular mechanisms active during embryogenesis, that lead to

smooth muscle diversity in the adult, come from recent studies in which the expression profile of a *lacZ* transgene under the control of the 5'-flanking minimal promoter for SM22α, a protein normally expressed in all smooth muscles, was examined during development in transgenic mouse embryos (Li et al., 1996; Moessler et al., 1996). The *SM22α-lacZ* transgene was expressed transiently in somites and the presumptive right ventricle in the early embryo and was subsequently seen, persisting to adulthood, in the arteries of the vascular system but was never expressed in visceral or venous smooth muscles. Endogenously expressed SM22α is present in all smooth muscles, hence, the divergent expression seen for the *SM22α-lacZ* transgene suggests that different transcriptional regulatory programs may distinguish visceral from vascular smooth muscle during development and in mature SMCs. Therefore, the myogenic program which determines the commitment of precursor cells to the smooth muscle lineage and which leads to different smooth muscle types is dependent upon the interplay between a specific repertoire of trans-acting transcription factors and regulation of their binding to cis-acting sites present in the sequences of DNA controlling transcription of smooth muscle-specific genes.

Studies are now being aimed at identifying the specific transcription factors, and their complementary *cis*-acting elements, which confer smooth muscle- and/or SMC phenotype-specific gene expression. Characterization of the gene promoters for several smooth muscle specific genes, including those for sm-α-actin (Blank et al., 1992), smMHC (Katoh et al., 1994; White and Low, 1996), SM22α (Solway et al., 1995) and calponin (Takahashi et al., 1996) has been carried out. Some of the promoters share

common *cis*-acting elements in their 5'-flanking regions. Triplet sets of CArG box elements, which bind Serum Response Factor (SRF), have been identified in the smMHC and sm-α-actin promoters and two consensus CArG box/SRF binding sites were identified in the SM22α promoter. Two proximal CArG boxes appear to be critical in conferring SMC-specific activity to the chicken sm-α-actin promoter in reporter gene constructs used in transient transfection experiments (Shimizu et al., 1995). In contrast, no CArG/SRF consensus sites were identified on the calponin 5'-flanking promoter.

White and Low (1996) recently reported evidence for a repressor element in the smMHC promoter which may play a role in conferring specificity of expression to mature, unmodulated SMCs. In addition, transient transfection analysis of the activity of various truncated smMHC promoter constructs showed evidence for differential gene expression in cultured airway *versus* vascular SMCs. A repressor sequence with capabilities similar to that of the smMHC promoter, is also thought to exist in the 5'-flanking promoter for sm-α-actin (Shimizu et al., 1995). Furthermore, a *cis*-acting repressor domain also exists in the 5' promoter sequence of the human calponin gene (Takahashi et al., 1996). Repression of calponin gene expression in non-muscle cells and in phenotypically modulated cells may be the result of methylation of cytosine bases in the 5' repressor element, thereby allowing for binding of the inhibitory transcription factor, methyl-cytosine binding protein.

Retinoic acid (RA) has been shown to be an inductive signal for producing and maintaining SMCs of the contractile phenotype (Blank et al., 1995; Colbert et al., 1996). It is interesting to note that in mice containing an RA response element/*lacZ* transgene,

RA-inducible gene transcription in vascular SMCs correlated with the expression of the smMHC isoform SM2, a definitive marker for the adult SMC phenotype, however, no such correlation was seen for visceral smooth muscle (Colbert et al., 1996). RA binds intracellular RA receptors (RAR) which affect gene transcription in two ways, firstly, they can associate with *cis*-acting hormone response elements in the target gene, and, secondly, they can associate with specific binding motifs present in other transcription factors, such as Hox proteins (Cardoso, 1995).

Several transcription factors which are members of families known to be involved with regulating development and differentiation have been found in SMCs. Myocyte-specific enhancer-binding factor-2 (MEF2) has been suggested as a candidate for regulating differentiation in all muscle types and potential MEF2 binding sites were identified in the rat and murine promoters for smMHC and SM22α. Firulli et al., (1996) report that mRNAs for several members of the MEF2 gene family were upregulated in proliferative vascular SMCs in the carotid neointima of a rat model of restenosis. Therefore, MEF2 does not appear to be involved with maintenance of the contractile adult SMC phenotype.

Homeodomain-containing transcription factors are known to be expressed in SMCs during embryogenesis and in adults. The DNA-binding specificity of these proteins has been shown to depend on their interaction with cofactors such as RA and they are important in control of patterning and positional signalling during development of body form (Cardoso, 1995). Homeobox genes expressed in adult or fetal SMCs include, *Mhox* (Blank et al., 1995), *Hox1.11* (Patel et al., 1992), *HoxA5, HoxA11, HoxB1,*

HoxB7 and *HoxC9* (Miano et al., 1996), *Gax* (Gorski et al., 1993) and *smLIM* (Jain et al., 1996). Both *Gax* and *smLIM* expression is down regulated in proliferative vascular SMCs suggesting a potential role for them in maintenance of a quiescent and/or mature phenotypic state (Gorski et al., 1993; Jain et al., 1996). In adddition, *HoxB7* and *HoxC9* appear only to be expressed in fetal vascular SMCs suggesting that they may be involved in presentation of this phenotypic state (Miano et al., 1996). However, no clear model for the role of these factors in determining the specifics of SMC differentiation and maturation has been described.

2.2.4 Smooth Muscle Cell Heterogeneity and the Disease State

The bulk of definitive work concerning the role of the SMC in disease pathogenesis has focussed on vascular remodelling associated with atherosclerosis and hypertension (Reviewed by Schwartz et al., 1986; Owens, 1989; Ross, 1993). It is now clear that SMCs within human atherosclerotic plaques and in neointimal lesions of endothelial-injury animal models, are phenotypically dissimilar to myocytes in the normal arterial media (Kocher and Gabbiani, 1986; Glukhova et al., 1988; Kocher et al., 1991). The pathogenic population of vascular SMCs are proliferative and secrete excess matrix components and growth factors (Rubin et al., 1988; Wilcox et al., 1988; Majesky et al., 1992). On the basis of ultrastructural characteristics and marker protein expression, SMCs orchestrating the formation of such lesions appear to be of a fetal phenotypic state (Manderson et al., 1989; Zanellato et al., 1990; Kuro-o et al., 1991; Aikawa et al., 1993). Figure 2 schematically represents the role played by arterial SMCs

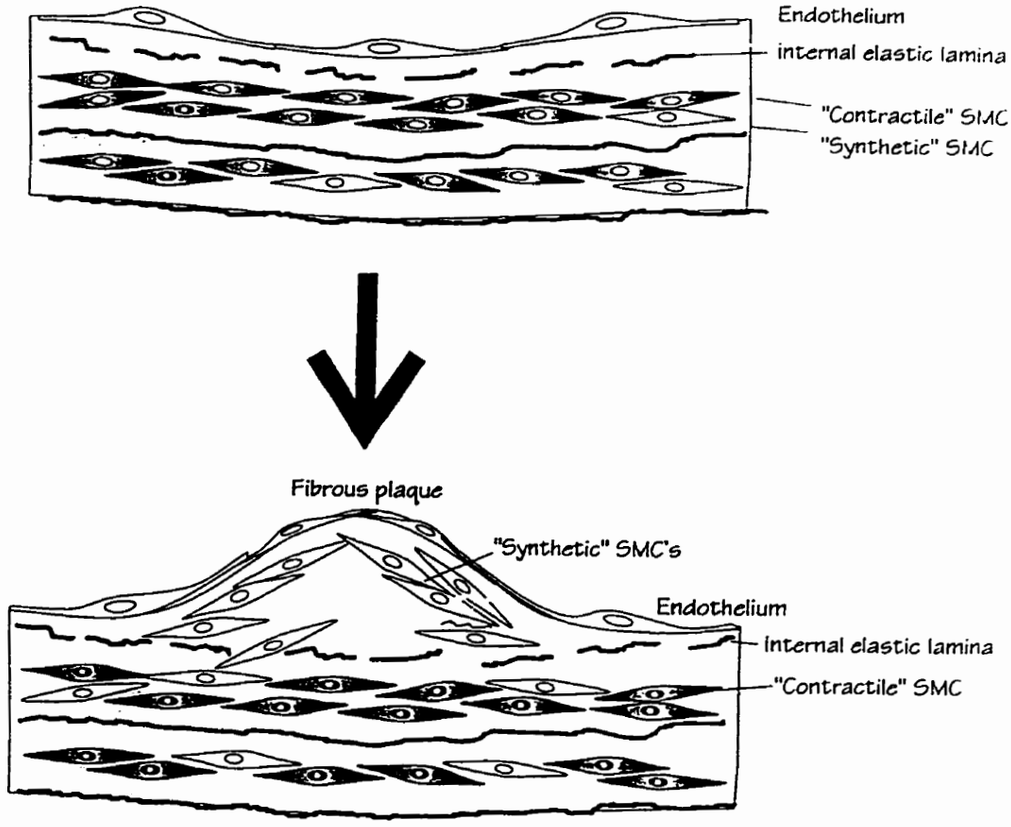
in the progression of an intimal lesion (Halayko and Stephens, 1994). The changes which occur in SMC phenotype in the formation of the fibrotic plaque do not appear to be permanent, based on observations that SMCs in vascular lesions reexpress contractile proteins after acute repair responses in animal injury models (Clowes et al., 1988; Kocher et al., 1991). Hence, much like SMCs in primary culture, in response to endothelial injury, arterial myocytes appear to modulate to a proliferative, synthetic state and then after normal cell-to-cell interactions are re-established, akin to reaching confluence in culture, protein markers of the mature state are reexpressed.

Currently, the question of the identity of the source of the myocytes responsible for plaque formation remains difficult to answer precisely. Knowledge of the existence of heterogeneous subpopulations of arterial SMCs, differing in proliferative capacity and matrix secretory function, has led many investigators to postulate that a distinct "stem cell" or myoblast subset, normally present in healthy vessels, is responsible for atherogenic thickening (Sartore et al., 1994; Giuriato et al., 1995; Bochaton-Piallat, 1996). A recent report supports this theory (Holifield et al., 1996) -- they observed that immature SMCs capable of rapid proliferation are found adjacent to the endothelium in the intimal layer of canine carotid arteries. Similarly, Schwartz et al. (1990) isolated a platelet-derived growth factor-independent, proliferative SMC population from adult rat aortae. These cells appear to be equivalent to a line of embryonic aortic SMCs which were isolated by Cook et al. (1994) from 13-17 day old rat embryos. The embryonic cells exhibited a capacity for self-driven growth independent of serum and growth factors. Of interest in this regard, during assessment of the heterogeneity of SMCs in

embryonic human aortae, Mironov et al. (1995) observed that in 18- to 28-week-old embryos, intimal and innermost medial cells had predominantly stellate shape and synthetic phenotype. However, no study using human material has demonstrated the existence of a myo-stem cell population of arterial medial origin which contributes to vascular lesion formation (Owens, 1995). To date, all data from animal models concerning the presence of an immature SMC population and the development of atheroma have been correlative in nature (eg. Giuriato et al., 1995). On the basis of the lack of a conclusive study in which the origin of intimal SMCs is/are clearly delimited using cell tagging, the possibility that neointimal SMCs may be recruited from the medial SMC populations, through phenotypic modulation and migration to the neointima (Campbell and Campbell, 1985; Schwartz et al., 1986), cannot be dismissed.

FIGURE 2. Changes which occur in smooth muscle cell phenotype during vascular wall remodelling associated with progression of an atherosclerotic lesion.

Smooth muscle cells (SMC's) migrate from the media of the vessel wall and accumulate in the developing lesion where they express a synthetic phenotype. These "synthetic" SMC's proliferate due to paracrine and autocrine stimulation and synthesize extracellular matrix thereby contributing to the fibroproliferative nature of the lesion.


Endothelium
internal elastic lamina
"Contractile" SMC
"Synthetic" SMC
Fibrous plaque
"Synthetic" SMC's
Endothelium
Internal elastic lamina
"Contractile" SMC

2.3 Smooth Muscle and Hyperresponsive Airways Disease

2.3.1 Airways Remodelling - Smooth Muscle Hyperplasia and Hypertrophy

Asthma, one of the most common diseases in industrialized countries, is a clinical state characterized by episodes of reversible dyspnea and wheezing. It is a disease of the airways, characterized symptomatically by persistent airway hyperresponsiveness to allergic and non-allergic stimuli (Barnes et al., 1988). That is, when airway responsiveness is measured against histamine or methacholine, using pulmonary function testing, there is a decrease in the dose required to elicit changes in airway resistance and an increase in the magnitude of airway constriction to the point where in moderate-to-severe asthmatics, the maximum response is elevated and fails to plateau (Woolcock et al., 1984). Recent interest in the disease has heightened with the realization that asthma-associated morbidity and mortality are increasing in spite of a rising use of anti-asthmatic medications, earlier diagnosis, and better care (Page, 1993; Barnes, 1993; Busse et al., 1993; Sears, 1996). At present, asthma is generally recognized as a chronic inflammatory disease highlighted by excessive airway narrowing in response to various stimuli (Page, 1993; Barnes, 1993; Busse et al., 1993; Sandford et al., 1996). However, neither the nature nor the site of the basic underlying defect of the disease has been established clearly.

Acute exacerbations of the disease are the result of airway obstruction resulting from spasm of airway smooth muscle and luminal plugging with excessive mucous secretions (Barnes et al., 1988). Contraction of the airway muscle is mediated primarily

by compounds released from mast cells in response to allergic and non-allergic stimuli (Beasley et al., 1989). Examples of causal and precipitating factors include exposure to allergens, airborne toxins, viral contaminants and mechanical stresses associated with exercise and cold air exposure (Barnes, 1993). Acute obstruction can be ameliorated with the use of $ß_2$-adrenergic bronchodilators, however, in the chronic disease a non-reversible component of hyperreactivity persists which is unresponsive to adrenergic therapy (Barnes et al, 1988). Severe airway inflammation is characteristic of patients who die of asthma (Dunnill, 1960); it also exists, though to a lesser degree, in patients with only mild forms of the disease (Laitinen et al., 1985; Beasley et al., 1989). The regular use of inhaled steroids is the treatment of choice to control chronic airways inflammation (O'Byrne, 1993).

It now appears that remodelling and thickening of the airway wall, which occur as a result of chronic inflammation, may represent a basic mechanism underlying irreversible airways hyperreactivity (James et al., 1989; Wiggs, et al., 1992). Sub-epithelial and adventitial fibrosis (Roche et al., 1989; Djukanovic et al,. 1990), and increased airway smooth muscle mass are characteristic pathological features of chronic asthma that contribute to airway wall thickening (Dunnill et al., 1969; Hossain, 1973; Ebina et al., 1990, 1993). Using computer modelling techniques, Wiggs et al. (1992) have demonstrated that for a particular degree of airway smooth muscle shortening, substantially higher airway resistance develops if airway wall thickening exists to the extent that has been measured in post mortem samples from asthmatic patients (James et al., 1989).

Fairly well defined structural changes associated with airways remodelling have been identified from autopsy samples of patients dying of status asthmaticus and from biopsy samples from subjects with milder disease (Laitinen et al., 1985; Ebina et al., 1993). These changes include infiltration with eosinophils and macrophages (Dunnill, 1960), edema, epithelial denudation (Laitenen et al., 1985), hyperplasia of mucous secreting glands (Dunnill, 1960), thickening of the basement membrane (Roche et al., 1989; Djukanovic et al,. 1990), subepithelial collagen deposition (Cutz et al., 1978), subepithelial accumulation of myofibroblasts (Brewster et al., 1990), and substantial thickening of the medial smooth muscle layers (Dunnill et al., 1969; Hossain et al., 1973; James et al., 1989; Ebina et al., 1990 and 1993).

Though the existence of medial smooth muscle hypertrophy in asthmatic airways has been well documented, little information is available about the mechanisms underlying the cellular growth response. Airway SMC hyperplasia and hypertrophy appear to occur in response to allergen challenge and airways inflammation (Hossain, 1973; Ebina et al , 1993; Panettieri et al., 1995). Ebina et al. (1993) have described different patterns of medial thickening in human airways from patients who died in status asthmaticus. In half of the subjects, referred to as Type I patients, increased smooth muscle mass was seen only in large bronchi. In the remainder, termed Type II patients, smooth muscle thickening was evident from large bronchi to small bronchiolar segments. Muscle thickening in Type I subjects resulted almost exclusively from cellular hyperplasia in large bronchi, whereas, in Type II subjects cellular hypertrophy occurred along the entire bronchial tree in addition to hyperplastic growth in large bronchi.

Regional disparity in the modality of airway myocyte growth suggests that 1) different mechanisms and/or stimuli might trigger smooth muscle growth in different areas of the lung and 2) heterogeneous populations of airway myocytes may exist which differ in distribution along the bronchial tree.

2.3.1.1 Paradigm for the Contribution of Airway Smooth Muscle Cells to Airways Remodelling

The bulk of definitive work concerning the contribution of SMCs to pathogenesis of fibroproliferative diseases has been focused on vascular smooth muscle and its role in vessel wall remodelling in hypertension and atherogenesis (Owens, 1989; Jackson and Schwartz, 1992; Ross, 1993; Schwartz and Liaw, 1993). The formation of an atherosclerotic lesion is an inflammatory response, likely due to endothelial injury induced by, among others, mechanical, immunologic, viral and cytotoxic stimuli. Interestingly, stimuli of a similar nature play causal and precipitating roles in the airway wall remodelling associated with chronic asthma (Barnes, 1993).

Persson and co-workers (Erjefält et al., 1994 and 1995) developed a novel *in vivo* animal model in which they produced reproducible zones of tracheal epithelial denudation, without causing basement membrane damage or bleeding, in order to study the temporal response of the airways to damage. With denudation a consistent, focal inflammatory response ensues -- the basal lamina is rapidly coated in a plasma-derived matrix containing fibrin, fibronectin, and growth factors. Adjacent goblet and mast cells degranulate, and ciliated epithelial cells flatten out and migrate over the wounded area

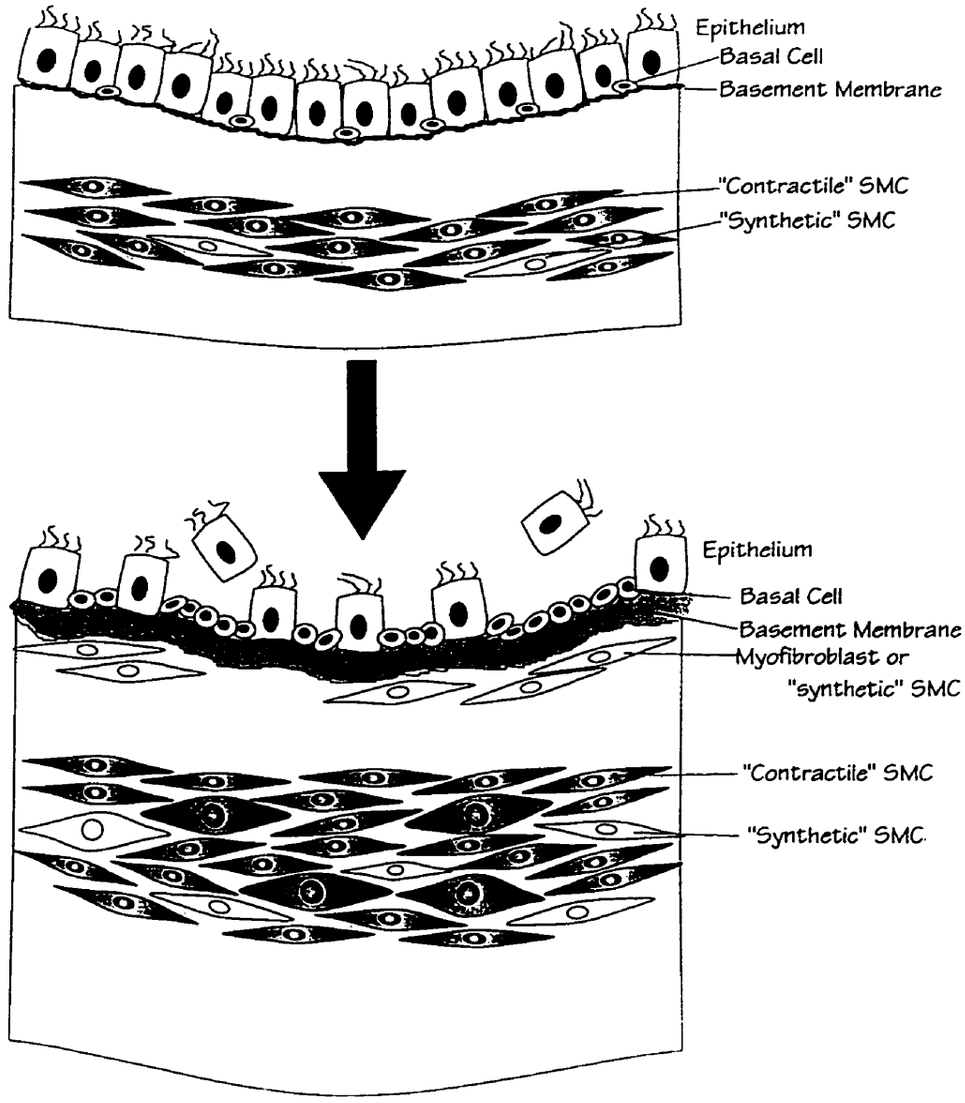
(800 μm x 30 mm), covering it with activated, undifferentiated epithelial cells within hours of the injury. Once the basal lamina is covered, proliferation increases substantially in the fibroblasts and SMCs lying beneath the wounded areas. Repeated denudation of the same area leads to thickening of the basement membrane and structural alterations similar to those catalogued for human asthmatic airways (Dunnill et al., 1969; Laitinen et al., 1985). The response of the airway to epithelial injury suggests that a sequence of inflammation-driven cellular events, similar to those described for vascular lesion formation after endothelial injury (Ross, 1993). In the process of arterial injury repair smooth muscle cells de-differentiate in a reversible fashion from a contractile phenotype to a synthetic phenotype which is capable of migration and proliferation (Ross, 1993; refer to section 2.2.4)

Reports detailing the extent and potential significance of phenotypic modulation of airway smooth muscle cells in inflammatory airway disease are not available though the proliferative effects of a few asthma-related mediators on cultured airway smooth muscle have been reported (Panettieri et al., 1990; Noveral et al., 1992; reviewed by Hirst and Twort, 1992). Specific studies regarding the effects of phenotypic modulation of airway smooth muscle and the potential contribution of this phenomenon to the pathology of chronic asthma have not been carried out. Figure 3 schematically depicts a proposed scheme for the involvement of airway SMCs in asthma-associated, airways remodelling (Halayko and Stephens, 1994). The scheme predicts that, in a fashion similar to that of vascular SMCs in atherogenesis, the phenotypes expressed by subsets of airway SMCs in asthmatic airways may play a critical role in determining the

pathophysiology of chronic asthma. The scheme presumes that airway remodelling, which includes thickening of the smooth muscle layer, could be the principal causative factor in chronic airway hyperreactivity (James et al., 1989; Wiggs et al., 1992). This scheme does not, however, negate the possibility that other suspected mechanisms for airways hyperresponsiveness, such as bronchoconstrictor hypersensitivity (Schellenberg and Foster, 1984; de Jongste et al., 1987), defects in autonomic regulation (Ayala and Ahmed, 1991) and altered non-adrenergic, non-cholinergic neuropeptide release and processing (Ollerenshaw et al., 1989), might have a synergistic effect.

FIGURE 3. Changes in smooth muscle cell phenotype which could occur in the course of remodelling of bronchial airway walls in chronic asthma.

In the healthy airway, smooth muscle cells (SMC's) present a primarily contractile phenotype though it is likely that a range in cellular phenotypes exists and that topographical differences in their distribution also exist. In chronic asthmatic airways, wall thickening develops which includes SMC hyperplasia and/or hypertrophy (denoted by large cells). Sub-epithelial fibrosis and basement membrane thickening develop while "synthetic" SMC-like myofibroblasts, which synthesize and deposit collagen and extracellular matrix components, appear in the interstitium. These cells could be derived from subsets of smooth muscle cells migrated from the media. The airway wall thickening which occurs results in increased airway resistance and contributes to the maintenance of chronic airway hyperreactivity.


Epithelium
Basal Cell
Basement Membrane
"Contractile" SMC
"Synthetic" SMC
Epithelium
Basal Cell
Basement Membrane
Myofibroblast or
"synthetic" SMC
"Contractile" SMC
"Synthetic" SMC

2.3.2 Regulation of Airway Smooth Muscle Cell Proliferation

2.3.2.1 Growth factors, cytokines and other molecules

The roles and identity of inflammatory cells and the substances they secrete in asthmatic airways is a well studied area (reviewed by Leff, 1991; Barnes, 1993). The chronic changes which occur in the bronchial wall of asthmatics (including hyperplasia and degranulation of mast cells, endothelial activation and oedema, sloughing of airway epithelium, and substantial infiltration by inflammatory cells including eosinophils, alveolar macrophages, lymphocytes and platelets), are likely to be perpetuated by, and may well be the consequence of, the release of pro-inflammatory mediators by a variety of "activated" cell types (Beasley et al., 1989 and 1993; Bradley et al., 1991). Naureckas et al. (1995) reported that the bronchioalveolar lavage fluid from immature rats with hyperoxia-induced airways inflammation was mitogenic for airway SMCs in culture. Hence, it appears that a wide range of growth factors and spasmogens with mitogenic properties for smooth muscle are present in affected airways and are capable of stimulating, directly or indirectly, hypertrophy, hyperplasia and/or phenotypic modulation of smooth muscle cells.

Examples of mediators known to be associated with airway inflammation which also possess mitogenic and growth inhibitory potential for SMCs are listed in Table 2. All the factors noted are known to be present in asthmatic airways, however, in some cases, data pertaining to effects on SMC growth have only been reported for myocytes of vascular origin. It should be kept in mind that the effects of individual factors will be

greatly influenced by the melange of other factors present at any time *in vivo*.

Several factors which are not noted in Table 2 also exist which could affect airway SMC growth. Lysosomal hydrolases and endoglycosidases secreted by macrophages have been shown to modify extracellular matrix components such as heparin sulfate and induce growth responses in cultured bovine tracheal myocytes (Lew and Rattazzani, 1991) and vascular smooth muscle cells (Campbell et al., 1992). In addition, mast cell and macrophage-derived proteases can affect smooth muscle cell growth by altering the integrity of tissue matrix (Clowes et al., 1990). Human asthmatic airways are deficient in vasoactive intestinal peptide (VIP)-containing nerve fibres (Ollerenshaw et al., 1989), this substance triggers elevation of cytosolic cyclic-AMP levels; elevated cyclic-AMP level is associated with inhibited vascular smooth muscle cell division (Orekhov et al., 1986) suggesting that an asthma-associated VIP deficiency could abrogate an endogenous anti-proliferative pathway.

TABLE 2: Asthma-associated factors and the smooth muscle cell growth response that they are known to effect.

Growth Factor/Inhibitor	SMC Growth Response	Cell Type	Reference
Histamine	Proliferation	Canine ASM	Panettieri et al, 1990
Endothelin-1	Proliferation	Rabbit ASM	Noveral et al., 1992
Prostaglandins D_2, E_2, $F_{2\alpha}$, I_2	Inhibited DNA synthesis; proliferation (D_2 only)	Rabbit VSM\Human ASM	Oreknov et al, 1986; Pietilä et al, 1980; Johnson et al., 1995
Thromboxane A_2	Proliferation	Rabbit ASM	Noveral and Grunstein, 1992
Leukotrienes B_4, C_4, D_4	Proliferation	Rabbit VSM\Rabbit ASM	Palmberg et al, 1989; Noveral et al., 1992
Neurokinin A	DNA synthesis	Rat VSM	Hultgragh-Nilsson et al, 1988
Substance P	DNA synthesis	Rat VSM	Nilsson et al,1985
Adenosine	Inhibited DNA synthesis	Rat VSM	Jonzon et al, 1986
Serotonin	Proliferation	Bovine VSM	Nemecek et al, 1986
Heparin	Inhibited replication	Canine ASM	Johnson et al., 1995
PDGF	Phenotypic modulation, migration and replication	Rabbit ASM	Hirst et al, 1992
EGF	DNA synthesis	Rat VSM	Hultgragh-Nilsson et al, 1988
FGF-2	Proliferation	Rat VSM	Lindner and Reidy, 1991
IL-1	Proliferation via enhanced PDGF expression	Human VSM\Rabbit ASM	Raines et al, 1989; De et al., 1993
TGF-β_1	Matrix secretion, hypertrophy, cell & culture dependent replication inhibition/stimulation	VSM	Majesky et al, 1991; Owens et al,1988

Abbreviations: ASM = airway smooth muscle; VSM = vascular smooth muscle; SMC = smooth muscle cells; PDGF = platelet-derived growth factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; IL-1 = interleukin-1; TGF-β_1 = transforming growth factor β_1.

2.3.2.2 Mechanism of Heparin-Mediated Inhibition of Smooth Muscle Cell Proliferation

Heparin and its derivatives have been studied extensively for their ability to inhibit restenosis in vascular-endothelial animal models (reviewed by Weissberg et al., 1993). Investigations have revealed that heparin inhibits migration and proliferation of SMCs (Clowes and Karnovsky, 1977) and also inhibits phenotypic modulation of vascular SMCs in culture and *in vivo* (Clowes et al., 1988). Johnson et al. (1995) and Kilfeather et al. (1995) demonstrated that heparin also has anti-proliferative activity for airway SMCs.

Numerous studies have attempted to elucidate the molecular mechanisms by which heparin mediates its growth-related effects. Inhibition of proliferation appears to be dependent upon receptor-mediated binding and internalization of heparin by SMCs (Bârzu et al., 1996; Letourneur et al., 1996). Heparin appears to exert an inhibitory effect on multiple signalling pathways involving PKC (Castellot et al., 1989) and casein kinase II (Sing et al., 1993) and mitogen activated protein (MAP) kinase (Ottlinger et al., 1993). It has been proposed that heparin modulates gene transcription by inhibiting *trans*-activation of phorbol-inducible genes by AP-1 nuclear transcription factor (Busch et al., 1992). However, this seems unlikely as internalized heparin does not localize to the nucleus (Au et al., 1994; Bârzu et al., 1996) and, in a recent study, Au et al. (1994), did not observe a direct effect of heparin on the *trans*-activating nuclear factor AP-1. Heparin may act at several points in the cell cycle; it has been shown to inhibit early G_0/G_1 transition events (Castellot et al., 1985) and late G_1 phase progression (Reilly et al.,

1989) in cultured vascular SMCs. Expression of the immediate-early protooncogenes, *c-fos* and *c-myc*, appear to be repressed by heparin in cultured vascular myocytes (Wright et al., 1989; Pukac et al., 1992). This is in contrast, however, to results obtained in a rabbit endothelial-injury model where protooncogene induction in SMCs was unaffected by heparin *in vivo* (Hamon et al., 1996). Grainger et al. (1993) reported that heparin-mediated inhibition of cultured vascular SMC proliferation was due to an extension in the length of time required for progression through G_2 phase and into mitosis. Consistent observations that heparin inhibits phenotypic modulation of vascular myocytes in culture and *in vivo* after arterial injury have been reported (Grainger et al., 1993; Clowes et al., 1988; Desmoulière et al., 1991).

Several investigators have reported the isolation of vascular SMC populations that differ in heparin sensitivity (Letourneur et al., 1995; Bârzu et al., 1996). Heparin sensitive subpopulations differ from insensitive subpopulations in their ability to upregulate expression of heparin receptors when exposed to the inhibitory agent (Letourneur et al., 1995). Heparin-sensitivity also appears to be phenotype state-dependent; growth arrested, cultured vascular SMCs are much more sensitive to heparin effects than are SMCs that have already reached a proliferative state (Castellot et al., 1987). This result compares well with those from *in vivo* studies in which it has been observed that heparin must be administered within 18 hours of induced arterial injury in order to achieve maximal inhibition of restenotic lesion formation (Majack and Clowes, 1984).

2.3.3 Alterations in Airway Smooth Muscle Properties in Asthma

No conclusive evidence is at hand to clarify whether a change in the intrinsic properties of airway smooth muscle, as the result of changes in smooth muscle cell phenotype, might persist in chronic asthma. Several studies exist concerning the isometric responsiveness of human asthmatic smooth muscle preparations to various spasmogens (Schellenberg and Foster, 1984; de Jongste et al., 1987; Björk et al., 1992). However, these types of pharmacological investigations do not address potential differences in functionally important modalities of smooth muscle contractility such as auxotonic shortening capacity and relaxation. For example, Schellenberg and Foster (1984) have reported that the *in vitro* isometric force generating capacity of human asthmatic airway preparations is increased in response to histamine. There are also several reports of altered human airway smooth muscle responsiveness after passive sensitization *in vitro* with serum from atopic patients (Black et al., 1989; Ben-Jebria et al., 1993; Villanove et al., 1993; Mitchell et al., 1994). Passive sensitization increased contractile responsiveness to a number of spasmogens including, potassium chloride, histamine and tachykinins. Relaxation in response to vasoactive intestinal peptide, Ca^{2+}-antagonists and potassium channel blockers was also reduced in these preparations. In addition, Mitchell et al. (1994) demonstrated that maximum shortening velocity and shortening capacity of human bronchial strips were increased after passive sensitization suggesting that fundamental changes in airway smooth muscle characteristics developed.

Unfortunately, difficulty in obtaining viable samples from asthmatic patients has precluded phenotypic analysis of human asthmatic airway smooth muscle contractility.

Bramley et al. (1993), however, have recently been able to measure the contractility of bronchial smooth muscle preparations from a resected lung lobe of a chronic, mildly asthmatic patient. The bronchial wall showed evidence of inflammation and subepithelial fibrosis but no significant increase in muscle mass. Importantly, maximum shortening capacity and force generating capacity of the asthmatic muscle were greater than for those from normal human lungs. In contrast, passive tension was reduced in the asthmatic preparation. These findings suggest that dynamic contractile properties of smooth muscle from asthmatic airways might be enhanced and that changes in tissue matrix elastance might be a contributing factor.

The density of β_2-adrenergic receptors may be increased in smooth muscle from patients who died of asthma (Bai et al., 1992). In contrast, there is a reduced response to β-agonists in isolated smooth muscle from fatal asthmatics suggesting that β_2-receptor function appears to be compromised (Goldie et al., 1986; Bai 1990). However, reduced responsiveness to β-agonists is not a consistent feature of airway smooth muscle obtained from resected lungs of patients with less severe asthma (Whicker et al., 1988). Point mutations in the β_2-adrenergic receptor are thought to modulate the severity of human asthma , however, they do not appear to play a primary role in the pathogenesis of the disease (Reihsaus et al., 1993; 137 Sandford et al., 1996).

There are several animal models of bronchial hyperresponsiveness in which the fundamental *in vitro* contractile properties of airways smooth muscle appear to be altered (Ishida et al., 1990; Stephens et al., 1991; Stephens et al., 1993). Measurement of force-velocity relationships for airway smooth muscle from a canine model of allergic

bronchial hyperresponsiveness, which mimics early asthma prior to the onset of bronchial inflammation, has revealed an intrinsic increase in muscle shortening velocity and capacity. Similar changes have been measured for tracheal smooth muscle from inbred rat and mouse models of airways hyperresponsiveness (Holme and Piechuta, 1981; Stephens et al., 1991b; Fan et al., 1996). The enhanced dynamic properties of smooth muscle from these animal models has been shown to be associated with increased levels of regulatory myosin light chain phosphorylation (Kong et al., 1990) and myosin light chain kinase activity (Jiang et al., 1992). In addition, the activity of acetylcholinesterase, which is likely secreted by the smooth muscle cells, is reduced (Mitchell et al., 1991). In another animal model, tracheal SMCs derived from guinea pigs receiving repeated antigen challenge generated greater amounts of inositol triphosphate, and intracellular Ca^{2+} was higher, following stimulation with carbachol and leukotriene D_4 in comparison to SMCs from controls (Salari et al., 1992). These data suggest that fundamental alterations in the signalling pathways regulating airway smooth muscle contraction may underlie changes in mechanical properties.

In a recent report, Nyce and Metzger (1997), presented data obtained using a dust mite-conditioned rabbit model of human asthma that indicated adenosine AT1 receptor number on bronchial smooth muscle cells became increased after sensitization. Furthermore, treatment of animals with aerosolized antisense cDNA for the AT1 receptor, blocked the development of bronchial hyperresponsiveness normally seen in response to dust-mite allergen sensitization. These findings correlate well with previous observations that human airway smooth muscle from asthmatics contracted in response to

exogenous adenosine whereas bronchial smooth muscle from non-asthmatic human lungs did not (Björk et al., 1992). These observations indicate that membrane receptor expression may be altered in airway smooth muscle from hyperresponsive animal models and humans.

Indirect evidence that phenotypic modulation of smooth muscle cells might be an effector of altered contractility has been obtained from a variety of tissues. Differences in the relative content of the 17 kDa-essential myosin light chain isoforms, 17a and 17b, are correlated with differences in maximum shortening velocity of various smooth muscles, indicating that changes in contractile protein isoform expression patterns can effect changes in contractile function (Malmqvist and Arner, 1991). Regulatory myosin light chain phosphorylation was shown to be significantly increased in areas of the carotid artery prone to vasospasm, 5 weeks after induction of endothelial injury, suggesting that qualitative changes occur in the contractile nature of medial myocytes in areas of neointimal lesions (Seto et al., 1993). In contrast, in a model for experimental colitis, reduced levels of regulatory myosin light chain phosphorylation and reduced shortening velocity have been reported in colonic smooth muscle (Xie et al., 1992). These data indicate that changes in smooth muscle properties, at sites of inflammation, may be smooth muscle lineage-dependent and unique to the anatomic site of the affected muscle. Changes in smMHC isoform distribution and actin-activated myosin ATPase activity associated with smooth muscle hypertrophy have also been demonstrated in affected bladder and, arterial smooth muscle (Stephens et al., 1991a; Samuel et al., 1992; Upadhya et al., 1993). In addition, biochemical changes correlated with decreased

shortening velocity have been reported for basic metabolic mechanisms; expression of lactate dehydrogenase isoforms that favour anaerobic capacity are increased in hypertrophic bladders (Malmqvist et al., 1991b). Though these data are not from airway smooth muscle sources, they do support the possibility that contractility of smooth muscle from asthmatic bronchi could be altered due to inflammation-induced proliferation.

The proliferative response in the asthmatic airway likely includes differentiation and de-differentiation of fibroblasts, pericytes and myofibroblasts. However, the possibility that cells of smooth muscle origin might be involved to a broader degree in the pathogenesis of asthma, through recruitment and modulation to functionally different phenotypes, has not been explored. For example, myofibroblasts increase in number in some bronchi of asthmatic patients (Roche et al., 1989). These cells are thought to contribute to sub-epithelial fibrosis via enhanced collagen deposition (Roche et al., 1990). There is debate as to the source or stem cell of the myofibroblast, indeed, the smooth muscle cell is considered a candidate (Sappino et al., 1990). Modulation of SMCs to an immature, myofibroblast-like cell is associated with focal fibrous intimal thickening seen in atherosclerosis (Campbell et al., 1991; Babaev et al. 1990). The composition of the extracellular matrix itself has been shown to regulate the phenotype expressed by SMCs (Majack et al., 1986; Fager et al., 1992). Hence, changes in basement membrane characteristics associated with asthma-related bronchial fibrosis could influence airway myocyte growth and phenotype. Changes in cellular function of myocytes associated with intimal thickening include increased production of collagen,

elastin, fibronectin, and glycosaminoglycans (Campbell and Campbell, 1987). These types of changes are in line with some of those described in fibrotic asthmatic bronchi (Roche et al., 1989; Rennard, 1996).

2.3.4 The Airway Smooth Muscle Cell in Culture

A wide range of cytokines, growth factors and other molecules capable of stimulating smooth muscle cell proliferation and hypertrophy are present in the asthmatic airway (28). Cultured airway myocytes provide a convenient model system to study: 1) the effects of specific mediators on smooth muscle cell proliferation and differentiation, 2) secondary signalling pathways which regulate contraction and proliferation, 3) membrane receptor and ion channel properties, 4) regulation of gene expression and post-translational processing of functionally important smooth muscle proteins, and 5) the effects of therapeutic interventions on the properties of airway myocytes. The number of studies using primary cultures of tracheal and bronchial myocytes has risen dramatically in the past ten years. Primary cultures of airway myocytes have been used as a model system for the proliferative response of airway SMCs associated with airway remodelling in chronic asthma. Cells from human airway smooth muscle have been used by several investigators (Panettieri et al., 1989; Twort and Van Breeman, 1989; Hall et al., 1992; Johnson et al., 1995). In addition, numerous studies using airway myocyte primary cultures of canine (Avner et al., 1981; Tom-Moy et al., 1987; Halayko et al., 1996), guinea pig (Salari et al., 1992; Pyne and Pyne, 1993), ovine (Farmer et al., 1991), bovine (Lew et al., 1992) and lepine (Chopra et al., 1991) origin have been reported.

2.3.4.1 Cell Isolation and Seeding

The majority of protocols used for preparing airway myocytes for primary culture entail the use of acute enzymatic dissociation of cells from tissues (Hall and Kotlikoff, 1995). Airway tissues are usually isolated, cleaned of adventitia by dissection then minced and the individual myocytes isolated using a digestion solution containing, chiefly, collagenase and elastase. Enzyme dispersal of cells is, in fact, a harsh treatment after which the cells often become rounded in shape and the number of viable cells may be compromised by as much as 50% (Avner et al., 1981). In response to enzymatic dissociation the sarcolemma appears to be highly active as evidenced by the loss of a definitive basal lamina on the cell's surface and, extreme degrees of folding associated with numerous caveolae and pinocytotic vesicles (Avner et al., 1981).

Dispersed cells are easily grown on uncoated plastic tissue culture plates in a range of culture media supplemented with fetal bovine serum (FBS) and a variety of antibiotics (Hall and Kotlikoff, 1995). Once plated, myocytes begin adhering to the culture surface within 1-3 hours and become flattened in appearance over the subsequent 24-48 hours (Mitchell and Halayko, 1996). Normal plating densities range from 1 to 10 x 10^3 cells per cm^2, and little change in cell number occurs over the first three to four days after plating (Halayko et al., 1996). During this time, few cell-to-cell contacts exist as cell density is low (Blennerhassett et al, 1987; Tom-Moy et al., 1987). The cells display morphological characteristics typical of cultured vascular smooth muscle cells (Chamley-Campbell et al., 1979) appearing spindle or ribbon-shaped with a large, oval, central nucleus marked by distinct nucleoli and having well-defined, perinuclear granules

(Tom-Moy et al., 1987). Immunofluorescent staining for smooth muscle specific marker proteins is often used to confirm the identity of the smooth muscle cells; for cultures obtained from tracheal sources, usually greater than 95% of the attached cells are positive for smooth muscle-α-actin (sm-α-actin) and smMHC (Panettieri et al., 1989 and 1990; Halayko et al., 1996). Approximately 5 days after initial seeding, cell number begins increasing and continues until confluence is reached, usually 8 to 10 days after initial seeding and the rate of increase in cell number diminishes drastically post-confluence (Tom-Moy et al., 1987). At confluence, the cells exist in several layers and the cultures exhibit a "hill-and-valley" pattern which is typical of smooth muscle cells in culture (Chamley-Campbell et al, 1979; Tom-Moy et al., 1987).

2.3.4.2 Phenotypic Changes of Airway Smooth Muscle Cells in Primary Culture

Principally, studies utilizing cultured airway SMCs have addressed questions relating to mechanisms coupling extracellular stimuli and sub-cellular signalling pathways in the regulation of airway myocyte proliferation or contraction/relaxation (Panettieri et al., 1990; Hirst et al., 1992; Smith et al., 1994; Johnson et al., 1995; Kelleher et al., 1995). Directly relating data obtained using cultured smooth muscle cells to *in vivo* circumstances is likely to be confounded by the tendency for phenotypic modulation of myocytes under normal culture conditions. It is now well established that in primary culture, serum stimulated smooth muscle cells undergo spontaneous, reversible modulation from the contractile to the synthetic phenotype similar to the changes seen during injury repair and proliferative responses *in vivo* (Chamley-Campbell

et al., 1979; Bowers and Dahm, 1993). Culture technique have been used to exploit this phenomenon using vascular SMCs, in order to characterize differences in protein expression and cellular function in smooth muscle cells expressing different phenotypes (eg. Shanahan et al., 1993).

It appears that modulation of airway SMCs occurs in culture. Data obtained using immunoblotting techniques (Panettieri et al., 1989; Halayko et al., 1996) have provided the most thorough and quantitative analysis of the changes occurring in protein expression of primary cultured airway myocytes. Assessment of the temporal changes occurring in the content of proteins which compose and regulate the contractile apparatus in canine tracheal myocytes (Halayko et al., 1996) indicates that the phenotype of cultured cells modulates rapidly when cultured in the presence of serum. The cells also demonstrate phenotypic plasticity as levels of contractile proteins increase again post-confluence (Halayko et al., 1996). Similar data have been described in numerous reports concerning vascular smooth muscle cells in culture and has led to the establishment of specific contractile and cytoskeletal proteins as markers of smooth muscle phenotype (Shanahan et al., 1993; Sartore et al., 1994). Freshly dispersed, contractile airway myocytes are relatively rich in proteins associated with the contractile apparatus (Halayko et al., 1996). These proteins include sm-MHC, sm-α-actin, calponin, desmin, ß-tropomyosin, myosin light chain kinase (MLCK) and *h*-caldesmon. Conversely, the content of these proteins is diminished significantly in cultured, proliferating tracheal myocytes (Panettieri et al., 1989; Halayko et al., 1996). Proliferating myocytes are, however, enriched in: 1) the non-sarcomeric isoforms of nonmuscle myosin heavy chain

(nm-MHC), 2) the nonmuscle, *l*-caldesmon isoform, 3) protein kinase C which is involved with the regulation of pathways mediating cell division, and 4) CD44, an integral membrane receptor for extracellular matrix components (Halayko et al., 1996).

Few studies have assessed the contractile performance of cultured airway myocytes (eg. Avner et al., 1981). Notwithstanding the tendency for cultured airway myocytes to lose their contractile proteins rapidly in culture, the adherent nature of the cells in culture precludes assessment of myocyte shortening as the plastic substrate to which they are attached presents a load which cannot be overcome. Receptor expression and coupling have been examined in cultured airway myocytes (reviewed by Hall and Kotlikoff, 1995). The profile of agonist receptors and their intracellular messenger pathways differ considerably between intact airway smooth muscle and cultured cells. There is a marked decrease in the inositol phosphate response to muscarinic receptor stimulation (Panettieri et al., 1989; Yang et al., 1991). The receptor subtypes, for example H_1 receptors (Daykin et al., 1993), mediating Ca^{2+} and inositol phosphate responses to some agonists appear to be the same in cultured cells and intact tissue. Subcultured human airway smooth muscle cells are also know to express functionally coupled receptors which are linked to adenylyl cyclase (Hall et al., 1992; Widdop et al., 1993). The distribution of charybdotoxin-sensitive K^+ channels having different conductance properties has also been assessed in cultured human bronchial SMCs using patch clamp techniques, and was found to be differentiation-state dependent, with freshly dispersed SMCs possessing the greatest number of high conductance channels (Snetkov et al., 1996). Cultured airway myocytes appear to express different profiles of receptor

subtypes depending on culture conditions, hence, this system provides a useful tool to study regulation of gene expression for the various proteins involved with excitation-contraction coupling.

2.3.4.3 Ultrastructure of Cultured Airway Smooth Muscle Cells

Ultrastructural characterization of cultured airway SMCs in different phenotypic states may provide insight into the functional changes which may occur in cells *in vivo*. Morphologic changes of vascular SMCs associated with growth and development in culture and *in vivo* have been well documented (Chamley-Campbell et al., 1979; Campbell et al., 1987). No systematic study of the morphologic changes in airway myocytes in hyperreactive airway disease has been reported. However, immunocytochemical (Tom-Moy et al., 1987; Panettieri et al., 1989; Mitchell and Halayko, 1996) and electron microscopic (Avner et al., 1981; Tom-Moy et al., 1987) analyses of cultured airway myocytes have been completed. These studies reveal that the changes known to occur in the cytostructural morphology of vascular myocytes in primary culture (Chamley-Campbell, 1979) also occur in cultured airway myocytes.

2.3.4.3.1 Organelles

The most comprehensive analysis of the ultrastructural properties of cultured airway myocytes has been performed using transmission electron microscopy of canine tracheal SMCs (Avner et al., 1981; Tom-Moy et al., 1987). These studies revealed that: 1) the cultured cells take on the morphologic appearance of SMCs of the so-called

synthetic phenotype, 2) even in long term cultures, however, several morphologic features unique to smooth muscle cells *in vivo* are maintained, and 3) considerable ultrastructural heterogeneity exists between cells in culture. Within several days of primary culture, in media supplemented with fetal serum, cytoplasmic organelles associated with cells that express the synthetic phenotype increase in number (Tom-Moy et al., 1987). These organelles include chiefly Golgi apparatus, sarcoplasmic reticulum, free ribosomes and mitochondria and their increase in density is associated with a concomitant disappearance of myofilaments in the cells. In line with the contention that these cells are synthetic in nature, considerable deposition of extracellular matrix, in the form of collagen fibrils and other amorphous materials, develops between adjacent cells (Tom-Moy et al., 1987). In post-confluent cultures these features are most striking, though not exclusively, in cells growing in "valleys".

2.3.4.3.2 Cell-to-cell junctions

Sites of cell-to-cell attachment known as *zona adherens* are a common feature of airway myocytes *in vivo* (Gabella, 1990). These contacts are disrupted during enzymatic dispersal of tissues in the preparation of airway SMCs for primary culture. Few cell-to-cell contacts can be seen in newly plated primary cultures as space between cells are very large. However, considerable cell-to-cell contact, in the form of intermediate junctions and gap junctions, begins to reform as cultures near confluent densities (Avner et al., 1987). Studies using cultured rat aortic smooth muscle cells demonstrate that in multi layered, confluent SMC cultures, gap junctions connect cells to lateral and vertical

neighbours (Blennerhassett et al., 1987). The gap junctions seen between cultured airway myocytes occur in both "hill" and "valley" cells and appear as classical multilayered structures by transmission electron microscopy (Tom-Moy et al., 1987). Gap junctions in canine tracheal myocyte cultures appear to provide functional metabolic and electrical coupling between cells as low molecular fluorescent tracer solutions microinjected into a single myocyte of confluent primary cultures spreads to neighbouring cells within several minutes (Mitchell and Halayko, 1996).

2.3.4.3.3 Cytocontractile structures

Thick and thin myofilaments comprise the cytocontractile apparatus in SMCs (Bagby, 1990). Thick filaments have been visualized, using transmission electron microscopy, in both acutely dissociated and cultured canine tracheal myocytes (Avner et al., 1981). The orientation of thick filaments in these preparations is more variable than that seen in cells of intact tissue. This is thought to be an effect of disrupting the normal integrity of the cell's shape upon enzymatic dispersal (Avner et al., 1981). Thin filaments are also seen in areas of the cytoplasm occupied by thick filaments. The volume fraction of the cytoplasm occupied by myofilamentous structure becomes dramatically reduced in phenotypically modulated cells in culture and filaments are localized to the periphery of myocytes (Campbell and Campbell, 1987).

The effects of cyclic mechanical strain on the volume fraction of myofilaments in canine tracheal smooth muscle cells cultured on flexible silastic membranes has been examined (Smith et al., 1994). The concentration of myofilaments in stretched cells is

increased and the myocytes become aligned parallel to the direction of stretch. Cytoplasmic and membrane-associated dense bodies, morphologic features typical of cells from intact tissues, are preserved in most cultured airway myocytes (Tom-Moy, et al., 1987). These dense areas are thought to represent areas where intermediate and actin-microfilaments are anchored (Bagby, 1990).

Localization of contractile proteins in cultured canine tracheal smooth muscle cells using immunofluorescent staining techniques shows the presence of filamentous structures oriented along the long axis of the cells (Bagby, 1990). Thick filaments are composed of sarcomeric smMHC which exists in two isoforms in airway SMCs (Kong et al., 1990). Non-sarcomeric, nonmuscle (nm-MHC), forms of myosin heavy chain are also expressed in airway smooth muscle cells (Halayko et al., 1996). Immunostaining of cultured tracheal myocytes with antibodies recognizing either sm-MHC or the A isoform of nm-MHC demonstrates that these proteins are localized into cytoplasmic filaments (Mitchell and Halayko, 1996). Structures which are positive for sm-MHC can be seen in tracheal myocytes through three subcultures, however, the intensity of staining is greatest in confluent plates and diminishes with each passage. Conversely, nm-MHC staining increases during the course of the initial primary culture and that level is maintained in subcultures thereafter (Halayko et al., 1996). In addition, staining of filamentous structures using an antibody which recognizes the A isoform of nm-MHC appears to be intermittent suggesting that this isoform may be localized only within specific segments of the filaments (Mitchell and Halayko, 1996). Filamentous structures similar to those seen with sm-MHC staining can be seen if cells are immunostained with antibodies

which recognize either sm-α-actin or *h*-caldesmon (Mitchell and Halayko, 1996). Actin is the primary constituent of thin filaments and caldesmon is a thin filament-associated regulatory protein; dual staining of cells for actin and caldesmon confirms that the proteins co-localize to the same cytoskeletal structures. It is not clear whether the structures visualized by these methods in de-differentiated, cultured airway myocytes represent contractile structures or whether they constitute cytoskeletal structures involved with changes in cell shape and cytokinesis.

2.4 General Perspectives - Airway Smooth Muscle Cells

The preceding literature review demonstrates that considerable insights into the nature of smooth muscle cell function and phenotype exist. However, it is evident that the mechanisms that regulate, development of smooth muscle lineages and maturation, modulation of the phenotypic state in the adult and expression of smooth muscle specific genes remain unclear. The major contributions in understanding the diversity and cell biology of smooth muscle cells have been achieved by investigators interested in vascular biology. Indeed, current, state of the art studies, addressing mechanisms regulating smooth muscle cell biology are being performed on myocytes of vascular origin as a result of the large base of knowledge concerning their nature and diversity. There is a paucity of fundamental descriptive information relating to airway smooth muscle development, growth, and phenotypic state and diversity. Hence, these are clearly areas which must be addressed for airway smooth muscle prior to embarking on more mechanistic investigations of airway myocyte cell and molecular biology.

3. MATERIALS AND METHODS

3.1 MATERIALS

All media, antibiotics, sera and other reagents used for cell culture experiments were obtained from Life Technologies - Gibco/BRL (Burlington, ON) unless otherwise noted. Redu-Ser (insulin-transferrin-selenium) was obtained from Upstate Biotechnology (Lake Placid, NY). Plastic tissue culture plates were from Corning Costar (Cambridge, MA). Collagenase used for the isolation of cells from tissue for primary culture and flow cytometry was purchased from Life Technologies - Gibco/BRL (Burlington, ON). Elastase and Nagarse protease were obtained from Sigma Chemicals (St. Louis,MO). Sodium heparin, purified from porcine intestinal mucosa (*Mr* 6-30 kDa, 184.6 anticoagulant units / mg), was purchased from Life Technologies - Gibco/BRL (Burlington, ON).

All reagents for protein electrophoresis were obtained from Bio-Rad (Mississauga, ON). Nitrocellulose membrane (0.22 μm pore size), used for Western blotting, was manufactured by Micron Separations Inc. (Westboro, MA). The Enhanced Chemiluminescence reagents (Amersham Life Science Inc., Oakville, ON) were used for chemiluminescent detection of proteins on immunoblots -- chemilumigrams were developed on HyperFilm ECL film (Amersham Life Science, Oakville, ON). Biotinylated secondary antibodies, horseradish peroxidase-conjugated antibodies and horseradish-conjugated stretavidin were also obtained from Amersham Life Science Inc.

Paraformaldehyde used for immunocytochemistry was purchased from TAAB Laboratories Equipment Ltd. (Reading, Berks, England). Fluorescien (FITC)-conjugated mouse, monoclonal anti-BrdU antibody was purchased from Becton

Dickinson (San Jose, CA). All other FITC- and indocarbocyanine (Cy3)-conjugated antibodies used in fluorescence immunocytochemistry and flow cytometry studies were obtained from Jackson ImmunoResearch Labs Inc. (BioCan Scientific, Mississauga, ON). Non-immune sera and non-specific Ig stocks, used for negative controls in flow cytometric assays, were also purchased from Jackson ImmunoResearch Labs Inc. Propidium iodide was purchased from Molecular Probes (Eugene, OR). Nylon filters used in preparation of cells for flow cytometry were purchased from BioDesign Inc. (Carmel, New York). Bromodeoxyuridine was obtained from Amersham Life Science (Burlington, ON).

3.2 Cell Culture

3.2.1 Canine Airway SMC Primary Culture.

Tracheas were excised from Nembutal-anaesthetized (30 mg/kg), 6-12 month old mongrel dogs (obtained from Central Animal Care Services, University of Manitoba) and placed into ice-cold Dulbecco's Modified Eagles Medium (DMEM) containing 100 units/ml penicillin, 100 mg/ml streptomycin and 50 μg/ml gentamycin. Trachealis muscle was removed and cleaned of serosa and epithelia at room temperature then washed four times, under aseptic conditions, in Hank's Balanced Salt Solution (HBSS) containing antibiotics as noted above. The muscle (500mg - 1gm) was thoroughly minced with fine scissors, then cells were isolated by resuspending the tissue slurry in 12 mls of digest buffer (HBSS containing: collagenase -- 600 units/ml; Type IV elastase -- 8 units/ml; and, Type XXVII Nagarse Protease -- 1 unit/ml). Cells were incubated at 37°C

for 45 minutes in a shaking water bath after which tissue pieces were disrupted with gentle trituration using a 1mm bore size borosilicate Pasteur pipette. The remaining debris was allowed to settle and the supernatant, containing isolated cells, was removed using a Pasteur pipette. The supernatant was immediately diluted 1:1 with DMEM containing 10% foetal bovine serum (FBS) and antibiotics and then the cells were pelleted by centrifugation (600 x *g*, 5 minutes). The resulting supernatant was discarded and the pelleted cells were resuspended in 5 mls of fresh DMEM / 10% FBS / antibiotics and stored on ice.

A fresh aliquot (12 mls) of digest buffer was added to the tube containing the debris that remained after the first digestion and, after mixing, it was placed back in the shaking water bath for a further 40 minutes to isolate the SMCs remaining. After the second digestion the tissue pieces were triturated again and the remaining debris allowed to settle. The supernatant was carefully removed using a Pasteur pipette and diluted 1:1 with DMEM / 10% FBS / antibiotics, centrifuged and resuspended in fresh DMEM in the same manner that the first digest was handled. The first and second digest fractions were subsequently pooled and filtered through 70μm nylon mesh. The cell suspension was pelleted by centrifugation then diluted in DMEM / 10% FBS / antibiotics. Cell number was estimated by counting using a Model Z_{BI} Coulter Counter or a haemocytometer after which the cells were plated onto Corning Tissue Culture Treated plastic dishes at a density of 1×10^4 cells/cm^2. Cells were grown at 37°C in a humidified atmosphere consisting of 95% air /5% CO_2, culture media was replaced every 72 hrs. For all studies, only unpassaged cultures were used unless otherwise stated.

To passage confluent cultures, plates were first washed three times (eg. using 2 mls for a 100mm diameter plate) with Ca- and Mg- free phosphate bufferred saline (PBS) (2.7 mM KCl / 1.2 mM KH_2PO_4 / 138 mM NaCl / 8.1 mM Na_2HPO_4, pH 7.4) which had been pre-warmed to 37°C. Following the final wash, PBS was aspirated. Cells were lifted by adding a pre-warmed (37°C) solution containing trypsin (0.05%) -EDTA·4Na (0.53 mM) in PBS (2 mls was used for a 100mm diameter plate). After 1.5 minutes cells were dislodged from the plastic plates by trituration using a Pasteur pipette. The cell suspension was transferred to a conical, polystyrene centrifuge tube and diluted 1:1 with DMEM / 10% FBS / antibiotics then centifuged (600 x *g*, 5 minutes) to pellet cells. The supernatant was dicarded and cells were resuspended in fresh DMEM / 10% FBS / antibiotics. When cells were passaged, a 1-to-4 split was employed (i.e. the cells from one confluent plate were diluted and replated into four new plates).

For experiments in which growth arrested cells were required the following protocol was employed. Culture media (DMEM / 10% FBS / antibiotics) was aspirated and the plates were subsequently washed three times with PBS which had been pre-warmed to 37°C. After removing the final PBS wash, serum-free arrest media, which was composed of Ham's F-12 Media containing Redu-Ser (final concentrations -- insulin 5 µg/ml, transferrin 5 µg/ml, selenium 5 ng/ml) and antibiotics, was added and the plates were returned to the cell culture incubator. All cultures were arrested for 96 hours prior to being used in subsequent experiments (Note: flow cytometric ananlysis revealed that a minimum of 72 hours arrest time was required to achieve >80% arrest and after 96 hours approximately 95% of all cells were arrested in the G_0/G_1 phase of the cell cycle).

For long term storage, cells were lifted from culture plates in the same manner described earlier in this section in the description of cell passage methods. The best viablilty for stored cells was seen when cells from confluent cultures were used. After cells were pelleted and the supernatant was removed, the cells were reusupended, by gentle trituration using a Pasteur pipette, in an ice cold solution composed of 90% FBS / 10 % dimethyl sulfoxide (DMSO) (1 ml of this solution was used for every 3 confluent plates from which cells were lifted). The cell suspensions were aliquoted into 2ml screw-cap cryovials and the vials were further sealed with Parafilm wrap and then placed on ice for 30 minutes. After this time tubes were transferred to a -80°C freezer and stored for up to 18 months. After long term storage, vials were removed from the freezer and warmed at room temperature. Once thawed the cell suspensions were transferred to 15 ml conical suspension tubes, diluted 3:1 with DMEM / 10% FBS / antibiotics and subsequently pelleted by centrifugation (600 x *g*, 5 minutes). Pellets were resuspended in culture media and the cells plated onto fresh plates (one vial was used to seed 10 plates).

3.2.2 MCH6 Fibroblast Cell Line

Cultures of MCH6 human foreskin fibroblast cells were maintained in McCoy's media containing 10% FBS, 100 units/ml penicillin and 100mg/ml streptomycin at 37°C in a humidified culture chamber. The cell cultures were a generous gift from Dr. K. Wrogemann, Department of Biochemistry and Molecular Biology, University of Manitoba. Media was changed every 72 hours.

3.2.3 Effect of Various Agents on Smooth Muscle Cell Cultures

The effects of various agents on the growth and phenotypic expression of tracheal SMC primary cultures were examined. Treatments were started 5 days after the initial seeding of isolated cells for primary culture. Treatments included: (1) 10nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 96 hours to stimulate protein kinase C mediated pathways, or; (2) 0.1μM histamine, a canine airway SMC spasmogen and putative mitogen (Panettieri et al., 1990), for 96 hours, or; (3) 0.1μM carbamyl choline chloride, a SMC spasmogen, for 96 hours, or; (4) 20μM 5-fluoro-2'-deoxyuridine, an inhibitor of thymidilate synthetase (Ellwart and Dormer, 1985), for 96 hours. Immediately after completion of the prescribed treatment, total crude protein extracts were obtained from the cells on the culture plates and the extracts were analyzed as described in the following sections.

3.3 Analysis of Cellular Constituents of Smooth Muscle Cells

3.3.1 Freshly Isolated and Cultured Airway Smooth Muscle Cells

A fraction of the tracheal SMCs isolated for culture were removed prior to plating and used to prepare crude protein homogenates. The isolated cells were washed by resuspension in ice cold, Ca^{2+} and Mg^{2+}-free PBS following centrifugation (600 x *g*, 5 min). This step was repeated twice, after the second resuspension step, cells were repelleted by centrifugation and protein extracts were prepared by lysing the cells in extraction buffer (100 μl / 10^6 cells) which contained: Tris-HCl (50 mM, pH 7.6), 0.3% sodium dodecyl sulfate (SDS), 0.6M β-mercaptoethanol, 20 μg/ml leupeptin, 250μm

phenylmethylsulfonylfluoride (PMSF), 50 mg/ml soybean trypsin inhibitor, and DNAse I (10 μg/ml). Cells were disrupted using a plastic pestle and the extracts then heated at 95°C for 2 minutes to fully solubilize and denature the samples. Samples were stored at -20°C until used for electrophoretic and immunoblot analysis.

To prepare extracts from cultured cells, first the media was aspirated, then plates were rinsed three times with cold PBS. Cells were lysed and proteins solubilized by adding 750 μl extraction buffer to each 100mm dish. Cells were further disrupted by scraping with a rubber policeman, then the extracts were placed into tubes and fully denatured by heating at 95°C for 2 minutes. Samples were stored at -20°C until used for electrophoretic and immunoblot assay.

3.3.2 Pulmonary Arterial Smooth Muscle Cells

Main pulmonary arteries were excised from anaesthetized mongrel dogs at the same time that tracheas were removed and were immediately placed into ice cold DMEM. The adventitial layers of the arteries were carefully removed on ice and the arteries were cut open longitudinally. After rinsing in fresh DMEM, the medial layer was exposed by rubbing away the endothelial layer sequentially using the blunt end of forceps and a cotton wool bud. The excised tunica media was minced with fine scissors and resuspended in a mild digest buffer (HBSS containing collagenase -- 300 units/ ml, Type IV elastase -- 5 units/ ml) for 40 minutes with shaking at 37°C. After trituration using a 1mm bore size borosilicate pipette the cell suspension was filtered through 100μm nylon mesh. Cells were then centrifuged (600 x *g*, 5 min) and the pellets resuspended in cold

PBS. This step was repeated twice. Homogenates were prepared from the pelleted cells by adding extraction buffer (10µl/mg original wet weight of the tunica media). Cells were disrupted using a plastic pestle and the extracts were heated at 95°C for 2 minutes. Samples were stored at -20°C until used for electrophoetic or immunoblot analysis.

3.3.3 Protein Assay

The protein concentration of crude extracts was estimated spectrophotemetically using the Bio-Rad Protein assay kit which is based on the coomassie dye-binding protocol first described by Bradford (1976). Absorbance of 595 nm light was measured using a Milton Roy Spectronic 1001 Plus spectrophotometer. Bovine serum albumin (BSA), Fraction V (Sigma Chemicals, St. Louis) was used as a relative protein standard for all assays. Assay results were used to calculate the dilution required to bring all sample to a protein concentration of 2 mg/ ml prior to storage at -20°C.

3.3.4 Protein Electrophoresis: SDS-PAGE

Proteins in crude extracts were size fractionated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system first described by Laemmli (1970). A vertical slab mini-gel apparatus was used for all applications. All gels measured 8 x 10 cm and were cast at 0.75 mm thickness. Lower gels (separating) ranged between 7.5% to 12% acylamide, depending on which protein constituents were of interest; 3.5% acrylamide stacking gels (upper) were used with all gels. Both upper and lower gels were prepared from a 30% acrylamide / *bis*-acrylamide monomer stock

solution in which the concentration of crosslinker (*bis*-acrylamide) was 3.7% w/w. The final composition of separating gels included: 1) acrylamide / *bis*-acrylamide in the concentraction required (7.5-12%), 2) 0.375 M Tris-HCl, pH 8.8, and 3) 0.1% (w/v) SDS. The final compostion of stacking gels included: 1) 3.5% (w/v) acrylamide / *bis*-acrylamide, 2) 0.125 M Tris-HCl, pH 6.8, and 3) 0.1% (w/v) SDS.

Samples were prepared by diluting crude extracts 1:3 in sample buffer (1.0 M Tris-HCl, pH 6.8, 8% (w/v) SDS, 45% (v/v) glycerol, 2.86 M β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and heating at 95°C for 1 minute. Proteins were fractionated by running the gels at constant voltage (200 V) for 45 minutes-to-1 hour at room temperaure. The electrode buffer used had the following composition -- 25 mM Tris, 0.192 M glycine, 0.1% (w/v) SDS. Protein loads in single lanes ranged from 2-15 μg total protein depending upon which protein constituent was of interest.

Some gels were stained for total protein using Coomassie Brilliant Blue R 250. Gels were fixed and stained immediately after electrophoresis by incubating the gels in glass trays contining 45% ethanol / 10 % acetic acid / 0.08 % Coomassie Brilliant Blue R 250 overnight. Gels were then destained in 35% ethanol / 10 % acetic acid until maximum resolution was obtained. Gels were then equillibrated in distilled water and subsequently air-dried between sheets of BioGel Wrap (BioDesign Inc., New York).

3.3.5 Western Blot Analysis of Protein Homogenates

The proteins in the samples obtained were size fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 8cm x 10cm mini-gels as

described in a previous section (3.3.4). Quantitative profiles of protein markers for the discrimination of cytodifferentiation state were obtained by electroblotting SDS-PAGE fractionated proteins to nitrocellulose as described by Towbin and Gordon (1984). Electroblotting was carried out at 4-8°C in 25 mM Tris / 192 mM glycine / 20% methanol (pH 8.3). Transfer times were 75 minutes using a constant voltage of 100 V. Immediately after transfer, blots were washed for 5 minutes in Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20. Blots were subsequently stained for total protein using either India Ink or Ponceau S or were utilized for immunodetection of specific protein constituents.

India Ink staining was performed as described by Hancock and Tsang (1983). Blots were placed in a staining solution consisiting of TBS / 0.1 % Tween-20 / 0.1 % (v/v) India Ink for 30 minutes to 1 hour. After staining, blots were rinsed with distilled water and incubated in fresh water for 30 minutes prior to being air dried. Ponceau S staining was achieved by placing blots, immediately after transfer, in a 0.15% solution of trichloroacetic acid containing 100 µg/ ml Ponceau S (Sigma Chemicals, St. Louis). After 15 minutes blots were rinsed in distilled water to remove background staining and visualize bands. Blots were destained by washing the blots in water until no color remained, then blots were incubated in TBS-0.1% Tween-20 (TBST) for 30 minutes and were subsequently used for immuodetection of protein constituents.

For immunodetection, blots were blocked overnight at 4°C in TBS / 0.1% Tween 20 (TTBS) containing 3% non-fat dried milk powder. Blots were then incubated in primary antibody diluted in TTBS containing 1% non fat dried milk powder for 2-3 hours

at room temperature. A variety of primary antibodies were used, these are summarized in Table 1 along with the dilutions used for each. Blots were subsequently rinsed and incubated for 40 minutes at room temperature with biotinylated secondary antibodies (Amersham Life Science, Oakville, ON) diluted 1:1000 in TTBS / 1% milk powder. Blots were rinsed again and finally incubated for 40 minutes at room temperature with streptavidin-horseradish peroxidase conjugate diluted 1:5000 in TTBS. Semi-quantitative staining of specific proteins was achieved using chemiluminescence detection; blots were washed in TBS and were subsequently dipped, for one minute, into luminol substrate solution (Amersham Life Science, Oakville, ON). Chemilumigrams were developed on Hyperfilm-ECL; the normal exposure times ranged from 30 s to 5 min.

3.3.6 Laser Densitometry

An LKB Ultroscan XL laser densitometer was employed to scan the developed films for estimation of protein content. All values obtained were normalized to total protein loaded onto the gels to allow for comparison between samples. The entire widths of the bands on chemilumigrams were scanned by consecutive parallel and adjacent passes, which covered a 8 μm path width each time. Data for each scan was captured using Gel Scan XL 2400 software. Absorbances within individual scanning profiles were collected, and customized software, Scanplot (Cunningham Engineering, Calgary), was used to convert the raw data into *X*- and *Y*-plane positional data and the *Z*-axis into absorbance data. After subtraction of background absorbance, integration of the three-

dimentional raw data yielded a volume (absorbance units (AU) x mm^2) that correlated qualitatively with the amount of stained protein in a particular band. Linearity of thedetection of the chemiluminescent signal from the blots by Hyperfilm ECL was determined prior to any experments by running blots with a wide range of known protein concentractions. The numbers obtained revealed that the response of the film was linear from 0.08 to 1.9 AU maximum, hence all scanned bands had to fall into this range in order to be included in subsequent statistical analysis.

3.3.7 Fluorescent Immunohistochemistry

3.3.7.1 Collagen Coating of Glass Coverslips

Round (12 or 22 mm diameter) glass coverslips were precleaned by washing in acidified aclohol (0.5% concentrated HCL in 75% ethanol) for 15 minutes then coverslips were rinsed once in sterile, double-distilled water and allowed to air dry on clean Whatman filter paper in a culture hood -- the coverslips were also irradiated with ultraviolet light while drying. Coverslips were then placed into culture dishes and approximately 100µl of sterile PBS, containing 0.1% rat tail collagen (Upstate Biotechnology Inc, Lake Placid, NY), 100 units/ml penicillin and, 100 mg/ml streptomycin was layered onto the coverslips. Coverslips were left uncovered in the culture dishes, under ultrviolet, until the collagen solution has dried completely, thus, leaving a thin, dry, fibrillar collagen coating. Once dry, the plates containing coverslips were sealed in sterile plastic bags and were stored at 4°C for up to 2 weeks proir to use.

TABLE 3. Primary antibodies used in immunoblotting to study marker protein content in isolated and cultured SMCs.

Antigen Specificity	Name	Dilution	Source
sm-α-actin	mouse Mab, asm1	1:10,000	Boehringer Mannheim Canada, Laval, PQ.
h- & *l*-caldesmon	rabbit PAb	1:10,000	Clark et al., 1986
calponin	rabbit PAb	1:1,000	Walsh et al., 1993
CD44 (HCAM)	mouse MAb, OX-49	1:250	PharMingen, San Diego, CA.
desmin	mouse Mab, DE-U-10	1:250	Sigma Immunochemicals, St. Louis, MO.
nm-MHC (NMA & NMB isoforms)	rabbit PAb	1:10,000	Kawamoto and Adelstein, 1986
sm-MHC (SM1 & SM2 isoforms)	rabbit PAb	1:10,000	Gröeschel-Stewart et al., 1976
MLCK	mouse Mab, K36	1:10,000	Sigma Immunochemicals, St. Louis, MO.
PKC (α, $ß_1$ & $ß_2$ isoforms)	mouse Mab, MC5	1:100	Amersham Canada, Oakville, ON.
tropomyosin (ß isoform)	mouse MAb, TM228	1:1,000	Sigma Immunochemicals, St. Louis, MO.
vimentin	mouse MAb, ascites fluid	1:100	Amersham Canada, Oakville, ON.

Abbreviations: MAb = monoclonal antibody; PAb = polyclonal antibody; sm/SM = smooth muscle; nm/NM = non muscle; MHC = myosin heavy chain; MLCK = myosin light chain kinase; PKC = protein kinase C.

3.3.7.2 Fluorescent Immunostaining of Cells

Freshly isolated cells were plated in dishes containing glass coverslips which had previously been coated with rat tail collagen. At predetermined times after cell plating the coverslips were removed from culture dishes, rinsed with PBS and the attached cells, were then fixed in 1% paraformaldehyde / PBS (pH 7.6) for 15 min at 4°C. Cells were subsequently permeabilized by replacing the fixing solution with 0.1% Triton X-100 / PBS and incubating for 15 mins at 4°C. Coverslips were then rinsed 3 times with PBS and were used immediately for immunostaing or were stored in a humidified chamber at 4°C for no longer than 72 hours. All subsequent steps were carried out at 4°C unless otherwise indicated.

For immunostaining, individual coverslips were transferred into separate 35 mm plastic dishes and then rinsed twice with 2 ml of PBS / 0.1% Tween-20. The coverslips were then coated with approximately 100 µl of blocking solution (PBS / 1% BSA / 5% normal animal serum / 0.1% Tween-20) and incubated for for 2-4 hours at 4°C in a humidified chamber. The animal species from which the normal serum was used, was determined by the identity of the secondary antibody used in later steps (eg. if the secondary antibody to be used was from sheep, then normal sheep serum was used in the blocking solution). The blocking solution was removed by rinsing the coverslips once with 2 ml of PBS / 1% BSA / 0.1% Tween-20 in a 35 mm plastic dish. All subsequent antibody incubations were carried out in humidified staining chambers at 4°C unless otherwise stated.

Approximately 75 µl of PBS / 1% BSA / 0.1% Tween-20 containing

appropriately diluted primary antibody was added on to each coverslip to cover the attached cells and were subsequently incubated overnight. A variety of primary antibodies were used over a range of concentrations -- these are summarized in Table 4. Negative controls were incubated in PBS / 1% BSA / 0.1% Tween-20 containing without primary antibody added. All subsequent washing and staining steps were identical for cells to be stained and their matched, negative controls. Coverslips were washed three times using 3-4 ml of PBS / 1%BSA / 0.1% Tween-20 and then secondary antibody solutions were added.

All secondary antibodies were diluted 1:100 in PBS / 1% BSA / 0.1% Tween-20 according to manufacturer's instructions. The secondary antibodies used included: 1) FITC-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, Code Number 515-095-003), 2) FITC-conjugated donkey anti-rabbit Ig (Amersham Life Science, Catalogue Number N1034), 3) Cy3-conjugated goat anti-mouse IgG and IgM (Jackson ImmunoResearch, Code Number 115-165-044), and 4) Cy3-conjugated sheep anti-rabbit IgG (Jackson ImmunoResearch, Code Number 111-165-003). The secondary antibody used or combination of secondary antibodies used was determined by the identity of the specific primary antibody or combination of primary antibodies employed. Approximately 75 µl of secondary antibody solution was added to each coverslip and the cells were subsequently incubated in the dark, for 1-2 hours at 4°C.

After secondary antibody incubation, coverslips were washed three times with 5 ml of PBS then twice with double-distilled water. Coverslips were then either mounted, cell side down, on glass slides using anti-fade media (85% glycerol / 1 mM *p*-

phenylenediamine / 100 mM Tris-HCl, pH 7.4) or were subjected to DNA-staining prior to mounting on a slide. To stain cells for DNA, after the final water wash noted above, the individual coverslips were placed in a 35 mm plastic dish and immersed in 3 mls of Hoechst 33342 dye (20 µg/ml bisbenzamide, Catalogue Number H-33342 , Sigma Chemicals, St. Louis) for 30-45 seconds. Coverslips were then removed, rinsed twice in 3 ml of double-distilled water and subsequently mounted onto microscope slides using mounting media described above. Prepared slides were stored in the dark at -20°C until they were viewed and photographed using a Nikon Diaphot microscope equipped with epifluorescence optics and a 35mm camera.

3.3.8 Immunoperoxidase Staining - Cytospin Analysis

For cytospin analysis suspensions of SMCs obtained by enzymatic dispersal from intact trachealis, from cell cultures, or from cell sorting experiments were used. Initial cell suspensions were prepared at 4°C in PBS at a density of 65,000 cells / ml as determined by cell counting using a hemocytometer. Approximately 250 µl of the cell suspension was spun (5 minutes at 500 x *g*) onto aminoalkylsaline (AAS)-coated glass microscope slides using a Shandon Cytospin 2. Immediately after spinning, cells were fixed by submerging microscope slides into acetone for 10 minutes. Slides were then air dried and stored at 4°C for up to 5 days prior to immunostaining .

TABLE 4. Primary antibodies used in immunocytochemical analyses of cultured canine tracheal SMCs.

Antigen Specificity	Name	Dilution	Source
sm-α-actin	mouse MAb, asm1	1:1000	Boehringer Mannheim Canada, Laval, PQ
h- & *l*-caldesmon	rabbit PAb	1:1000	Clark et al., 1986
nmMHC (NMA & NMB isoforms)	rabbit PAb	1:1000	Kawamoto and Adelstein, 1986
NMA isoform of nmMHC	rabbit PAb	1:350	Gift from R.Adelstein, NHLBI - National Institutes of Health, Bethesda, MD
sm-MHC (SM1 & SM2 isoforms)	rabbit PAb	1:1000	Gröeschel-Stewart et al., 1976
sm-MHC (SM1 & SM2 isoforms)	mouse MAb	1:250	Sigma Immunochemicals, St. Louis, MO
α-actinin	mouse Mab, clone BM-75.2	1:100	Sigma Immunochemicals, St. Louis, MO
vimentin	mouse MAb, ascites fluid	1:50	Amersham Canada, Oakville, ON
desmin	mouse Mab, ascites fluid	1:50	Amersham Canada, Oakville, ON

Abbreviations: MAb = monoclonal antibody; PAb = polyclonal antibody; sm/SM = smooth muscle; nm/NM = non muscle; MHC = myosin heavy chain

AAS coating of slides was achieved using a modified version of the protocol described by Maples (1985). In slide staining chambers, slides were dipped into dry acetone for several seconds then were transferred to AAS solution (15 ml 3-aminopropyltriethoxysilane added to 750 ml dry acetone) for 2 minutes (Note: dry acetone is prepared by adding approximately 100 gms of Anhydrous Cupric Sulfate fine powder to 1 L of acetone then mixing vigorously -- the stock is stored, with intermittent mixing, for at least 48 hours at room temperture prior to using). Slides were then rinsed at least three times with double distilled water and allowed to dry overnight. Slides were subsequently boxed and stored until used at room temperature.

Cytospin preparations were immunostained using either fluorescent or immunoperoxidase staining techniques. Immuofluorescent staining was achieved using the same protocol and antibody dilutions described in a previous section which detailed the staining protocol used for cultured cells on coverslips (Refer to Section 3.3.7.2). For other slides immunoperoxidase staining methods were employed as described hereafter.

The unlabelled antibody peroxidase-anti-peroxidase (PAP) technique was used to detect either sm-α-actin or desmin in cytospun SMCs. Prior to staining, samples were rimmed with a circle using a PAP Pen (Dimension Laboratories). This provided a hydrophobic barrier which ensured antibody solutions would remain covering the cells by preventing spreading of the solutions over the whole slide. Samples were rehydrated by submersion, for 15 minutes, in 0.05 M Tris, pH 7.6 / 1.5% (w/v) NaCl (TCL) at room temperature. Samples were then placed into humidified chambers in which all subsequent incubation steps were completed unless stated otherwise. Samples were

covered with TCL containing 3% normal goat serum and were incubated at room temperature for 30 minutes. This solution was shaken off the slides and replaced with approximately 75 µl of diluted primary mouse antibodies for sm-α-actin or for desmin (Refer to Table 4). Antibodies were diluted in TCL / 1% normal goat serum. Negative controls were incubated inTCL / 1% normal goat serum. Slides were incubated overnight at 4°C then were washed by immersing them in TCL three times. After returning slides to the humidified chamber, samples were covered with 75 µl of goat anti-mouse IgG (Sternberger Monoclonals Inc., Baltimore, MD, Code Number 501), diluted 1:100 in TCL / 1% normal goat serum. The slides were incubated for 60 minutes at room temperature then they were washed three times by immersion in TCL. After returning slides to the humidified chamber, samples were covered with 75 µl of PAP immune complex (Sternberger Monoclonals Inc., Baltimore, MD; Code number 405 - Mouse ClonoPAP) diluted 1:100 in TCL / 1% normal goat serum. Slides were incubated at room temperature for 60 minutes then they were washed three times by immersion in TCL. Samples were then covered with 100 µl of freshly prepared substrate solution (TCL containing 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) and 0.02% H_2O_2) and color was allowed to develop for 5-30 minutes depending on intensity. The staining reaction was stopped by immersing the slides in TCL and then washing three times thereafter in double-distilled water.

Following immunoperoxidase staining, the samples were counterstained with Harris' Hematoxylin (Accustain HHS-80, from Sigma Diagnostics, St. Louis, MO). The samples were covered with the staining solution, which was added dropwise, and

incubated for 2 minutes. Slides were then rinsed in running tap water for 3 minutes and then submerged in 0.5% acid alcohol (0.5% concentrated HCl in 75% ethanol) for 10 seconds and subsequently were washed gently in running tap for 3 minutes to blue the hematoxylin. Following counterstaining the samples were dehydrated by passing the slides through graded alcohols (25%, 50%, 75%, 95% and 100% respectively). Slides were then cleared in xylene (3 minutes) and coverslips were mounted over the samples using Permount mounting media.

3.3.9 Measurement of Mitogen Activated Protein Kinase Activity

Activation of mitogen activated protein (MAP) kinase was assessed in tracheal SMC cultures. For all experiments, cells were grown at 37°C in 100 mm culture dishes. All cultures used in these assays were grown to 75% confluence then proliferation was arrested by maintaining the cells in serum-free media. Growth arrested cells were stimulated by replacing serum-free media with DMEM / 10% FBS -- the effect, on MAP kinase activation, of including 100 μg/ml sodium heparin in the arrest and stimulating media was measured. After stimulating the cells, plates were randomly selected, at pre-determined times ranging from 0 to 60 minutes, to assess the temporal pattern of MAP kinase activation in cultured cells in the absence (control) and presence of heparin (experimental). For each experiment, duplicate plates were removed from the incubator at every time point for control and experimental groups; seven separate experiments were completed.

The media in each plate was discarded and then immediately washed three times

with 5-7 mls of ice cold PBS. All subsequent steps were performed at 4°C unless otherwise noted. Plates were placed on ice and protein homogenates were obtained by adding 300 µl of extraction buffer (50 mM β-glycerophosphate / 2 mM EGTA / 0.5% Triton X-100 / 1 mM sodium orthovanadate / 0.02 mg/ml aprotinin / 0.5 mM phenylmethylsulfonylfluoride (PMSF) / 0.1 mM bacitracin) and scraping the cells vigorously with a rubber policeman. The extracts obtained from both plates in each group were then pooled and transferred to a microcentrifuge tube and were then further disrupted by sonication for 30 seconds. The samples were then cenrifuged (10, 000 x *g*) for 20 minutes and the supernantant was transferred to a new tube. Protein content was determined using the BioRad Protein Assay Kit and samples were stored frozen (-80°C) until used to measure MAP kinase activation by an in-gel assay.

A modification of the "activity gel method" (Kameshita and Fujisawa, 1989) was employed to estimate MAP kinase activity. A 10% SDS-PAGE gel was prepared as described in Section 3.3.4, however, myelin basic protein (MBP) was also included (5 mg/ml) in the separating gel mixture. Cell extracts were thawed and then diluted 3:1 with loading buffer -- final loading buffer composition was 0.18 M Tris, pH 6.7 / 6% (w/v) SDS / 28% glycerol / 3% bromophenol blue. Fifteen to 20 µg of total crude protein extract was loaded into each well and the gels were electrophoresed at 24 mA per gel at 4°C for one hour. After electrophoresis gels were washed twice for one hour in 50 mM Hepes, pH 7.4 / 5 mM β-mercaptoethanol / 20% (v/v) isopropanol. The proteins in each gel were then denatured by incubating the gels in 50 mM Hepes, pH 7.4 / 5 mM β-mercaptoethanol / 6 M guanidine-HCl at room temperature for one hour. Proteins were

renatured by tranferring the gels to 50 mM Hepes, pH 7.4 / 5 mM β-mercaptoethanol / 0.04% Tween-40 and incubating, with shaking, overnight at 4°C. The renaturation solution was changed 5 times over the course of this incubation period. Each gel was then incubated, using a shaking water bath, for one hour at 37°C, in 20 ml of 50 mM Hepes, pH 7.4 / 10 mM $MgCl_2$ / 0.1 mM EGTA / 5 mM dithiothreitol / 25 mM ATP / 250 μCi [γ-^{32}P] ATP. Following this incubation the radioactivity not incorporated into MBP in the gels was removed by extensive washing in 5% (w/v) trichloroacetic acid / 1% (w/v) sodium pyrophosphate; usually 5-8 washes using 250 mls of washing solution for 20 minutes was used. Gels were then dried onto Whatman filter paper using a BioRad Gel Drying apparatus.

The incorporation of ^{32}P into the MBP substrate by the activity of kinases which had been separated in the gel was detected by autoradiography using Kodak XAR 2 Diagnostic Flm. Typically, film was exposed from 12-24 hours to obtain optimal autoradiograms. Activity was measured qualitatively by measuring the intensity of the bands on the autoradiograms using laser densitometry as described above (Section 3.3.6). In order to account for autophosphorylation reactions, samples were also separated in gels containing no substrate and, if present, contribution of this activity was subtracted from the values obtained using gels with MBP added.

The identity of the p42 and p44 MAP kinase bands was confirmed by immunoblotting as described in Section 3.3.5. ERK 1 (K-23) rabbit polyclonal anti-ERK1 (p44) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used to probe the blots -- this antibody regognizes both the p44 and p42 MAP kinase isoforms in

immunoblot protocols. Activity of p44 and p42 MAP kinase was confirmed by colocalization of the chemilumigram bands obtained after immunoblotting with those seen on autoradiograms obtained for the gel assay.

3.3.10 Analysis of Tyrosine Phosphorylation

Activation of tyrosine kinases was determined by measuring the content of phosphotyrosine in proteins extracted from cultured tracheal myocytes. The effect of heparin on activation of tyrosine kinase after serum stimulation of growth arrested tracheal SMCs was measured using immunoblot assay. Mouse monoclonal, 4G10 anti-phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY, Catalogue number: 05-321) was used to probe the blots; chemilumigrams were obtained as decribed in Section 3.3.5.

Tracheal SMC cultures were grown, in 100 mm diameter dishes with DMEM / 10% FBS, to 75% confluence, then the media was replaced with serum-free Ham's F-12 and maintained for 96 hours in order to achieve growth arrest (Refer to Section 3.2.1). Heparin was added (100 μg/ml) to half of the plates during the arrest period. Cells were stimulated to proliferate by replacing serum-free media with DMEM / 10% FBS -- heparin was included in this media for those cells which had been previously arrested in the presence of heparin; four separate experiments were completed.

To assay for tyrosine kinase activation, at predetermined time points after stimulating the cultures, two plates from each treatment group were removed from the incubator, rinsed twice with ice cold PBS and then placed on ice. Cells were lysed by

adding 500 µl of ice cold 20 mM Tris, pH 8.0 / 137 mM NaCl / 10 % glycerol / 1% Triton X-100/ 1 mM sodium vanadate / 1 mM PMSF / 0.02 mg/ml aprotinin to each plate. The plates were incubated, on ice, for 15 minutes on a rotary shaker and cells were then further disrupted using a rubber policeman. The extracts obtained from two plates were pooled, then triturated several times using a 1cc syringe equipped with a ½"-21 gauge needle. Samples were tranferred to microcentrifuge tubes then were centrifuged for 10 minutes (13,000 x *g*) at 4°C. Supernatants were tranferred to fresh tubes and protein content was determined using the BioRad protein assay dye reagent (See Section 3.3.3). Samples were then diluted with SDS sample buffer (see Section 3.3.4) and heated at 95°C for 2 minutes to fully denature proteins and prepare the samples for SDS-PAGE and immunoblotting; these samples were stored at -20°C or used immediately for phosphotyrosine immunoblot assay.

For phosphotyrosine analysis, 25 µg of crude protein was loaded per lane on 10% acrylamide gels for SDS-PAGE. Gel electrophoresis, immunoblotting and immunostaining were carried out as described in Sections 3.3.4 and 3.3.5. The anti-phosphotyrosine primary antibody was used at a concentration of 1 µg/ml and was incubated with blots overnight at 4°C. The extent of tyrosine phosphorylation in each sample was compared by measuring signal intensity in chemilumigrams using laser densitometry as described in Section 3.3.6.

3.3.11 Northern Blot Analysis

RNA was extracted from cells on culture plates using a guanidinium

isothiocyanate / phenol method (Chomczynski and Sacchi, 1987). Plates (100 mm diameter) were rinsed with PBS, and the cells were lysed in 2.0 mls GITC (4M guanidinium isothiocyanate, 0.5% n-lauryl sarcosine, 25 mM sodium citrate, pH 7.0, 0.7% β-mercaptoethanol). DNA and protein were then precipitated on ice by adding (200 mM) sodium acetate, pH 4.0 and water saturated phenol. Precipitates were pelleted and the supernatant, which contained RNA, was transferred to another tube. RNA was precipitated from the supernatant fraction by adding isopropanol and was subsequently pelleted by centrifugation. The pellet was resuspended in GITC; RNA was further purified by repeating the sodium acetate / phenol and isopropanol steps described heretofore. After the final isopropanol precipitation step, RNA was pelleted by centrifugation and re-solubilized in deionized formamide for storage at -70°C.

Northern blot analysis of the RNA samples was performed using standard methods including: fractionation of RNA on 1.5% agarose gels (7% formaldehyde), blotting to nitrocellulose membrane by capillary transfer and fixing RNA to membrane by baking at 80°C for 2 hr. Prior to detection of mRNA transcripts, blots were pre-hybridized at 42°C for 4-6 hours in 900mM NaCl, 90mM $Na_3C_6H_5O_7$, pH 7.0 (6X SSC) containing 0.5% w/v sodium dodecyl sulphate (SDS), 200μg/ml denatured salmon sperm DNA, 50% v/v deionized formamide and 5X Denhardt's reagent (5% w/v Ficoll [Type 400], 5% w/v polyvinylpyrrolidone, 5% w/v bovine serum albumin). After prehybridization, the blots were hybridized for at least 16 hours at 42°C with specific cDNA probes to detect 1) muscle and nonmuscle actins (Garfinkel et al., 1982), or 2) smooth muscle myosin heavy chain (Babij and Periasamy, 1989), and 3) glyceraldehyde

3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985). All cDNA probes were the generous gift of Dr. Muthu Periasamy, Dept. Cardiology, U of Cincinnati. Prior to hybridization, DNA probes (25ng) were random primer labelled with [α-^{32}P]dCTP (50μCi, 3000Ci/mmole) using the Prime-a-Gene Labelling System (Promega); the labelled probes were added directly to the pre-hybridization solution in which the blots were incubated. After hybridization blots were subjected to a series of washes to remove non-specifically bound probe and reduce background signal. Washes were routinely carried out twice for 5 minutes in 2X SSC at room temperature, twice for 15-30 minutes in 1X SSC, 1% w/v SDS at 65°C and finally once in 0.1X SSC for 15 minutes at room temperature. Blots were subsequently exposed to Kodak XAR-2 film at -70°C for 24-96 hours; the size and intensity of the specific mRNA bands obtained on the autoradiograms was assessed by two dimensional scanning laser densitometry using an LKB UltroScan XL densitometer. Levels of specific mRNA transcripts were obtained from 4 different primary cultures; data were normalized against the GAPDH signals for each lane.

3.4 Flow Cytometry

3.4.1 Analysis of Intracellular Proteins and DNA Content

3.4.1.1 Preparation of Single-SMC Suspensions from Intact Tissues

Samples of cervical trachea were obtained from adult, mongrel dogs and the smooth muscle was dissected clean of epithelium and adherent adventitia in ice cold,

aerated (95% O_2 / 5% CO_2), Ca^{2+}-free Krebs Henseleit. The muscle was then minced well with fine scissors and resuspended in 20 mls of Hanks Buffered Salt Solution (pH 7.4) containing collagenase (600 units/ml), type IV elastase (10 units/ml) and type XXVII Nagarse protease (1 unit/ml). The suspension was agitated in a shaking water bath at 37°C for 40 minutes after which time, remaining clumps were broken apart by trituration using a boroscillicate Pasteur pipette. The suspension was then filtered twice through 70 μm nylon mesh and cells were subsequently pelleted by centrifugation (600 x *g*, 5 minutes). Pellets were then resuspended by trituration in ice cold Ca^{2+}- and Mg^{2+}-free PBS and then centrifuged (600 x *g*, 5 minutes). This resuspension / centrifugation step was repeated then cells were resuspended in 500 μl of ice cold saline. SMCs were subsequently fixed and permeablized by adding the cell suspension, drop wise, to a tube being vortexed vigorously, which contained 5 mls of ice cold 70% ethanol. The tubes were kept on ice for 30-60 mins and then transferred to a -20°C freezer and stored overnight prior to staining.

3.4.1.2 Preparation of Single-SMC Suspensions from Primary Cultures

Cultured cells were lifted from plastic dishes using 0.05% trypsin- 0.05 mM EDTA as described in Section 3.2.1. Suspended cells were centrifuged (600 x *g*, 5 minutes) and then were resuspended in ice cold Ca^{2+}- and Mg^{2+}-free PBS and then centrifuged again (600 x *g*, 5 minutes). The resulting pellet was resuspended, by trituation using a boroscillicate Pasteur pipette, in 5 mls cold Ca^{2+}- and Mg^{2+}-free PBS. The cell suspension was filtered twice through 70 μm nylon mesh and then pelleted by

centrifugation (600 x *g*, 5 minutes). The resulting pellet was resuspended in 500 µl of ice cold saline and cells were fixed in 5 mls of ice cold 70% ethanol and stored as described in the previous section (3.4.1.1).

3.4.1.3 Fluorescent Staining of SMCs for Intracellular Proteins and DNA

Fixed cells, in 70% ethanol, were pelleted by centrifugation for 15 mins (1300 x *g*) at room temperature and the supernatant was discarded. Pelleted cells were resuspended in 3 ml of cold PBT (PBS / 1% (w/v) BSA / 0.1% Tween 20 (v/v)) by vigorously vortexing the tubes; remaining clumps were disrupted by gentle trituration using a 3cc syringe equipped with a 24g needle. Cells were counted using a haemocytometer; 2.5 x10^6 cells were aliquoted into separate tubes and each diluted to 3 mls by adding an appropriate amount of cold PBT. The cells suspensions were centrifuged (1000 x *g*, 5 min) and the resulting pellets were disrupted and resuspended by trituration, using a 1 cc syringe equipped with a 24g needle, in 1 ml of PTB which contained primary antibodies. Primary antibodies used included:

1) polyclonal rabbit anti-smMHC, a gift from Dr. Ute Gröschel-Stewart, Germany, diluted 1:1000,

2) monoclonal mouse anti-sm-α-actin, clone asm-1 (Boehringer Mannheim Canada, Laval, PQ) diluted 1:500,

3) monoclonal mouse anti- calponin, clone hCP, (Sigma Immunochemicals, St.Louis, MO), diluted 1:100, or,

4) monoclonal mouse anti-caldesmon, CALD-8, (Sigma Immunochemicals, St.

Louis, MO), diluted 1:500.

Negative controls were incubated with IgG from rabbit (for the smMHC control) or from mouse (for actin, calponin and caldesmon), diluted 1:500 in PTB.

Cells were incubated in primary antibody using constant, gentle agitation for 16-18 hours at 4°C after which, the suspensions were diluted with 5 mls of cold PTB and then the cells were pelleted (1000 x *g*, 7 mins). Pelleted cells were resuspended in 5 mls cold PTB and centrifuged again. Pellets were resuspended, by trituration using a 24g needle, in 1 ml PTB containing diluted (1:100) FITC-conjugated secondary antibody:

1) FITC-sheep anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, Code number 515-095-003), or

2) FITC-donkey anti-rabbit Ig (Amersham Life Science, Oakville, ON, Catalogue number N1034).

The cell suspension was agitated gently for 3-4 hours at 4°C then was diluted to 10 mls with PBT and centrifuged (1000 x *g*, 7 minutes). In order to stain the cells for the determination of DNA content, pellets were disrupted and resuspended, by gentle trituration with a 24 gauge needle, in 1ml of PBS / 20mM EDTA / 0.5% Tween-20 containing 15µg/ml propidium iodide (PI) and 50µg/ml RNAse. Cells were incubated for 15 minutes at room temperature then were placed on ice for about 1 hour prior to flow cytometric analysis of protein and DNA content.

3.4.1.4 Analysis of Cellular Constituents by Fluorescence Activated Cell Sorting (FACS)

Analysis of immunofluorescence was carried out using an EPICS Model 753 Fluorescence Activated Cell Sorter (Coulter Electronics, Hialeah, FL) equipped with a 488nm (500 mW) line from an argon laser. To enable the detection of FITC fluorescence (emission peak ~520 nm), a 540 ● 10 nm band pass filter was used. PI fluorescence (emission peak ~620 nm) was determined through a 610 nm long pass filter. Bivariate, fluorescence histograms of 64 channel resolution on ordinate and abscissa were collected for each sample. Tracheal SMC samples obtained from each animal were analyzed in triplicate. Forward *versus* 90° light scatter histograms were used to gate on intact cells and eliminate debris. Peak *versus* integrated PI fluorescence emission signals were used for doublet discrimination gating. Fluorescence histograms were collected for at least 5,000 cells which satisfied the light scatter and doublet discrimination gating criteria. The mean fluorescence intensities of each parameter, *i.e* channel number on the abscissa (PI fluorescence) and ordinate (FITC fluorescence), were determined for cell clusters seen in bivariate histograms using the PARA 1 analysis program (Coulter Corporation, Hialeah, FL).

3.4.2 Cell Cycle Analysis

3.4.2.1 Cell Preparation

Primary cultures of tracheal SMCs from adult mongrel dogs were prepared as described in Section 3.2.1. Only unpassaged cells were used in these studies. Cultures were grown at 37°C in DMEM / 10% FBS until 75% confluence was reached. Control

cultures were then growth arrested for 96hrs in serum-free Ham's F-12 medium supplemented with insulin (5μg/ml), selenium (5ng/ml) and transferrin (5μg/ml). Trial experiments showed that 95% of cells were in the G_0/G_1 cell cycle phase after 96 hours. Sodium heparin (100μg/ml) was also included in the arrest medium of treated cell cultures. After growth arrest, the media of control cultures was replaced with DMEM / 10% FBS to stimulate cell cycle entry of airway SMCs; the media of treated cultures was the same and also contained, from 1 to 100μg sodium heparin / ml. Cultures were returned to the incubator and thereafter plates for each group were randomly selected at predetermined time points (from 0 to 40 hours) to assess the cell cycle distribution of the SMCs. Five experiments, using different primary cultures in each, were carried out.

At each time point, cells were lifted from culture plates by trypsinization (refer to section 3.2.1). The cells from two plates for each treatment were pooled, then transferred to pre-chilled tubes and pelleted (5 min., 800 x *g*). Supernatants were discarded, then pellets were resuspended in ice cold PBS. Cell suspensions were pelleted again, supernatants were discarded and then pellets were resuspended in 200μl of ice cold saline. Cells were fixed by adding the suspensions, drop wise, to 5mls of ice cold 70% ethanol while vortexing vigorously. Fixed cell suspensions were stored for at least 24 hrs at -20°C until used for analysis of cell cycle distribution; maximum storage time used was 5 days.

3.4.2.2 FACS Analysis of Cell Cycle Distribution - PI Staining

Fixed cells were pelleted (10 minutes, 1000 x *g*) and then were stained for DNA

by resuspending in 1ml PBS / 20mM EDTA / 0.5% Tween-20 containing 15 μg/ml PI and 50μg/ml RNAse (refer to Section 3.4.1.3). Cell clumps were eliminated first, by triturating the cell suspension gently using a syringe equipped with a 24 gauge needle and, then by filtering through 70 μm nylon mesh. Cells were stained for about one hour at RT prior to initiating flow cytometric analysis of DNA content using an EPICS Model 753 Fluorescence Activated Cell Sorter (Coulter Electronics, Hialeah, FL) with a 488 nm (500 mW) line from an argon laser. Forward *versus* 90° light scatter histograms were used to gate on intact cells and eliminate debris, while peak *versus* integrated PI derived fluorescence signals were used for doublet discrimination gating. PI fluorescence was determined through a 610 nm long pass filter. Fluorescence histograms of 256 channel resolution were collected for at least 5,000 cells which satisfied the light scatter and doublet discrimination gating criteria. The distribution of cells in phases G_0/G_1, S, and G_2 of the cell cycle was determined using the PARA 1 DNA analysis program (Coulter Corporation, Hialeah, FL).

3.4.2.3 Cell Preparation - Bromodeoxyuride (BrdU) Incorporation

These studies were carried out to obtain a more detailed analysis of the effects of heparin on cell cycle ditribution during periods of rapid replication. Primary cultures of canine tracheal SMCs were prepared as described in Section 3.2.1. Only unpassaged cells were used. Cultures were grown at 37°C in DMEM / 10% FBS until 75% confluence was reached. Cultures were then synchronized by growth arrest and were subsequently stimulated to proliferate by adding 8 mls of DMEM / 10% FBS to each 100

mm plate, as described in Section 3.4.2.1. Heparin was included in half of the plates during growth arrest and was also included in the DMEM /10% FBS that was subsequently added to the same plates; only one concentration of soduim heparin, 100 μg/ml, which gave maximum inhibition of SMC replication, was used in these studies.

Earlier studies in which only PI staining had been used, revealed that cells completed first mitosis approximately 32-36 hours after serum stimulation. Since it was our intention to examine cell cycle progression during rapid growth periods cells were studied between 68 and 72 hours after serum stimulation. Sixty eight hours after serum stimulation, 2 mls of DMEM / 10% FBS supplemented with 50 μM 5-bromo-2'-deoxyuridine (BrdU) / 50 μM 5-fluoro-2'-deoxyuridine (BrdU Labelling Solution, Amersham Life Science, Oakville, ON; Catalogue number RPN.201), was added to each control plate. For plates which were receiving heparin treatment, sodium heparin (100 μg/ml) was also included in the labelling media added to the proliferating plates. The final concentraction of BrdU in each plate was 10 μM. Plates were incubated at 37°C for 4 hours after addition of the labelling solution. After this period (72 hours after the initial serum stimulation), culture plates were removed from the incubator and the cells were lifted by trypsin-EDTA treatment to make a single cell suspension as described in Section 3.2.1. The cells were then fixed in 70% ethanol and stored overnight at -20°C as described in Section 3.4.2.1. Five experiments, using different primary cultures in each, were carried out.

3.4.2.4 FACS Analysis of Cell Cycle Distribution - PI Staining / BrdU

Incorporation

Fixed cells were pelleted by centrifugation (1200 x *g*, 12 minutes, 4°C). Supernatant was discarded and the cell pellets were loosened by adding 500 µl of ice cold saline then vortexing vigorously for 30 seconds. Nuclear DNA was then denatured by adding 500 µl of 4 M HCl/ 1% Triton X-100 -- cells clumps were disrupted with gentle trituration using a syringe equipped with a 24 guage needle. The cell suspension was incubated for 30 minutes at room temperature after which 1 ml of fresh saline was added and the cells were pelleted with centrufugation (1000 x *g*, 5 minutes, room temperature). After discarding the supernatant the cell pellet was loosened by vortexing vigorously for 30 seconds, then the cells were neutralized by adding 1.5 mls of $Na_2B_4O_7$, pH 8.5. Cells were dispersed by trituration using a 24 guage needle. Cells were collected by centrifugation (1000 x *g*, 5 minutes, room temperature) and the cells in the resulting pellet were washed once by adding 1 ml of cold PBS / 1.0% BSA / 0.5% Tween-20 and triturating with a Pasteur pipette.

For immunostaining of the BrdU incorporated into nuclear DNA during S-phase, cells were collected by centrifugation and resuspended in 100 µl of cold PBS / 1% BSA/ 0.5% Tween-20 by trituration using a 24 guage needle, the cell suspensions were transferred to microcentrifuged tubes, then 20 µl of monoclonal, FITC-mouse anti-BrdU was added to each tube. Cells were incubated, covered in aluminium foil, in primary antibody solution for 2 hours at room temperature after which the suspension was tranferred to a 15 ml centrifuge tube and subsequently diluted by adding 3 mls of PBS / 1% BSA / 0.5% Tween-20. After vortexing the solution vigorously, cell suspensions

were filtered twice, through 70 μm nylon mesh and cells were subsequently pelleted by centrifugation (1000 x *g*, 7 minutes, room temperature). Supernatants were discarded and the cells were stained for DNA content by resuspending the SMCs in PI staining solution as described in Section 3.4.1.3.

Analysis of immunofluorescence was carried out using an EPICS Model 753 Fluorescence Activated Cell Sorter (Coulter Electronics, Hialeah, FL) equipped with a 488nm (500 mW) line from an argon laser. To enable the detection of FITC fluorescence (emission peak ~520 nm), a 540 ± 10 nm band pass filter was used. PI fluorescence (emission peak ~620 nm) was determined through a 610 nm long pass filter. Bivariate histogram data collection and analysis was carried out as carried out as described in Section 3.4.1.4. Gating parameters and collection and anlysis of PI-staining, DNA histograms were performed as described in Section 3.4.2.2.

3.4.3 Cell Sorting

Canine tracheal smooth muscle was obtained and cleaned as described for preparation of primary SMC cultures in Section 3.2.1. Cells were enzymatically dispersed from intact tissue under aseptic conditions, as described in Section 3.2.1. Tissue was minced using scissors and resuspended in a solution consisting of HBSS / 10 mM taurine / 400 units/ml collagenase (Life Technologies - Gibco/BRL, Burlington, ON) / 5 units/ml Type IV elastase (Sigma Chemicals, St. Louis, MO) / 1 unit/ml Nagarse protease (Sigma Chemicals, St. Louis, MO) / 100 mg/ml streptomycin / 100 units/ml penicillin / 50 μg/ml gentamycin. The suspension was incubated for 40 minutes at 37°C

in a shaking water bath after which cell clumps were disrupted by trituration using a Pasteur pipette. The cell suspension was filtered twice through 70 µm nylon and then diluted 1:1 with ice cold DFTA (DMEM / 10 mM taurine / 10% FBS / 100 mg/ml streptomycin / 100 units/ml penicillin / 50 µg/ml gentamycin). Cells were pelleted by centrifugation (600 x *g*, 5 minutes, room temperature) and resuspended in fresh, cold DFTA by trituration using a Pasteur pipette. Cell number was then determined using a hemocytometer. The cells were centrifuged again and then resuspended in an volume of DFTA which yielded a cell suspension consisting of 2×10^6 cells/ml. The cells were then filtered once more through 70 µm nylon mesh, aliquoted into new, sterile tubes and placed on ice for transport to the flow cytometer lab for cell sorting.

All flow lines, chambers and tubes used for flow cytometry were sterilzed with 70% ethanol or by autoclaving where appropriate. Flow cytometry was performed using an EPICS Model 753 Fluorescence Activated Cell Sorter (Coulter Electronics, Hialeah, FL). Forward and 90° light scatter of a 488nm (50 mW) line from an argon laser were collected. Forward *versus* 90° light scatter histograms were used to gate on intact cells and eliminate debris. Sorting parameters were set manually based on the distribution of cells observed on the bivariate light scatter histograms obtained for each sample. Sorting was carried out at rates between 500 and 1200 cells/sec. The cells in the sorting tube were agitated every 5-10 minutes in order to resuspend the SMCs which settled in the the tube during the experiment. Sorted cells were captured in pre-cooled tubes containing 5-7 mls of DFTA; typically $1 - 2 \times 10^6$ of each cell popultion were obtained for subsequent analysis. Sorted cells were stored on ice until used for cytospin analysis or for seeding in

cell culture.

3.5 Statistical Analyses

All measurements made from chemilumigrams or autoradiograms using laser densitometry were completed in triplicate from samples obtained from 4-6 different animals.

Values reported in graphs and tables for all data obtained represent means ± standard errors. Comparisons between idividual mean values from different treatments on SMCs and, between mean values of parameters measured by FACS analysis were done using unpaired two-tailed Student's *t*-test. Comparisons between multiple values from different treaments on SMCs were done by one-way Analysis of Variance combined with Duncan's new multiple range test. Statistical significance for all analyses was accepted at $P<0.05$.

4. MARKERS OF AIRWAY SMOOTH MUSCLE CELL PHENOTYPE

4.1 BACKGROUND

Specific proteins, associated with the function of contractile or cytoskeletal cyto-structures, have been identified as markers of the cytodifferentiation state of vascular SMCs (Shanahan et al., 1993). Quantitation of these marker proteins *in situ*, using immunohistchemical methods, has demonstrated that arterial SMCs modulate their phenotype during development and in the pathogenesis of atherosclerosis (Zanellato et al., 1990; McHugh, 1995). Vascular SMC cultures have been used to identify changes in phenotypic maker protein expression -- in primary culture, mature vascular myocytes spontaneously and reversibly, modulate from a contractile phenotype to a proliferative, secretory phenotype in primary culture in the presence of serum (Chamley-Campbell et al., 1979).

In culture, proliferative vascular SMCs exhibit a reduction in smooth muscle α and γ-actins (Shanahan et al., 1993), smooth muscle SM1 and SM2 myosin heavy chain isoforms (Rovner et al., 1986), the smooth muscle regulatory 20kDa myosin light chain isoform (Monical et al., 1993), the thin filament associated regulatory proteins calponin (Shanahan et al., 1993) and *h*- caldesmon (Ueki et al., 1987), the sarcoplasmic reticulum membrane associated protein, phospholamban (Shanahan et al., 1993), and the cytoskeletal proteins, desmin (Kocher et al., 1985) and meta-vinculin (Belkin et al., 1988). Furthermore, phenotypically modulated, cultured vascular SMCs demonstrate an increase in expression of foetal and non-muscle isoforms of several proteins including, β-isoactin (Fatigati and Murphy, 1984), non-muscle myosin heavy chains (Rovner et al., 1986), the non-muscle regulatory myosin light chain isoform (Monical et al., 1993), *l*-

caldesmon (Ueki et al., 1987), and the intermediate filament associated protein, vimentin (Kocher et al., 1985).

Airways smooth muscle (ASM) is the known effector of episodic, acute bronchospasm associated with asthma. Hyperplasia and hypertrophy of the medial myocytes comprising airway smooth muscle are a feature of airways remodelling in chronic asthma (Ebina et al., 1993). Fairly well defined structural changes associated with airways remodelling have been identified from autopsy samples of patients dying of status asthmaticus and from biopsy samples from subjects with milder disease (Laitinen et al., 1985; Ebina et al., 1993). These changes include infiltration with eosinophils and macrophages (Dunnill, 1960), oedema, epithelial denudation (Laitenen et al., 1985), hyperplasia of mucous secreting glands (Dunnill, 1960), thickening of the basement membrane (Roche et al., 1989; Djukanovic et al,. 1990), subepithelial collagen deposition (Cutz et al., 1978), subepithelial accumulation of myofibroblasts (Brewster et al., 1990), and substantial thickening of the medial smooth muscle layers (Dunnill et al., 1969; Hossain et al., 1973; James et al., 1989; Ebina et al., 1990 and 1993).

Molecular characterization of the phenotypic state of ASM has not been reported, perhaps due to a lack of reports which identify definitive markers for airway SMC phenotype. Primary cultures of airways smooth muscle cells (SMCs) from human and animal sources are now in wide use for the purpose of characterizing the mitogenic effects of various inflammatory mediators and the regulation of receptor-mediated myocyte responses. However, as was recently noted in a review by Hall and Kotlikoff (1995), no reports have systematically documented the morphological and biochemical

changes which occur in airway SMCs in primary culture.

Growth characteristics and gross morphology of airway SMC in primary culture have been reported in some studies; it appears that changes occuring in culture for airway SMC may mimic those described for vascular SMC (Chamley-Campbell et al., 1979). Two studies (Avner et al., 1981; Tom-Moy et al., 1987), in which morphological changes of canine tracheal myocytes in sub-confluent primary cultures were examined using transmission electron microscopy and immunofluorescence staining, revealed that the number of contractile filaments and the intensity of staining for smooth muscle myosin, decreased with time in culture. Similarly, Panettieri et al. (1989) reported that the number of smooth muscle α- and γ-actin positive filaments in cultured human tracheal myocytes was significantly decreased in the third passage and confirmed these findings semi-quantitatively using an immunoblot assay. No other quantitative or semi-quantitative studies of the changes in content of contractile and cytoskeletal proteins in cultured airway SMCs have been reported.

One the basis of the information summarized heretofore a specific hypothesis is proposed:

SPECIFIC HYPOTHESIS -- Airway smooth muscle cells undergo phenotypic modulation in primary culture, a phenomenon defined by specific, measurable changes in a number of biochemical and morphological properties.

Definitive markers of airways myocyte phenotype can be used to characterize the phenotypic state of SMCs in the airways of patients suffering from asthma and of cultured airway SMCs. This ability will enable elucidation of physiological properties of

both cultured airway SMCs and in determining the extent to which the phenotype presented by airway SMCs contributes to the pathology of airway diseases.

4.2. Specific Aims

1. To establish canine airway SMC primary cultures and characterize cellular morphology and growth properties *in vitro*.

2. To compare the content of specific smooth muscle proteins in cultured SMC of divergent phenotypes in order to establish the reliability of using these proteins as phenotype markers.

3. To compare the profile of phenotypic marker proteins in SMC from smooth muscles differing in physiologic properties.

4.3 Experimental Design

Canine tracheal SMC cultures were established using conventional techniques for the enzymatic dispersal of cells, standard culture media and conditions (Section 3.2.1). Growth of cells in culture was measured by cell counting at specific time points after cells were plated. Fluorescent immunocytochemical analysis of proteins markers in airway SMCs was carried out on cells grown on coverslips as described in Section 3.3.7.2.

In order to measure changes in protein marker content, associated with

phenotypic modulation of SMCs in culture, cell lysates were obtained in triplicate at specific time points from culture plates as described in Section 3.3.1. Cell lysates were also obtained from MCH6 fibroblasts using the same methods. The contents of specific proteins in the cell lysates were determined by Western blot analysis and laser densitometry as described in Sections 3.3.3 - 3.3.6. A total of six separate experiments were conducted for each protein marker measured. For analysis of myosin and actin mRNA content in cultured cells total RNA was extracted from plates in triplicate at specific time points (24 hours after plating and 7 days after plating) and pooled for Northern blot analysis (Section 3.3.11). Four experiments, using different primary cultures, were completed.

To assess the physiological relevance of the markers which appeared to be differentially expressed in a phenotype-specific fashion in airway SMCs, we compared their distribution between two pulmonary smooth muscle types, tracheal and pulmonary arterial, which are known to differ in contractile and secretory properties. The protein constituents in each tissue were compared by preparing cell lysates obtained from freshly dispersed SMCs (Sections 3.3.1 and 3.3.2) and subjecting them to immunoblot analysis as described in Sections 3.3.3-3.3.6. Six separate experiments were carried out to compare the isolated tracheal and pulmonary arterial SMC protein constituents.

4.4 RESULTS

4.4.1 Growth Characteristics and Morphology

Typically, 8 - 12 x 10^6 SMCs were isolated from the canine trachealis tissues (500

mg - 1 gm) used for generating primary cultures in these studies. Cell viability, assessed by trypan blue staining, ranged from 65-85%. The cell number in each culture dish began increasing approximately 4 days after plating until confluence was reached 10 days after initial seeding (Figure 4, upper panel). The rate of increase in cell number diminished post-confluence. At confluence cultures exhibited a "hill-and-valley" pattern which is common of SMCs in confluent cultures (Chamley-Campbell et al., 1979). The cells displayed morphological characteristics typical of cultured SMCs (Chamley-Campbell et al., 1979), appearing spindle or ribbon-shaped with a large, oval, central nucleus marked by distinct nucleoli and having well-defined, perinuclear granules and/or vesicles (Figure 4A). Immunofluorescent staining revealed that greater than 95% of the cells in the primary cultures were positive for both smooth muscle-α-actin and smooth muscle-specific myosin heavy chain (sm-MHC) six days after initial seeding (Figures 4B and 4C).

Cells stained for sm-MHC were frequently characterized by a punctate staining pattern which included intense perinuclear fluorescence (Figure 4C). Immunostaining with antibodies that recognized either sm-MHC or the A isoform of nm-MHC demonstrated that these proteins were localized to filaments within the cytoplasm of the cells (Figure 5A and 5B). Structures which are positive for sm-MHC can be seen in tracheal myocytes through three subcultures, however, the intensity of staining is greatest in confluent plates and diminishes with each passage. Conversely, nm-MHC staining increases during the course of the initial primary culture and that level is maintained in subcultures thereafter. In addition, staining of filamentous structures using an antibody

which recognizes the A isoform of nm-MHC appears to be intermittent (Figure 5C) suggesting that this isoform may be localized only within specific segments of the filaments.

Filamentous structures similar to those seen with sm-MHC staining can be seen if cells are immunostaining with antibodies which recognize either sm-α-actin or caldesmon (Figure 6). Actin is the primary constituent of thin filaments and caldesmon is a thin filament-associated regulatory protein; dual staining of cells for actin and caldesmon confirms that the proteins co-localize to the same cytoskeletal structures. Since the structures visualized by these methods were present in phenotypically modulated, cultured airway myocytes they likely represent cytoskeletal structures involved with changes in cell shape and cytokinesis rather than contractile myofilaments.

The cultured cells possess an extensive array of cytoskeletal intermediate filaments which could be visualized easily using immunofluorescent staining with antibodies against vimentin and α-actinin (Figure 7). Dense bands, dense bodies and focal ahesion plaques are rich in α-actinin, staining with anti-α-actinin revealed a punctate staining pattern which is likely associated with the distribution of these cellular structures (Figure 7).

The general profile of proteins in isolated tracheal myocytes varied considerably with time in culture (Figures 8A and 8B). Prominent bands around 43kDa and 200kDa were visible in freshly isolated cells using SDS-PAGE and Coomassie blue staining. Treatment, for 96 hours, of proliferating cells with, histamine, TPA, or with carbamyl choline did not alter the protein banding pattern normally seen for SMCs 7 days after

initiating primary cultures. Additionally no significant differences in the rate of increase in cell number per dish were seen after treatment with any of these compounds. Treatment, beginning five days after seeding, with 5-fluoro-2'-deoxyuridine, for 96hrs in the presence of 10% FBS virtually blocked any increase in cell number. Though the staining intensities of all bands on SDS-PAGE gels were decreased after this treatment no change in the distribution of the major bands was seen in these cells compared to the other treatments employed.

4.4.2 Temporal Expression of Marker Proteins

Western immunoblot assays for semi-quantitative measurement of the temporal changes in marker protein content of cells in primary culture revealed that the content of sm-α-actin and sm-MHC, declined to less than 25% of that in freshly isolated cells within 3 days of seeding (Figure 9). This time corresponded with the pre-proliferative phase of the growth curve (Figure 4). No change was seen in the abundance of putative markers of non-contractile SMCs, vimentin and nm-MHC, over the same time period. Minimum levels of α-actin and sm-MHC were reached during proliferative stages (Figure 9). The content of sm-α-actin increased when plates reached 75% confluence whereas sm-MHC remained at minimal levels until plates reached confluence. Vimentin and nm-MHC content increased at the onset of the proliferation and continued increasing until the cultures reached confluence (day 10). At confluence (day 10) the fractional content of these proteins compared to freshly isolated myocytes (which would have a value of 1) were 0.54±0.06 for α-actin, 0.15±0.03 for sm-MHC, 2.09±0.03 for vimentin

and 5.38±0.16 for nm-MHC. The content of sm-MHC increased post-confluence reaching a maximum fractional content of 0.55±0.13 compared to isolated cells. Vimentin content declined post-confluence and returned to levels similar to those of uncultured cells. Conversely, elevated nm-MHC content was maintained in post-confluent cultures 5 to 7-fold higher than that measured in uncultured cells.

Temporal patterns of sm-MHC and sm-α-actin expression were also measured in passaged cells. Qualitative similarities, including re-expression of these marker proteins post-confluence, were seen in passage 1 through 3 with to the pattern demonstrated in unpassaged cultures. However, the ability of the SMCs to express smMHC and α-actin was compromised with successive passage. Little or no smMHC was present in cultures of tracheal myocytes beyond passage 4. The cells still expressed α-actin after 9 passages, though the maximum seen at this time was <25% of that seen in freshly dispersed tracheal myocytes.

4.4.3 Marker Proteins in Freshly Dispersed and Cultured Airway SMCs

We compared the contents of other protein markers (Table 5) in contractile, freshly isolated tracheal myocytes with those in tracheal SMCs in primary culture. To assess differences between SMC's of divergent phenotypes, the contents of protein markers, normalized to protein load, in proliferating cells (7 days after seeding) were estimated using Western immunoblot assays and compared to acutely dissociated cells. The densitometric values obtained from chemilumigrams and the total protein loads onto the SDS-PAGE gels used are listed in Table 5. The ratio of values obtained for the

content of a protein in isolated tracheal SMCs and in proliferating, 7-day cultured tracheal SMCs (TSMC(d7)) is depicted in Figure 10. It should be noted that differences in specific affinities of the antibodies employed, negate comparisons of the absolute content of two different proteins in the cell populations.

Proteins which were decreased in content (Table 5) by more than 75% in cultured cells included sm-MHC, calponin, and desmin. Other proteins which were less abundant ($P<0.05$) in proliferating myocytes included, sm-α-actin, myosin light chain kinase, ß-tropomyosin and *h*-caldesmon. Conversely, contents of nm-MHC, α/ß-protein kinase C and CD44, an integral membrane glycoprotein which interacts with extracellular matrix components including hyaluronan, fibronectin, collagen and laminin (Jalkanen et al., 1991), were increased 5-fold or greater in proliferating cultured cells compared to isolated SMCs. The contents of both vimentin and *l*-caldesmon were also significantly greater ($P<0.05$) in the 7-day cultured cells.

The abundance of sm-MHC mRNA was decreased by 80% in proliferating cells (Figure 11). This alteration correlates well with the loss in sm-MHC protein, measured by immunoblotting, in the same cells (Figure 10). Northern blots demonstrated that the abundance of transcripts coding for different actin isoforms was altered in proliferating tracheal myocytes (Figure 11). Transcript coding for sm-α-actin (1.7 kilobases) comprised about 75% of the total actin mRNA present in mature tracheal SMCs but represented only 25% of the total actin mRNA in proliferating cells. In contrast, expression of mRNA coding the non-muscle actin isoforms, ß- and γ-, was elevated in cultured cells compared to isolated SMCs.

4.4.4 Marker Protein Expression in MCH6 Fibroblasts

Western blot analysis was utilized to determine the content of a variety of proteins in sub-confluent, proliferative MCH6 fibroblasts and compared to that measured in sub-confluent primary cultures of tracheal SMCs (Figure 12). Proteins which were undetectable in the MCH6 cells included sm-MHC, calponin and desmin. Significant quantities of the putative smooth muscle specific marker proteins, sm-α-actin and *h*-caldesmon, were detected in cell lysates from MCH6 cultures. Furthermore, two bands of *h*-caldesmon were detected at ~158 and 148 kDa in MCH6 lysates. This pattern was not seen in lysates obtained from primary cultured SMCs or in tracheal SMC cultures maintained through 12 or more passages. With the exception of this *h*-caldesmon doublet no protein examined was present exclusively in MCH6 cells in culture.

4.4.5 Marker Proteins in Freshly Dissociated Tracheal and Arterial SMCs

In order to assess the relationship between marker protein content and tissue properties we measured and compared the abundance of marker proteins in freshly dissociated SMCs obtained from canine trachea and from canine pulmonary artery. Figure 8B illustrates the Coomassie blue staining pattern of protein lysates from acutely dissociated pulmonary arterial SMCs and tracheal SMCs. The patterns obtained appeared to be quite similar excepting the appearance of a more intense band around 200kDa in the tracheal samples. Western blot analysis revealed that the content of proteins in the trachea and the pulmonary artery differed considerably (Figure 13). Isolated tracheal myocytes contained greater amounts ($p<0.05$) of contractile proteins,

including sm-MHC, sm-α-actin, calponin, myosin light chain kinase, ß-tropomyosin and *h*-caldesmon (Table 5 and Figure 13). Conversely, pulmonary arterial SMCs were enriched ($p<0.05$) in nm-MHC content compared to tracheal myocytes. The cell populations did not differ in content of intermediate filament-associated proteins, vimentin and desmin, nor did they differ in *l*-caldesmon content. No qualitative differences in the banding patterns for other proteins on Western blots were observed.

TABLE 5. Abundance of marker proteins determined by Western blot analysis.

Protein‡	Protein Abundance# (Arbitrary units)†				
	TSM	TSMC(d7)	PASM	TSM/TSMC(d7)	TSM/PASM
sm-α-actin (5)	8.4±1.3	2.8±0.5*	5.3±1.3*	3.0±0.4	1.6±0.5
h-caldesmon (10)	2.6±0.9	1.0±0.2*	1.0±0.4*	1.7±0.4	1.7±0.5
l-caldesmon (10)	1.5±0.4	2.5±0.5*	2.1±1.0	0.6±03	0.7±0.4
calponin (10)	1.6±0.5	0.1±0.04*	0.6±0.2*	13.2±3.9	2.7±0.8
CD44 (10)	0.6±0.2	2.9±0.6*	n.d	0.2±0.06	n.d.
desmin (10)	0.4±0.2	0.1±0.04*	0.3±0.1	4.5±1.8	1.3±0.2
nmMHC (7.5)	0.6±0.1	3.0±0.3*	1.5±0.3*	0.2±0.1	0.4±0.1
smMHC (5)	3.1±0.6	0.3±0.1*	1.6±0.5*	10.1±3.4	2.0±0.7
MLCK (7.5)	3.9±0.5	2.1±0.5*	2.0±0.7*	1.9±0.3	1.9±0.7
PKC (10)	0.2±0.1	1.9±0.7*	n.d.	0.1±0.04	n.d.
tropomyosin (10)	2.8±0.6	1.1±0.3*	1.3±0.4*	2.5±0.6	2.1±0.8
vimentin (7.5)	0.2±0.1	0.5±0.2*	0.3±0.1	0.4±0.2	0.8±0.2

Abbreviations: TSM- isolated tracheal smooth muscle cells; TSMC(d7)- cultured tracheal smooth muscle cells 7 days after seeding; PASM- pulmonary arterial smooth muscle cells.

‡ Numbers in brackets indicate the amount of crude homogenate protein (μg) that was loaded onto gels for Western blot analysis; this value was used to normalize densitometric data. # Determined by laser densitometry of chemilumigrams developed for each protein using Western blot analysis. † Results in each group represent mean ± SEM of six experiments. * Significantly different ($P<0.05$) from the abundance determined for isolated TSM. n.d. Not determined

FIGURE 4. Growth characteristics of cultured canine tracheal smooth muscle cells.

The top panel illustrates a typical growth curve for cells grown in DMEM containing 10% FBS; initial seeding density was 5 x10^3 cells/cm^2. Morphological characteristics of the cells 6 days after seeding were observed using **(A)** phase contrast microscopy, **(B)** immunofluorescent staining using a primary anti-sm-α-actin antibody and a Cy3-conjugated secondary antibody, and **(C)** immunofluorescent staining using a primary anti-sm-MHC antibody and a FITC-conjugated secondary antibody.

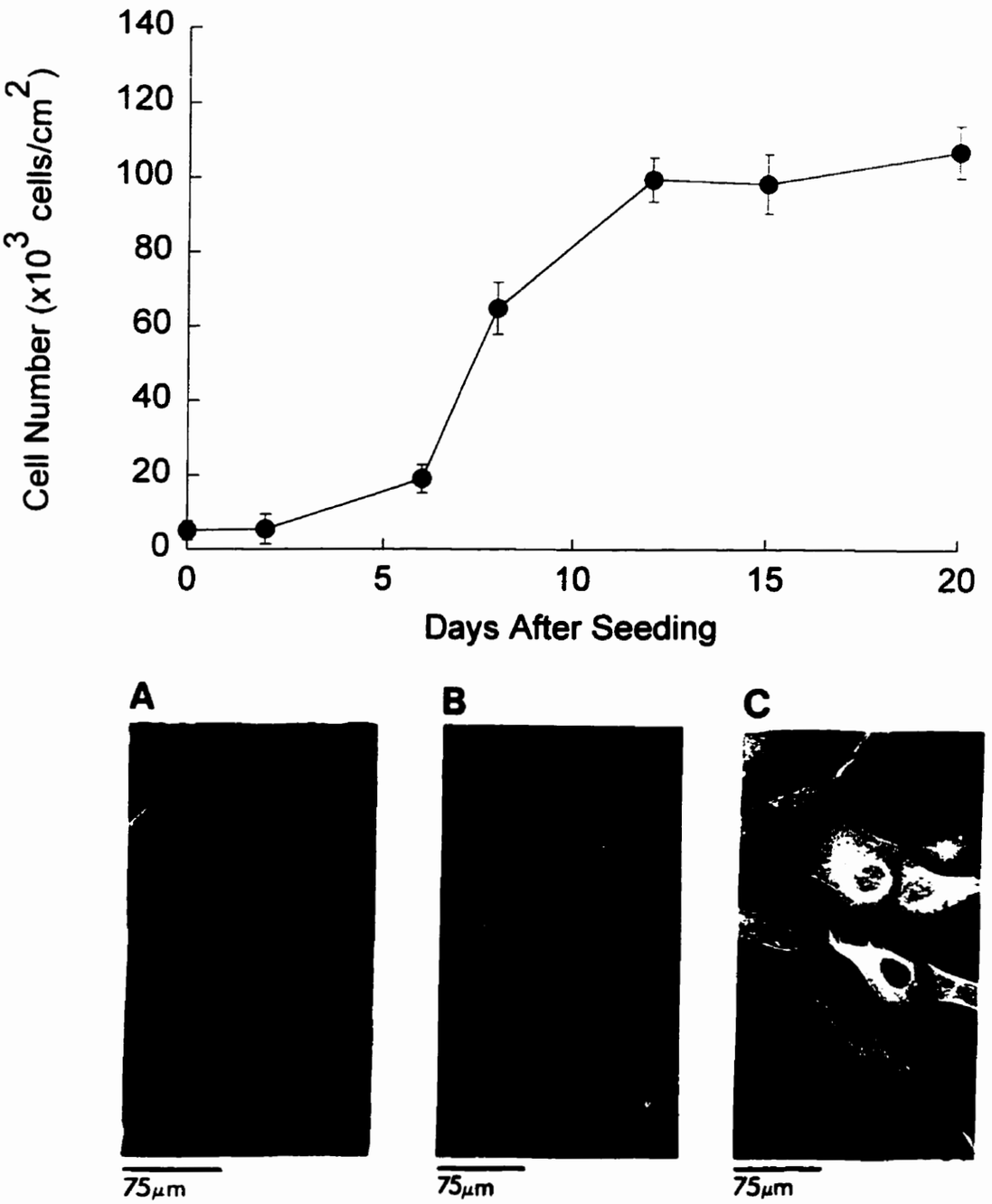

140
120
100
80
60
40
20
0
Cell Number (x10^3 cells/cm^2)
0
5
10
15
20
Days After Seeding
A
B
C
75μm
75μm
75μm

FIGURE 5. Indirect immunofluorescense staining, using FITC-conjugated secondary antibodies, of myosin in cultured canine tracheal SMC.

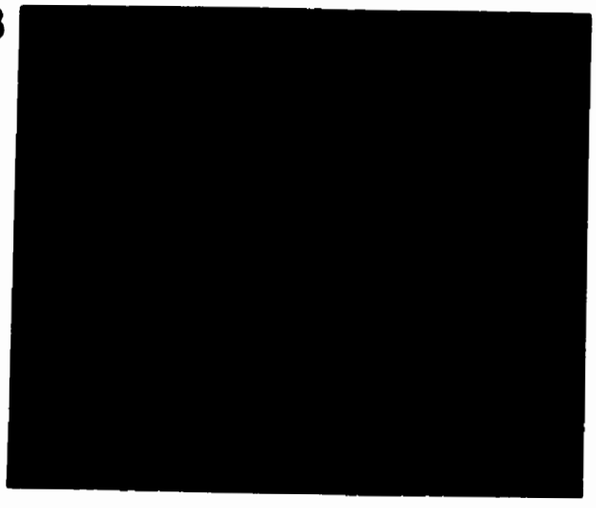
(A) A cell stained five days after initial plating for sm-MHC. The primary antibody employed recognized both the 200kDa and 204kDa isoforms. Numerous myosin labelled myofilaments are present and are oriented along the long axis of the myocyte; note that the number of filaments appears to be greater at the perimeters of the cell. (x 250).

(B) Cells stained seven days after initial plating using a primary antibody against the 196 kDa non-muscle myosin heavy chain isoform (nmA-MHC). Filaments running the full length of the long axis of the cells can be seen. (x200).

(C) Higher magnification (x400) of seven-day old myocytes stained for nmA-MHC. Note that the filaments stain in a regular, periodic pattern suggesting that they may be organized into discrete, repeating units.

A

B



C

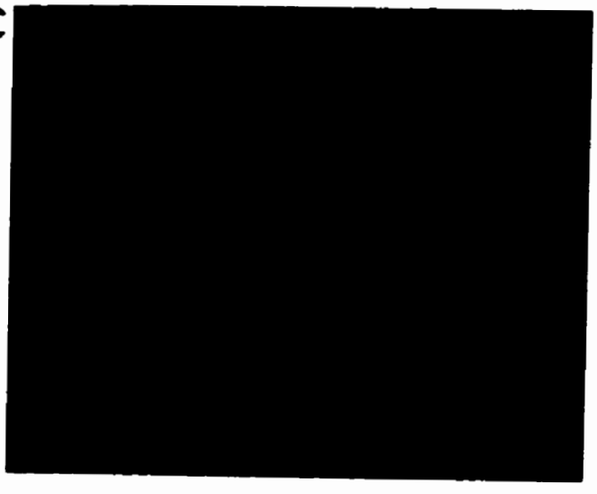


FIGURE 6. Indirect immunofluorescent staining, using FITC-conjugated secondary antibodies, of smooth muscle-α-actin and *h*-caldesmon in canine tracheal myocytes in primary culture.

(A) A cell stained for sm-α-actin five days after initial plating. Note that all cells stain positively; actin myofilaments can be clearly seen which run the full length of the long axis of the cells. (x200).

(B) Cells similar to those in plate A but stained for the actin filament-associated protein, *h*-caldesmon. Caldesmon positive filaments demonstrate a similar orientation and size as those seen when cells are stained for sm-α-actin.

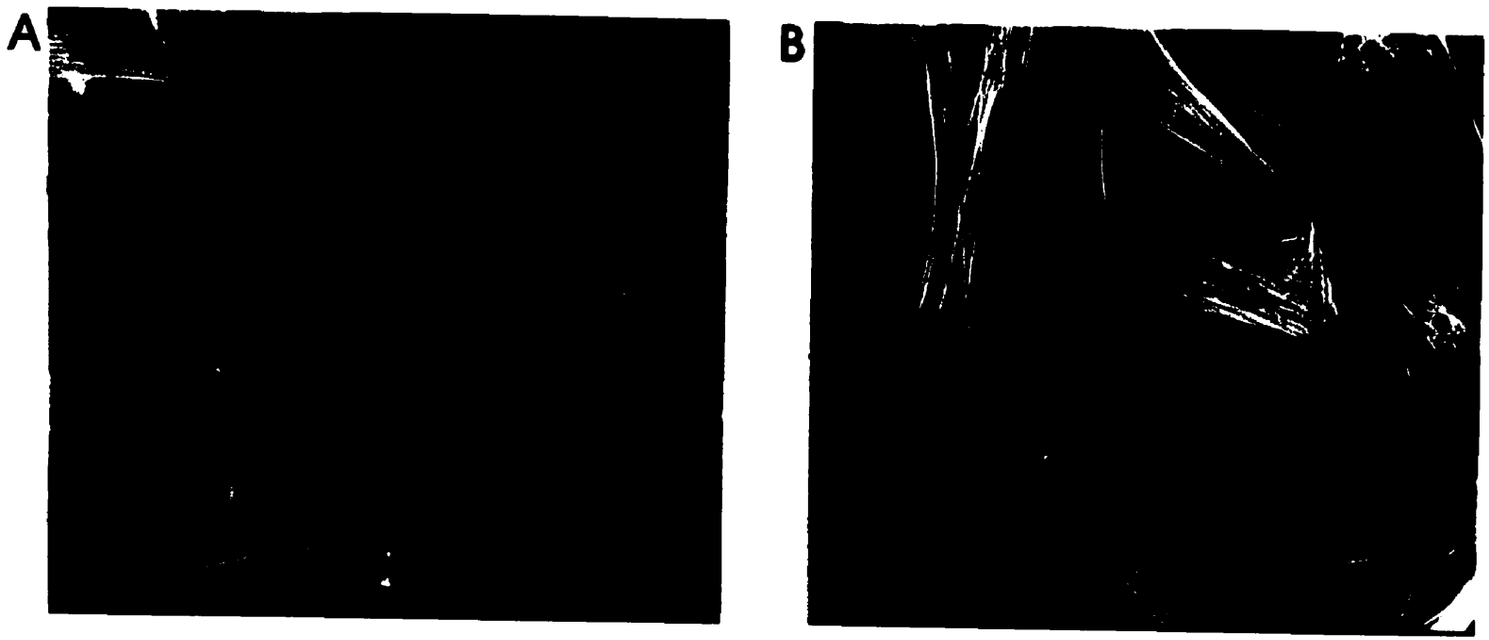

A
B

FIGURE 7. Demonstration of dense bodies / focal adhesion plaques and intermediate filaments in cultured canine tracheal SMC using indirect immunohistochemical techniques.

(A) Recently divided myocytes, six days after plating, stained for α-actinin, a structural protein constituent of dense bodies. Dense bodies appear as bright foci of staining in the cell's cytoplasm. Bright staining can be seen at the site of sharp filopodia along the sarcolemmal edges; these are sites of cell attachment to the culture plate and likely correspond to membrane dense focal adhesion plaques. Extremely bright peri-nuclear α-actinin staining can also be seen and is a common feature of cultured airway SMC.

(B) Tracheal SMCs, stained for vimentin using a commercial monoclonal antibody, four days after plating in primary culture. Vimentin is a stuctural protein constituent of intermediate filaments. The cells demonstrate an extensive intermediate filament network which extends throughout the whole cytoplasm and surrounds the nucleus

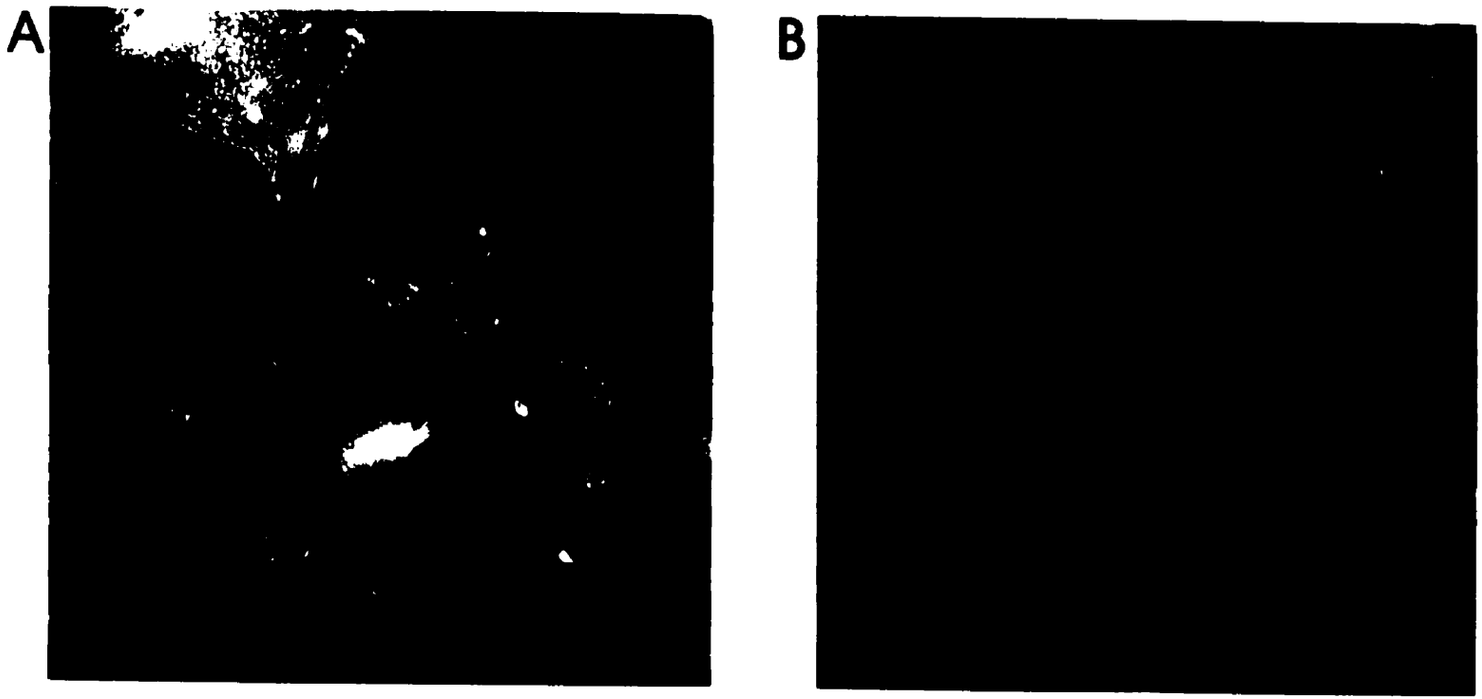
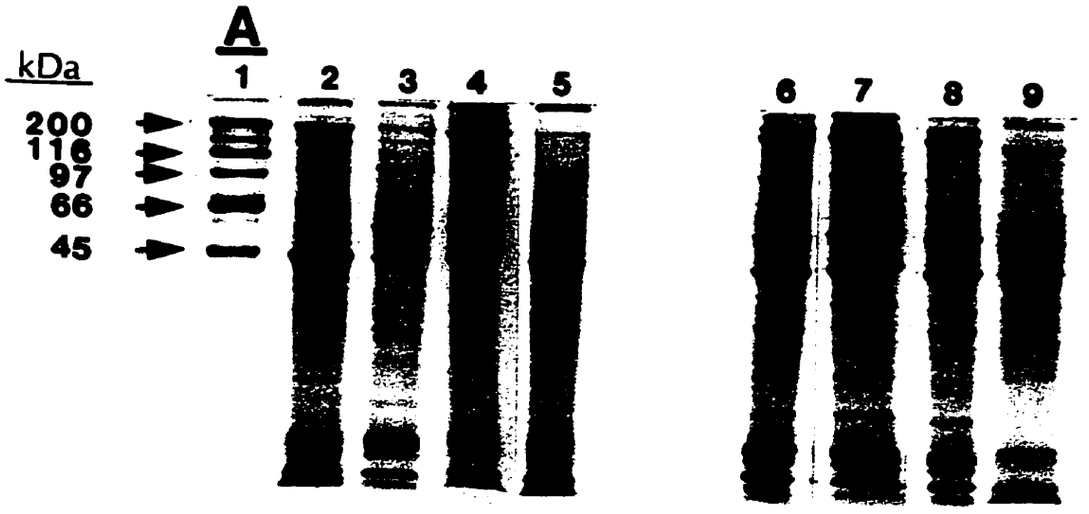

A
B

FIGURE 8. Coomassie blue stained 10% SDS-PAGE gels showing the profiles of total protein in crude homogenates of acutely dissociated tracheal and pulmonary arterial SMCs.

(A) Proteins extracted from isolated canine tracheal smooth muscle cells were loaded onto lane 2. Lanes 3 through 5 represent proteins (12μg loading) extracted from cultured tracheal myocytes at 7 days, 12 days and 18 days after seeding respectively. Proteins extracted from cells (12 μg loading) 7 days post-seeding and having been treated for 96 hours with either 10nM TPA (lane 6), 0.1μM histamine (lane7), 0.1μM carbamyl choline (lane 8) or with 20μM 5-fluoro-2'-deoxyuridine (lane 9) are also shown. Molecular weight marker proteins were loaded into lane 1.

(B) Profile of proteins extracted from freshly isolated pulmonary arterial smooth muscle cells (lane 2), from freshly isolated tracheal smooth muscle cells (lane 3) and from cultured tracheal smooth muscle cells 8 days after seeding (lane 4). 12μg of total crude protein was loaded to each lane. Molecular weight markers were loaded onto lane 1.


A
kDa
1
2
3
4
5
6
7
8
9
200
116
97
66
45

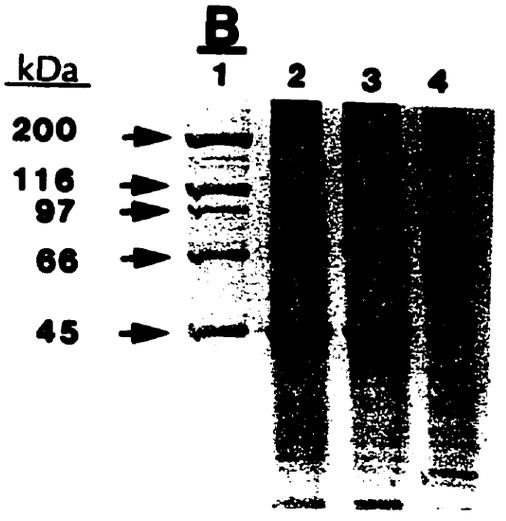
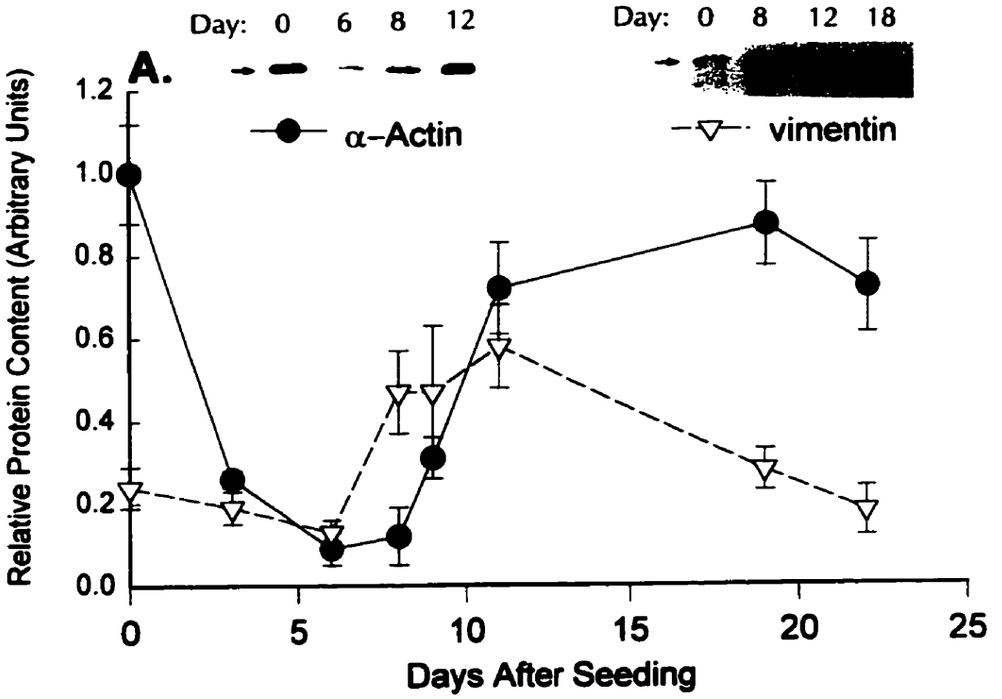

B
kDa
1
2
3
4
200
116
97
66
45

FIGURE 9. Temporal changes in abundance of specific smooth muscle proteins in cultured tracheal smooth muscle cells as assessed by Western blotting and laser densitometry.

(A) sm-α-Actin (43kDa), ●, and vimentin (57kDa), ∇, were separated using 10% SDS-PAGE. Total protein load for each lane was 5μg for actin analysis and 10μg for vimentin.

(B) nm-MHC (196-198kDa), ●, and sm-MHC (200-204kDa), ∇, were separated using 7.5% SDS-PAGE. Total protein load for each lane was 7.5μg for nm-MHC and 5μg for sm-MHC.


A.
Day: 0 6 8 12
Day: 0 8 12 18
α-Actin
vimentin
Relative Protein Content (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Days After Seeding

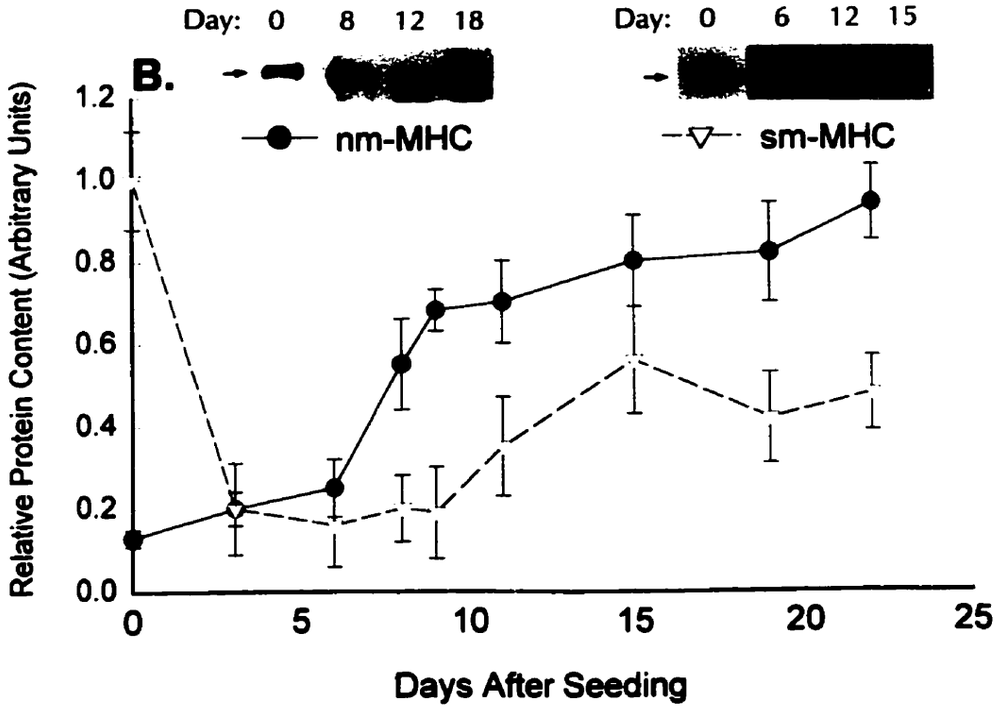

B.
Day: 0 8 12 18
Day: 0 6 12 15
nm-MHC
sm-MHC
Relative Protein Content (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Days After Seeding

FIGURE 10. Comparison, using Western blot analysis and scanning laser densitometry, of the abundance of specific smooth muscle proteins in freshly isolated tracheal smooth muscle cells with those in primary cultures of tracheal smooth muscle cells 7 days after seeding.

7.5% SDS-PAGE was used to separate sm-MHC (200-204kDa), MLCK (138kDa), *h*-caldesmon (150kDa), *l*-caldesmon (80kDa) and nm-MHC (196-198kDa). 10% SDS-PAGE was used to separate sm-α-actin (43kDa), calponin (34kDa), ß-tropomyosin (36kDa), desmin (53kDa), vimentin (57kDa), PKC (79kDa) and CD44 (85-90kDa). Total protein loads used for each protein are indicated in Table 5.

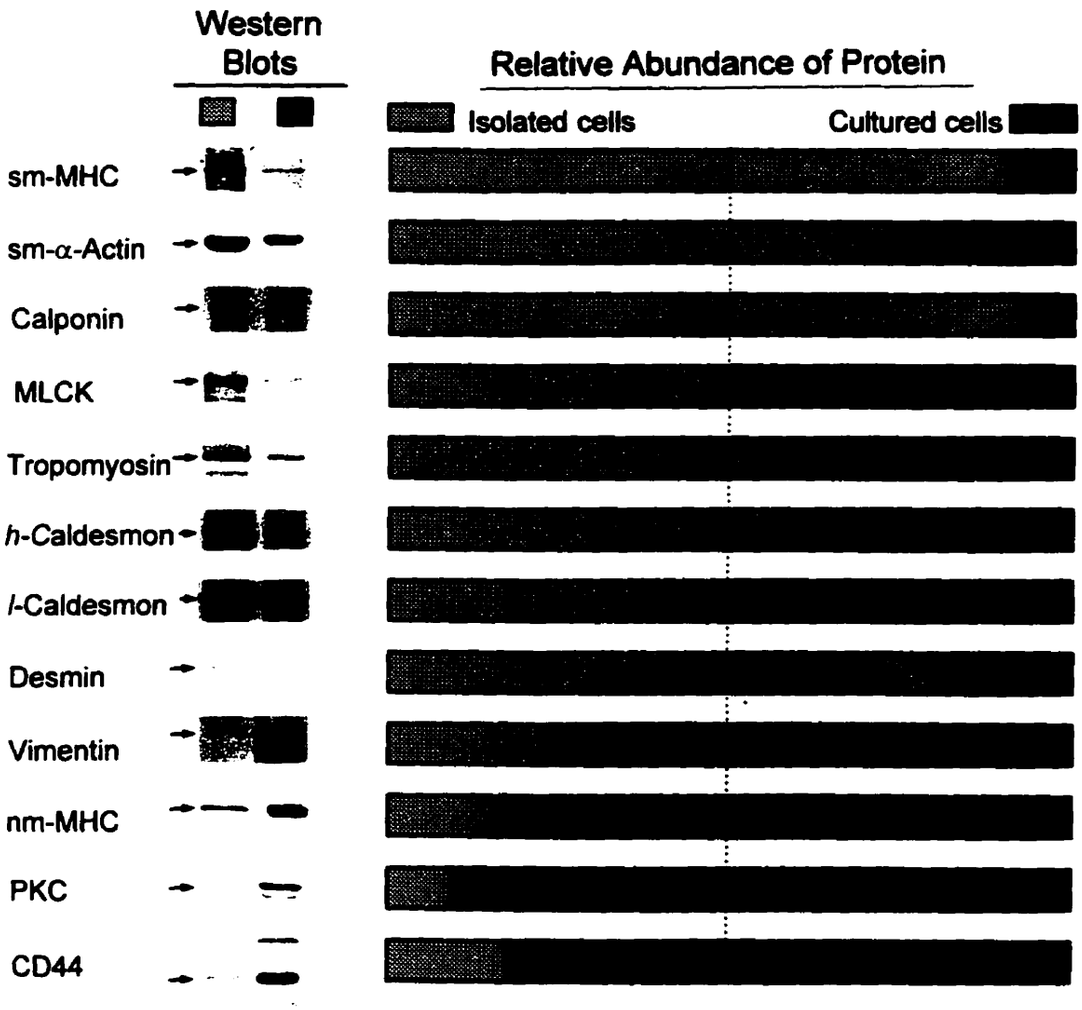

Western Blots
Relative Abundance of Protein
Isolated cells
Cultured cells
sm-MHC
sm-α-Actin
Calponin
MLCK
Tropomyosin
h-Caldesmon
l-Caldesmon
Desmin
Vimentin
nm-MHC
PKC
CD44

FIGURE 11. Northern blot analysis of steady state smMHC and actin mRNA contents in freshly isolated tracheal smooth muscle cells and in primary cultured tracheal smooth muscle cells 7 days after seeding.

(A) Autoradiograms showing signal intensity for bands corresponding to sm-MHC mRNA (6.5kb), ß and γ actin (2.1kb), sm-α-actin (1.7 kb) and for GAPDH (1.4kb) in total RNA extracts. Total RNA loaded to each lane was 50μg.

(B) Bar graph depicting the distribution, determined by scanning laser densitometry, of isoform specific actin mRNA in total RNA extracts.

(C) Histogram comparing the abundance of sm-MHC RNA, determined by laser densitometry and normalized to the amount of GAPDH message, in total RNA extracts.

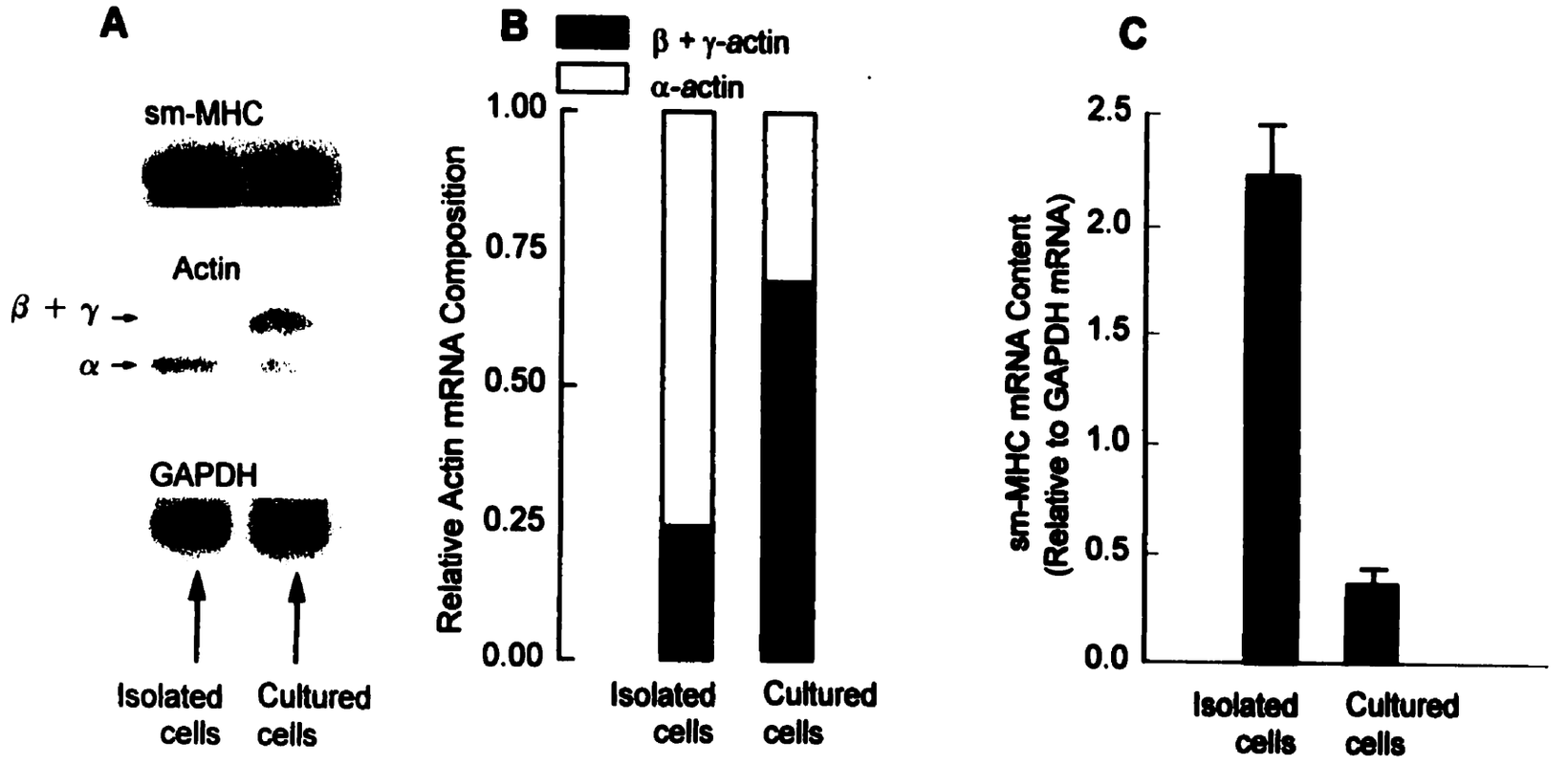
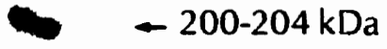

A
sm-MHC
Actin
β + γ
α
GAPDH
Isolated cells
Cultured cells
B
β + γ-actin
α-actin
Relative Actin mRNA Composition
1.00
0.75
0.50
0.25
0.00
Isolated cells
Cultured cells
C
sm-MHC mRNA Content (Relative to GAPDH mRNA)
2.5
2.0
1.5
1.0
0.5
0.0
Isolated cells
Cultured cells

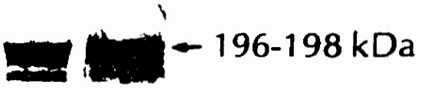
FIGURE 12. Comparison of the presence of specific protein markers in total homogenates of primary cultured tracheal smooth muscle cells (5 days after seeding) and in homogenates from cultured human foreskin fibroblasts, cell line MCH6, by Western blot analysis.

Proteins in panels a),b),f) and h) were separated using 7.5% SDS-PAGE, proteins in all other panels were separated using 10% SDS-PAGE. Total protein loaded to each lane was 10μg except for gels for blots in panel a) and d) which were loaded with 5μg total protein per lane.

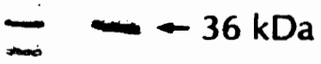
a) sm-MHC



b) nm-MHC

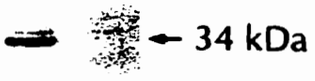


c) Tropomyosin

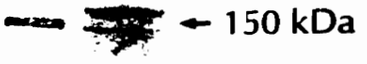


d) sm-*α*-Actin

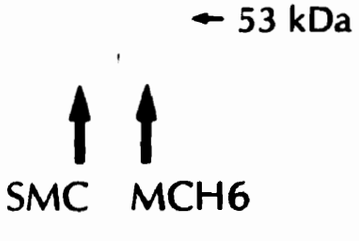
e) Calponin



f) *h*-Caldesmon



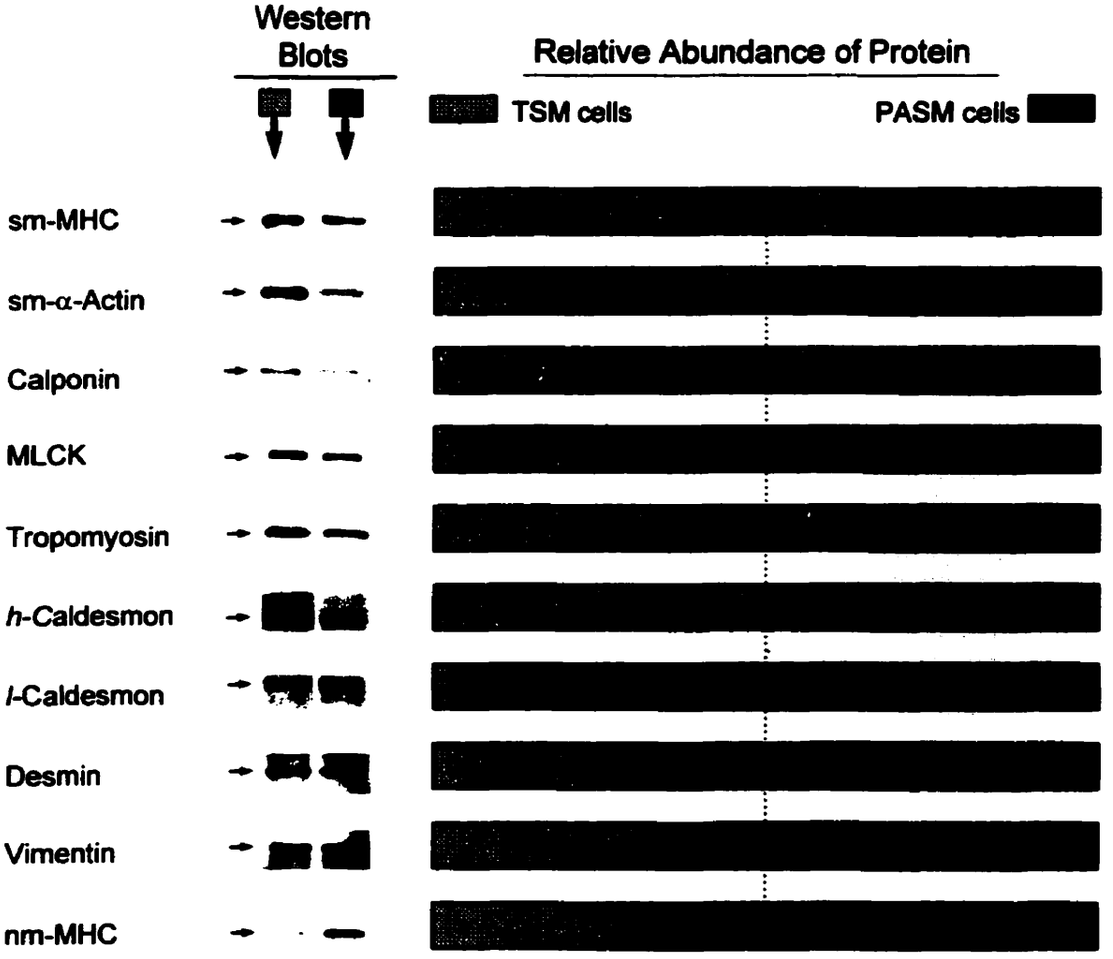
g) Desmin



h) *l*-Caldesmon

FIGURE 13. Comparison, using Western blot analysis and scanning laser densitometry, of the abundance of specific smooth muscle proteins in freshly isolated tracheal smooth muscle (TSM) cells with those in freshly isolated pulmonary arterial smooth muscle (PASM) cells.

All values are normalized to total protein loaded per lane (Table 5). 7.5% SDS-PAGE was used to separate sm-MHC (200-204kDa), MLCK (138kDa), *h*-caldesmon (150kDa), *l*-caldesmon (80kDa) and nm-MHC (196-198kDa). 10% SDS-Page was used to separate sm-α-actin (43kDa), calponin (34kDa), ß-tropomyosin (36kDa), desmin (53kDa) and vimentin (57kDa). Protein loaded per lane was the same for TSM and PASM for specific marker proteins


Western Blots
Relative Abundance of Protein
TSM cells
PASM cells
sm-MHC
sm-α-Actin
Calponin
MLCK
Tropomyosin
h-Caldesmon
l-Caldesmon
Desmin
Vimentin
nm-MHC

4.5 Discussion

The data obtained from these studies describe temporal changes in the expression and content of proteins composing the contractile apparatus, regulating the contractile apparatus and associated with the cytoskeleton of cultured canine tracheal myocytes. Tracheal SMCs underwent rapid phenotypic modulation in primary culture. The content of specific proteins in cultured cells was dependent upon time in culture and cell density. Similar studies concerning vascular SMCs (Shanahan et al., 1993) have established specific contractile and cytoskeletal proteins as markers of SMC phenotype. We report that contractile airway SMCs are relatively rich in contractile proteins, including sm-MHC, sm-α-actin, calponin, desmin, ß-tropomyosin, MLCK, and *h*-caldesmon. On the other hand, proliferating tracheal myocytes were enriched in nm-MHC, *l*-caldesmon, protein kinase C and CD44 in primary culture. The cells also demonstrated an ability for phenotypic plasticity as evidenced by re-expression of sm-MHC and sm-α-actin, in post-confluent cultures. The culture conditions used in our studies (*i.e.* cell dissociation protocol, plating density, choice of culture media and serum supplementation) are typical of most studies of airway myocytes from various species, hence, the temporal changes we report should correlate well with observations made by other investigators.

One of the cytostructural features of mature, contractile SMCs is the presence of a high volume fraction of thick and thin myofilaments which constitute the contractile apparatus of the cells (Chamley-Campbell et al., 1979; Campbell and Campbell, 1987). Vascular SMCs are known to express genes coding the smooth muscle and non muscle

isoforms of myosin heavy and light chains (Rovner et al., 1986; Kawamoto and Adelstein, 1987; Monical et al., 1993). Our studies demonstrate that both sm- and nmMHC are also present in contractile airway smooth muscle. In addition, the content of smMHC protein depleted rapidly prior to the onset of proliferation of the SMCs in primary culture and was correlated with >75% reduction in smMHC mRNA also. Concomitantly, nmMHC protein began increasing at the onset of the proliferative phase of the growth curve. This pattern of expression has been described previously for both heavy and light chain myosin isoforms in cultured vascular SMCs (Rovner et al., 1986; Kawamoto and Adelstein, 1987; Monical et al., 1993). Furthermore, using rat aortic SMC primary cultures, Grainger et al. (1991) determined that loss of smMHC was dependent on time in culture whereas increase of nmMHC was correlated with the passage of cells through mitosis. Accumulation of nmMHC appears to be obligatory for vascular SMC cell division as proliferation of cultured cells is blocked using antisense nmMHC oligonucleotides (Simons and Rosenberg, 1992).

In post-confluent tracheal SMC cultures we observed a re-accumulation of smMHC protein whereas the elevated content of nmMHC was maintained. This difference suggests that regulation of smMHC content during phenotype switching may not be linked directly with that for nmMHC. The increase of nmMHC and its maintenance post-confluence in cultured vascular SMCs is thought to be due to an increase in protein half-life at first M phase which is sustained thereafter in culture (Grainger et al., 1991). Though we did not systematically analyze specific smMHC and nmMHC isoform expression in our cultures we have previously demonstrated (Kong et

al., 1990) the existence of the SM1 (204kDa) and SM2 (200kDa) smMHC isoforms in intact canine airway smooth muscle. Studies (Rovner et al., 1986; Kawamoto and Adelstein, 1987) employing vascular SMCs in primary culture revealed that the SM2 isoform disappeared more rapidly than the SM1 isoform. Immunohistological analysis of myosin isoform expression in the developing rat lung suggests that the SM2 isoform may be a marker for mature airway myocytes as it was seen only in fully developed airways (Woodcock-Mitchell et al., 1993).

Actin is a globular protein which can polymerize to form thin myofilamentous structures which are important for contraction, cell motility and determining cell structure. Six isoforms of actin have been identified of which at least three isoactins, α-,ß- and γ, exist in tracheal smooth muscle at a ratio of 37:21:42 respectively (Fatigati and Murphy, 1984). Northern blot analysis of acutely dissociated tracheal SMCs confirmed that transcripts coding for sm-α-actin and ß/γ-non muscle actin are present and exist in a ratio of approximately 75:25. Immunoblot and northern analysis of sm-α-actin content in cultured tracheal myocytes revealed that both protein and mRNA levels diminished by more than 70% as SMCs became proliferative in culture. Coomassie blue staining of polyacrylamide gels did not, however, suggest a decrease in staining of the band corresponding to total actin in cultured cells. This inconsistency was clarified by Northern analysis which revealed that reduction of sm-α-actin mRNA was compensated for by an increase in abundance of ß- and γ-isoactin transcripts. Using 2-d gel protein electrophoresis, Fatigati and Murphy (1984) have also shown, for swine aortic SMCs, that the fractional content of sm-α-actin decreased with time in primary culture whereas

ß- and γ-isoactin content increased. Though sm-α-actin does appear to be more highly expressed in contractile SMCs, it does not appear to be specific for SMCs as we were able to detect substantial levels of the protein in lysates of MCH6 fibroblasts. Numerous reports exist confirming the presence of sm-α-actin in a variety of nonmuscle cell types (Sappino et al., 1990).

The interaction of myosin and actin filaments determines the contractile properties of smooth muscle and is regulated by a number of signal transduction pathways (Somlyo and Somlyo, 1994). Myosin light chain kinase activates actomyosin ATPase activity via phosphorylation of the regulatory myosin light chain to initiate crossbridge cycling and contraction. The content of MLCK was reduced by >50% in proliferative tracheal SMCs in culture. Using rat aortic SMCs, Monical et al. (1993) showed there are no changes in relative phosphorylation of the sm- and nm-regulatory myosin light chain isoforms in culture suggesting that the loss of MLCK is not associated with alterations in substrate specificity. Tropomyosin is a thin myofilament associated protein involved with organization of the filaments into functional arrays and with regulating actin-myosin interactions and myosin ATPase (Phillips and Chacko, 1996). The content of ß-tropomyosin in proliferating tracheal myocytes is reduced to ~40% of that seen in acutely dissociated cells. Interestingly, immunoblots comparing lysates from cultured MCH6 cells and tracheal SMCs indicated tropomyosin content was similar. This observation could imply that the tropomyosin present in fibroblasts and SMCs is associated with similar non-contractile cytostructural assortments.

Calponin, another thin filament-associated regulatory protein, is thought to be a

specific marker for SMCs (Frid et al., 1992; Walsh , 1994). Indeed, we were unable to detect calponin in MCH6 cells using immonoblotting even at relatively high loads (25 μg) of crude lysate. Calponin was easily detected in acutely dissociated tracheal SMCs but cell content dropped dramatically in SMCs cultured for 6 days. Frid et al. (1992) showed that human visceral smooth muscles, including the trachealis, were calponin immunoreactive in 10 and 20 week old fetuses whereas aortic medial cells, which express the contractile phenotype after reaching the neonatal stage (Campbell and Campbell, 1987), were not immunoreactive for calponin. In light of these data, our observations suggest that calponin may be a marker of mature airway SMCs in culture and *in vivo*.

Caldesmon co-localizes with the contractile actin domain in smooth muscle which is consistent with its role as a thin filament-associated regulatory protein of contraction. Two major types of caldesmon have been identified, high M_r *h*-caldesmon (120-150 kDa as determined by SDS-PAGE) and low M_r *l*-caldesmon (70-80 kDa by SDS-PAGE) (Walsh, 1994). Both forms of caldesmon were detected in acutely dissociated and cultured tracheal SMCs. However, *h*-caldesmon predominated in the former whereas *l*-caldesmon predominated in the latter. Similarly, 150 kDa caldesmon content in vascular SMCs has been shown to decrease until cells began proliferating whereas 80 kDa caldesmon content increased over the same time (Ueki et al., 1987). The same investigators have also shown that SMCs in culture can re-express *h*-caldesmon, with a concomitant loss of *l*-caldesmon, after growth arrest. We detected two bands (~158 and 148 kDa) of *h*-caldesmon in MCH6 cells, but *l*-caldesmon was much more prominent. In the fibroblastic 3T3 cell line and chicken embryo fibroblasts, *h*-caldesmon

also appears as a doublet and the content of *l*-caldesmon was predominant (Bretscher and Lynch, 1985). The data presented here indicate that predominance of *h*-caldesmon is likely a good indicator of the contractile airway smooth muscle phenotype, but, in culture it is not present exclusively in SMCs.

The expression patterns of vimentin and desmin, proteins associated with intermediate filaments, have been well described for SMCs in developing vasculature. In the rat, there is an increase from the fetus to 12 weeks after birth in the number of aortic SMCs containing desmin with a concomitant decrease in the number of cells containing only vimentin (Kocher et al., 1985). Data obtained with airway SMCs indicate that when the cells modulate their phenotype in primary culture, desmin expression is reduced greatly and is virtually undetectable in proliferating cells. Conversely, vimentin content had increased ~2-fold in the same cells. Vimentin content began increasing at the onset SMC proliferation and peaked as plates reached confluence. Vimentin levels then decreased post confluence suggesting that loss of the protein might be an indicator of phenotypic switching of the cells. Desmin is considered to be a marker of mature muscle cells but is not specific for smooth muscle as it is present in cardiac and skeletal muscles as well. The findings in the present study are consistent with the concept that desmin is a marker of mature smooth muscle cells, as we detected only minimal quantities in proliferative tracheal SMCs and could not detect it at all in MCH6 lysates.

Activation of protein kinase C is known to be a concomitant feature of cellular proliferation. The enzyme is represented by a family of at least 11 isoenzymes of which 5, namely α, β, δ, ϵ and ζ are reportedly expressed in smooth muscle cells (Andrea and

Walsh, 1992; Assender et al., 1994). Using PCR and immunoblotting, Assender et al. (1994) showed in cultured human and rat vascular SMCs, that expression of PKC-α was independent of SMC phenotype but PKC-β was not present in proliferating cells. The antibody we employed in our studies recognizes only the α and β isoenzymes. A single band of M_r 79 kDa was detected in both acutely dissociated and cultured tracheal SMCs, however, the protein detected was 8-fold greater in abundance in proliferating cells.

Hematopoietic, neural, epithelial and mesenchymal cell types, including smooth muscles, express the integral membrane proteoglycan, CD44 (Picker et al., 1989; Jalkanen et al., 1991). Also known as the lymphocytic homing cellular adhesion molecule (HCAM), CD44 exists in cultured fibroblasts as both a 80-90 kDa and a 200 kDa form (Picker et al., 1989). Our immunoblot assays revealed that both acutely dissociated and cultured tracheal myocytes expressed a $\sim$85 kDa form of CD44. Cultured cells, however, possessed $>$5-fold more of the protein than did non-cultured cells. This differential expression of CD44 is likely an indicator of functional differences between the cell populations. CD44 binds extracellular matrix components and its cytoplasmic domain associates with actin-rich cytoskeletal filaments (Jalkanen et al., 1991). Hence, CD44 can act as a mediator of cell motility, a phenomenon commonly associated with cultured cells.

Broad differences in the contractile properties of smooth muscles from various organs exist. Differences appear to be related to variation in the expression of ion channels and isoforms of contractile and regulatory proteins including the 17kDa

"essential" myosin light chain , myosin heavy chain and actin (Somlyo and Somlyo, 1994). We sought to determine if differential expression, *in vivo* , of the proteins we identified as markers for SMC phenotype, was related to differences in the contractile nature of the tissues. Studies performed previously (Antonissen et al., 1979; Kong and Stephens, 1981) demonstrated that the contractile properties of canine tracheal smooth muscle are different from those of canine pulmonary arterial smooth muscle. Using a similar myograph in each case, values measured for tracheal and pulmonary arterial preparations respectively were: 1) maximum zero-load shortening velocity, 0.30 and 0.11 lo/sec, and, 2) maximum force (corrected for muscle cross-sectional area), 1.7 and 1.1 kg/cm^2. Comparison of the profiles of the content of marker proteins in SMCs dissociated from the trachealis and the pulmonary artery revealed that the former were enriched by 1-fold or more in sm-α-actin, *h*-caldesmon, calponin, sm-MHC, MLCK and tropomyosin. These data suggest that the extent of the contractile cytostructural array may be greater in tracheal SMCs. Comparative electron microscopic analyses are consistent with this contention; they have shown the cellular volume fraction of myofilaments to be reduced in SMCs of large elastic arteries (Campbell and Campbell, 1987). However, direct comparisons of the protein profiles of SMCs derived from different tissues may be confounding due to recent evidence of phenotypic heterogeneity of SMCs within the vascular wall (Frid et al., 1994). Indeed, we have obtained morphological and immunohistochemical evidence that SMC phenotypic heterogeneity also exists within airway smooth muscle (refer to Chapters 6 and 7 in this thesis). Therefore, differences in marker protein content

between tissues may reflect variation in the distribution of phenotypically distinct subpopulations moreso than indicating that all of the SMCs from different sources are phenotypically dissimilar.

There is good evidence suggesting that the requisite factors to trigger airways SMC proliferation are present in asthmatic airways (Halayko and Stephens, 1994). However, the influence of the state of airway smooth muscle phenotype on modulating abnormal growth and contractility, as it might relate to asthma, has not been investigated. Subtle changes in the characteristics, or phenotype, of smooth muscle can greatly affect its physiological role. We have found (Jiang et al., 1992) shortening velocity and capacity of airway smooth muscle to be increased in ragweed sensitized dogs. These changes have been shown to be correlated with increased levels of regulatory myosin light chain phosphorylation, increased content and activity of MLCK and reduced acetylcholinesterase activity (Mitchell et al., 1991). Difficulties in obtaining viable smooth muscle samples from asthmatic patients have greatly limited the study of airway smooth muscle contractility directly. It may be feasible to apply knowledge concerning the relationship between marker protein expression and airway SMC phenotype, using immunohistochemical analysis of biopsies obtained from asthmatics, to determine if a change in airway smooth muscle phenotype is a feature of asthma.

The results of the studies reported herein confirm that airway SMCs are capable of phenotypic modulation which is manifest as differences in expression of phenotypic marker proteins. Our studies did not address the question of expression

profiles of membrane ion channels or receptors, however, it seems likely that differential changes in the content of these proteins will also be a fundamental characteristic of cultured cells. Interpretation of any data obtained using cultures of airway SMCs needs to carefully consider the knowledge that these cells demonstrate plasticity in phenotypic expression which is dependent upon culture conditions, time in culture and cell density.

4.6 SUMMARY

Airways smooth muscle plays a principal role in the pathogenesis of asthma. Primary cultures are being used to investigate airways myocyte proliferation and cellular pathways regulating contraction. Airway SMCs modulate from a contractile to a non-contractile phenotype in culture but no systematic study of the concomitant changes in expression of contractile and cytoskeletal proteins has been reported. Temporal changes in protein marker expression of canine tracheal SMCs in primary culture were measured using specific antibodies and cDNA probes. Immunoblot analysis revealed when cells became proliferative after five days of culture, the content of smMHC, calponin, sm-α-actin and desmin diminished by $>75\%$; myosin light chain kinase, *h*-caldesmon and ß-tropomyosin had also decreased significantly ($p<0.05$). Northern blots revealed that mRNA levels for smMHC and sm-α-actin were also significantly reduced in proliferative SMCs. Conversely, immunoblotting demonstrated the content of non-muscle myosin heavy chain, *l*-caldesmon, vimentin, α/ß-protein kinase C and CD44 to be increased up to 6-fold as cells became

proliferative. The content of sm-MHC and sm-α-actin protein increased post-confluence suggesting that cultured airway SMCs are capable of phenotypic plasticity. Marker protein contents were also compared, by immunoblot assay, between SMCs dissociated from trachealis or pulmonary arterial media. Contractile protein content was higher in the trachea, which shortens faster than the pulmonary artery. The identification of these markers provides potentially useful tools for assessing the phenotype of airways SMCs in culture and the airways of healthy and asthmatic patients.

5. HETEROGENEITY IN THE RESPONSE OF AIRWAY SMOOTH MUSCLE CELLS TO HEPARIN-MEDIATED INHIBITION OF PROLIFERATION

5.1 BACKGROUND

Heparins are highly charged, anionic, natural glycosaminoglycans composed of alternating residues of either β-D-glucuronic or α-L-iduronic acid and α-D-glucosamine linked by 1,4 glycosidic linkages with O- and N-sulfation/acetylation (Garg and Lyon , 1991). They have been known to possess anti-inflammatory properties for some time (Dolowitz and Dougherty, 1960) and have been extensively studied for their ability to inhibit arterial SMC migration and proliferation *in vitro* and during arterial remodelling after injury (Clowes and Karnovsky, 1977; Majack and Clowes, 1984; Au et al., 1992). Heparin has been shown to affect the binding of PDGF and the expression of receptors for epidermal growth factor in arterial SMCs (Fager et al., 1992; Reilly et al., 1987). Binding and internalization of heparin, via receptor-mediated endocytosis, appears to be a requirement for anti-proliferative activity (Letourneur et al., 1995). Internalized heparin is dispersed throughout the cytosol but localizes near the nucleus, with no evidence of nuclear uptake, and at sites of cell contact or cellular adhesion (Bârzu et al., 1996). Heparins appear to exert their effects on vascular SMCs by inhibiting a number of mitogenic signal transduction pathways including those involving PKC, casein kinase II, and mitogen activated protein (MAP) kinases (Castellot et al., 1989; Ottlinger et al., 1993; Singh et al., 1993). There is evidence that heparin-insensitive mitogen signalling pathways also exist, for example, heparin does not inhibit MAP kinase activation in vascular SMCs stimulated with epidermal growth factor (Ottlinger et al., 1993). Flow cytometric analysis of cell cycle progression of cultured arterial SMCs revealed that heparin slowed entry to S-phase somewhat but its major effect on proliferating cells was

to inhibit the rate that SMCs progressed from G_2-phase through mitosis (Grainger et al., 1993). A concomitant property of heparins on arterial SMCs is a potency for inhibition of phenotypic modulation of the myocytes in primary culture and *in vivo* in response to arterial injury (Chamley-Campbell and Campbell, 1981; Majak and Bornstein, 1984; Clowes et al., 1988).

Recent reports indicate that heparin-sensitive and -insensitive arterial SMC subpopulations exist (Caleb et al., 1996; Bârzu et al., 1996), and clonal cell lines of each have been isolated (San Antonio et al., 1993). Heparin-sensitive arterial SMCs appear to differ from their heparin-insensitive counterparts in the ability to up regulate expression of heparin receptors when exposed to exogenous heparins (San Antonio et al., 1993; Letourneur et al., 1995). Increased numbers of receptors on heparin-sensitive arterial SMCs results in increased internalization of heparin and subsequent inhibition of cellular replication, migration and modulation of expression of genes for extracellular matrix proteins (Majak and Clowes, 1984; Lyons-Giordano et al., 1989). Heparin-resistant arterial cells display lower levels of immunostaining for sm-α-actin, are smaller and proliferate more rapidly than control SMC cultures in response to serum and phorbol ester stimulation (San Antonio et al., 1993).

Asthma is characterized by airway remodelling, a pathological feature which includes thickening of the airway smooth muscle layer due to SMC hypertrophy and hyperplasia (Dunnill et al., 1969; Ebina et al., 1990 and 1993). Hence, numerous studies defining the factors that regulate airway SMC proliferation in culture have been reported (reviewed by Hirst and Twort, 1992; Halayko and Stephens, 1994). Inhibitors of airway

SMC proliferation have been identified including PGE_2 (Florio et al., 1994), β-adrenergic agonists (Tomlinson et al., 1994) and heparin (Johnson et al., 1995; Kilfeather et al., 1995).

Heparin, is produced and stored in considerable quantities in pulmonary mast cells (Schwartz and Austen, 1980) and appears to prevent antigen-induced airway hyperresposiveness in *Ascaris suum*- sensitized sheep (Abraham et al., 1994; Ahmed et al., 1996). The amount of heparin released from activated mast cells in antigen-challenged, sensitized lung slice preparations appears to be sufficient to inhibit airway SMC proliferation based on *in vitro* experiments (Green et al., 1993; Johnson et al., 1995). Maximum inhibition of serum-stimulated, cultured airway SMC proliferation and DNA synthesis appears to be approximately 40% using high doses of heparin (<500 μg/ml) (Johnson et al., 1995; Kilfeather, et al., 1995) suggesting, as appears to be the case for arterial SMCs, heparin-insensitive airway SMC subpopulations may exist. Though the existence of endogenous anti-mitogenic factors are recognized, the role they play in determining the responsiveness of airway myocytes to mitogenic stimuli *in vivo* and *in vitro* are not clearly established. Indeed, other than quantitative information concerning the dose responsiveness of cultured airway SMCs to heparin treatment, no information is available concerning the cellular mechanisms and pathways by which heparins mediate reduced proliferation of airway SMCs.

A specific hypothesis was established, based on information noted heretofore concerning the role of SMC phenotype in determining heparin responsiveness:

SPECIFIC HYPOTHESIS -- Exogenous heparins inhibit replication of heparin-sensitive

subsets of cultured airway smooth muscle cells through defined pathways which regulate different steps of the cell cycle.

Understanding of the mechanism of inhibition of SMC replication by heparin will be of benefit in elucidating the role of the endogenous glycosaminoglycan in determining the pathology of the airways in chronic asthma.

5.2 Specific Aims

1. To measure the effects of exogenous heparin on proliferation and phenotypic modulation of airway SMCs in culture.

2. To characterize the progression of airway myocytes through replication and elucidate the specific actions of exogenous heparins on proliferation of individual SMCs by measuring cell cycle transit and DNA synthesis using flow cytometry.

3. To measure the effects of exogenous heparins on activation of mitogen activated protein kinases and tyrosine kinases, two important enzyme systems involved in mitogenic signalling, in serum-stimulated, cultured airway SMCs.

5.3 Experimental Design

To determine the effects of exogenous heparin on proliferation and phenotypic modulation of airway SMCs, primary cultures were established and the plates randomly divided into two groups. One group (control) was grown under normal conditions in the

media supplemented with 10% FBS. The media of the heparin-treatment group was replaced with control media containing sodium heparin (100 μg/ml), 36 hours after initial cell seeding and the cells were grown under those conditions thereafter. Proliferation was assessed by counting cell number and by measuring total protein in cell lysates obtained from individual culture plates at pre-determined time points (from 0 to 20 days after cell seeding). Temporal patterns of phenotypic marker protein content were determined at each time point by quantitative immunoblotting (Sections 3.3.1, 3.3.3, 3.3.4, 3.3.5 and 3.3.6) using protein lysates obtained from individual plates in triplicate. A total of four different experiments was completed.

To characterize the progression of airway myocytes through the cell cycle, culture plates were growth arrested for 96 hours in order to synchronize the SMCs (note: preliminary experiments showed that 96 hours was required to arrest >95% of the cells in G_0). Plates belonging to the heparin-treatment group contained heparin during growth arrest. Cells were stimulated to proliferate by replacing serum-free, growth arrest media with serum-supplemented media -- heparin (from 1 - 100 μg/ml) was included in the treatment group media. Cells were harvested at predetermined time points (0 to 48 hours), from two plates of each group for cell cycle analysis using propidium iodide-staining and flow cytometry. Each sample was analysed in duplicate on the flow cytometer to determine the distribution of cells throughout the cell cycle (Section 3.4.2).

To measure DNA synthesis, control and heparin-treated cells were arrested and subsequently serum-stimulated as just described -- for these experiments only one heparin dose (100 μg/ml) was used. Cells were allowed to proliferate for 68 hours then

were pulsed for 4 hours with the thymidine analogue, BrdU, for 4 hours during which BrdU could be incorporated into newly synthesized DNA of cells in S-phase of the cell cycle. After the BrdU pulse, cells were harvested and analysed for BrdU incorporation and cell cycle distribution by FACS analysis (Sections 3.4.2.3 and 3.4.2.4). Three different plates were analysed in each group in each experiment -- 5 experiments were completed in total.

To measure the effects of exogenous heparins on activation of mitogen activated protein (MAP) kinase and tyrosine kinase in serum-stimulated, cultured airway SMCs cells were divided into control and heparin-treated groups and growth arrested as described above. Only one dose of heparin (100 μg/ml) was used in these studies. Cell lysates were obtained from duplicate plates, for each group, for MAP kinase assay at pre-determined time points (0 - 60 minutes) -- seven experiments were completed. MAP kinase assay was carried out as described in Section 3.3.9 and the relative activity in each group compared. Cell lysates were obtained in duplicate, for each group, for tyrosine kinase assay at pre-determined time points (0 - 18 hours). Phosphotyrosine assay was carried out as described in Section 3.3.10 -- four experiments were completed.

5.4 RESULTS

5.4.1 Effects of Heparin on Growth Characteristics and Phenotypic Modulation

To measure the proliferative response of canine airway SMCs, cell number and total protein in each culture plate were measured. Cells grown in control conditions (10% FBS) showed typical growth characteristics for cultured SMCs. Cell number started

to increase approximately 5 days after plating and at confluence, approximately 9 days after seeding the primary cultures, cell density was between 7.5 and 10.4 x 10^4 cells/cm^2 (Figure 14A). At confluence these cultures exhibited a typical "hill-and-valley morphology" and possessed numerous areas of multi-layered, focal overgrowth. Total protein extracted from each plate increased temporally in a pattern similar to that seen for the increase in cell number (Figure 14B).

Inclusion of 100 μg/ml of heparin to the culture media delayed the onset of rapid SMC proliferation, measured by cell number. Onset occurred 8 days after initial seeding, a delay of 3 days from that seen for control cultures (Figure 14A). Confluence was reached 4 days after cell numbers first began to increase, this period of time was comparable in time to the length of the proliferative phase seen in control cultures. Interestingly, at confluence (day 12), the mean cell density of heparin-treated plates was only between 5.4 - 6.8 x 10^4 cells/cm^2, approximately 35% lower than that seen in control plates ($p < 0.01$, $n= 4$). At confluence heparin-treated cultures did not exhibit a distinct "hill-and-valley" morphology, instead the cultures had a cobblestone appearance and lacked the focal, multi-layered overgrowths seen in control airway SMC cultures. The growth curves for heparin-treated cultures remained flatter post-confluence than control cultures indicating a greater tendency for cessation of proliferation after cell-to-cell contacts had been made. In contrast to the differences between heparin-treated cells and control cells in cell number, the temporal pattern of protein accumulation was nearly the same for the first 10 days in primary culture. However, after confluence was reached total protein continued to increase slowly in control cultures but did not change in

heparin-treated cultures (Figure 14B).

Temporal expression of the airway SMC phenotypic markers, smMHC, sm-α-actin, MLCK, nmMHC, and vimentin was assessed by immunoblot assay. In control cultures contractile SMCs modulated to an immature phenotype as was indicated by a gradual loss of smMHC and sm-α-actin and an accumulation in cellular content of nmMHC and vimentin (Figure 15). Cells began accumulating smMHC and sm-α-actin after confluence was reached and cellular vimentin content began to decrease, indicating that the SMCs reverted partially to their original phenotypic state. Inclusion of heparin had no effect of the loss or accumulation of smMHC, sm-α-actin, nmMHC and MLCK in the cultured cells (ANOVA, n=4). These data suggest strongly that heparin did not inhibit modulation from the contractile phenotype of cultured, airway SMCs. In contrast, the temporal pattern of cellular vimentin content in heparin-treated SMCs was different from controls (ANOVA, $p < 0.05$, n=4). Indeed, cellular vimentin content was approximately 50% higher at confluence (day 12) in heparin-treated airway SMCs (Figure 15E).

5.4.2 Effects of Heparin on Airway SMC Cell Cycle Progression

Effects of heparin on cell cycle progression of airway SMC were examined by flow cytometry. Subconfluent (70%) cultures were growth arrested for 96 hours in serum free media. The data reported hereafter were obtained from experiments in which heparin was included in the arrest media. Three studies were, however, conducted in which heparin was not included in the arrest media, but was added at the time of serum

stimulation after growth arrest. The data obtained using both protocols were not different, hence, for brevity, only data obtained from cultures in which heparin was included in the arrest media are presented. Cells were then stimulated with DMEM, 10% FBS containing heparin and subsequently harvested and cell cycle distribution was determined by measuring cellular DNA content using propidium iodide staining (Figure 16). The maximum fraction of tracheal SMCs in control cultures which traversed the first cell cycle in response to serum stimulation, as determined by the decrease in the number of SMCs in G_0/G_1, was 70% (Figures 16A and 17). This finding suggests that 30% of all the cultured tracheal myocytes cells were refractory to mitogenic factors normally present in FBS. Hence, the cells that entered the first cell cycle after serum stimulation will be distinguished, called serum-responsive SMC (srSMC), from those cells that did not progress through the first cell cycle after serum stimulation, called serum-insensitive SMC (siSMC).

Serum-responsive tracheal SMCs began entering S-phase approximately 18 hours after serum stimulation. The cells entered G_2-phase about 25 hours after stimulation and returned to G_0/G_1 about 36-40 hours after serum had been added to the arrested cultures. Therefore, the cell cycle time for serum-stimulated, tracheal srSMC appeared to be approximately 38 hours (Figure 16A).

Heparin (100 μg/ml) appeared to have no effect on the rate of SMC transit through S-phase and into G_2, however, the total number of srSMCs which entered the cell cycle was inhibited by 40.3 ± 5.1% (Figures 16B and 17). This suggests that approximately half of the proliferative srSMC were heparin-sensitive and could be

blocked from cell cycle entry by exogenous heparin. The remaining srSMC appeared to be heparin-resistant, however, examination of the DNA histograms obtained for heparin-treated cells indicated that the number of cells in G_0/G_1 did not begin to increase again until 44-48 hours after serum-stimulation (Figures 16B and 17). Hence, the cell cycle time of srSMC that appeared to be heparin-resistant was, in fact, 6-10 hours longer than seen in control conditions. These findings suggest that proliferation of most srSMC may be affected by exogenous heparin, however, a heterogeneous response pattern exists in which about 40% of cells are blocked form cell cycle entry while the reminder enter the cell cycle but progress more slowly than usual through the replicative process.

The maximum number of heparin-insensitive srSMC that reached G_2/M phase was attained 28 hours after serum stimulation. Therefore, to assess the concentration-response relationship between heparin and inhibition of cell cycle entry, the fraction of SMCs in the cell cycle 28 hours after serum-stimulation, in the presence of different concentrations of heparin was compared (Figure 18). Concentration-response characteristics of heparin-mediated inhibition of cell cycle-entry of srSMC demonstrated an "all-or-none" character (Figure 18). That is, 1 μg/ml heparin was as potent as 50 μg/ml heparin at blocking cell cycle entry ($p<0.01$, $n=4$). In fact, the lowest dose of heparin used (1 μg/ml) was only 20% less potent than the concentration that exhibited maximum anti-proliferative activity (100 μg/ml).

Bivariate FACS analysis of cell cycle distribution and DNA synthesis indicated that factors regulating airway SMC proliferation were affected by exogenous heparin (Figure 19). Bi-variate dot plots demonstrated clearly that 68-72 hours after serum

stimulation, the proportion of SMCs in the latter phases of the cell cycle are increased in the presence of heparin (100 μg/ml). The proportion of cells between late-S phase and M phase was doubled in the presence of heparin (Figure 20). These data suggest that the time for proliferating cells to progress through G_2 and into M phase of the cell cycle is increased by heparin treatment. The cell cyle length for srSMC which traverse the cell cycle in the presence of heparin was increased by 6-10 hours over controls (Figure 16B and 17), the findings presented in Figure 20 may therefore indicate that the mechanism underlying this difference is related to alterations in regulatory pathways that determine G_2/M-phase transit time.

5.4.3 Effects of Heparin on Signalling Pathways

The effects of heparin on activation of MAP kinases were determined using an "in gel" assay. Primary cultures (75% confluent) were growth arrested for 96 hours in serum free media. The activation profile showed a bi-phasic response, first increasing rapidly and transiently after serum stimulation and then increasing again 60 minutes later (Figures 21 and 22). In control cultures stimulated with 10% FBS, the activity of both 42 kDa and 44 kDa MAP kinase increased rapidly within 2 minutes of serum addition (Figures 21 and 22). MAP kinase activity remained at a high level 10 -20 minutes after the addition of serum but returned to basal levels 20-30 minutes after serum stimulation. A second increase in enzyme activity was seen 60 minutes after serum was added, however, no samples were collected after that time so the temporal pattern of the second peak in activity is not known. Numerous other bands of activity could be seen on the

gels, some of which increased in response to serum stimulation and others which were evident whether cells were stimulated with serum or not. The identity of the enzymes in these bands was not ascertained. Heparin-treated cells demonstrated a qualitatively similar temporal pattern of MAP kinase activation as seen in control cultures , however, peak activation of both isoforms from 2-10 minutes after serum stimulation was inhibited by approximately by 30-50% (Figures 21 and 22). Activity 60 minutes after serum addition was also 30-40% lower in heparin-treated cultures.

The effects of heparin on pathways involving tyrosine phosphorylation were determined with an immunoblot assay using 4G10 anti-phosphotyrosine antibodies. Primary cultures (75% confluent) were growth arrested then were serum-stimulated with 10% FBS and the activity of tyrosine kinases was estimated at certain time points thereafter. Tyrosine phosphorylation was seen in protein bands corresponding to 190, 116, 70-80, 58 and 53 kDa within five hours of serum stimulation (Figure 23). The pattern of phosphotyrosine proteins detected up to 24 hours after serum was added did not differ significantly from that seen 5 hours after serum addition. Laser densitometry of the chemilumigrams obtained revealed that the level of tyrosine phosphorylated proteins was reduced from 20 - 40 % in extracts obtained from heparin-treated plates were reduced 5 hours and 18 hours after serum stimulation. Interestingly, the reduced levels of tyrosine-phosphorylated proteins is of the same magnitude as the reduction seen in the number of heparin-treated srSMC that entered the cell cycle after serum stimulation.

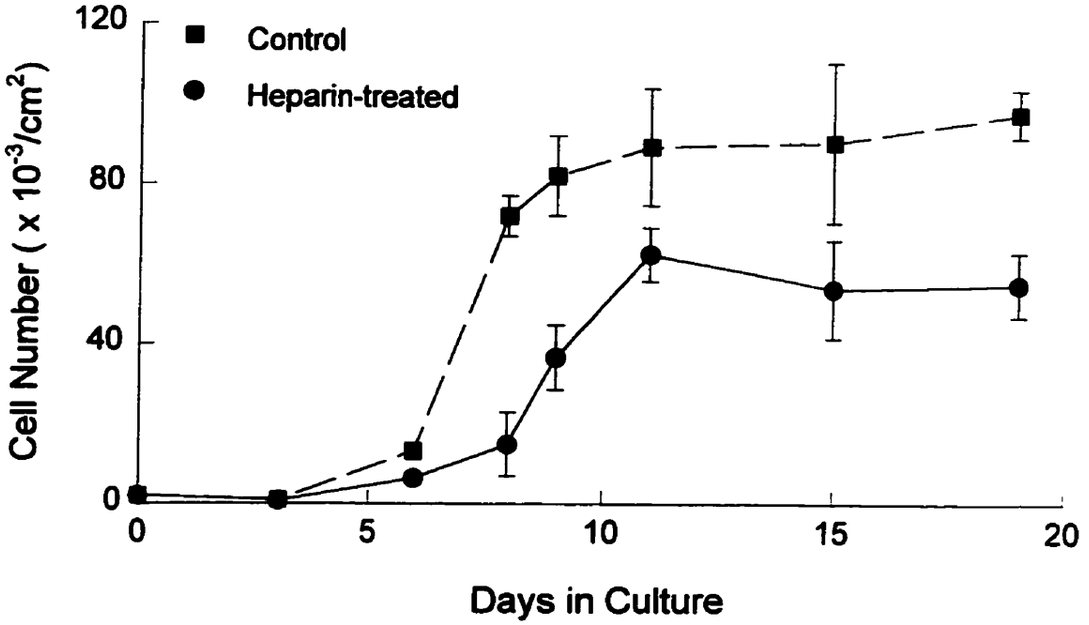
FIGURE 14. Effects of heparin on the growth characteristics of canine tracheal SMCs in primary culture.

Initial seeding density was 5×10^3 cells/cm^2. Heparin was added to treatment plates approximately 36 hours after initial seeding of the primary cultures. Error bars represent standard error of the means obtained form 4 experiments completed in triplicate.

A. Growth curves for tracheal SMCs form in DMEM containing 10% FBS in the presence (●) and absence of 100 μg/ml sodium heparin (■).

B. Total protein extracted in cell lysates from culture plates of tracheal myocytes in grown in primary culture in the primary culture in the presence (●) and absence of 100 μg/ml sodium heparin (■).

A: Cell Number



B: Total Protein

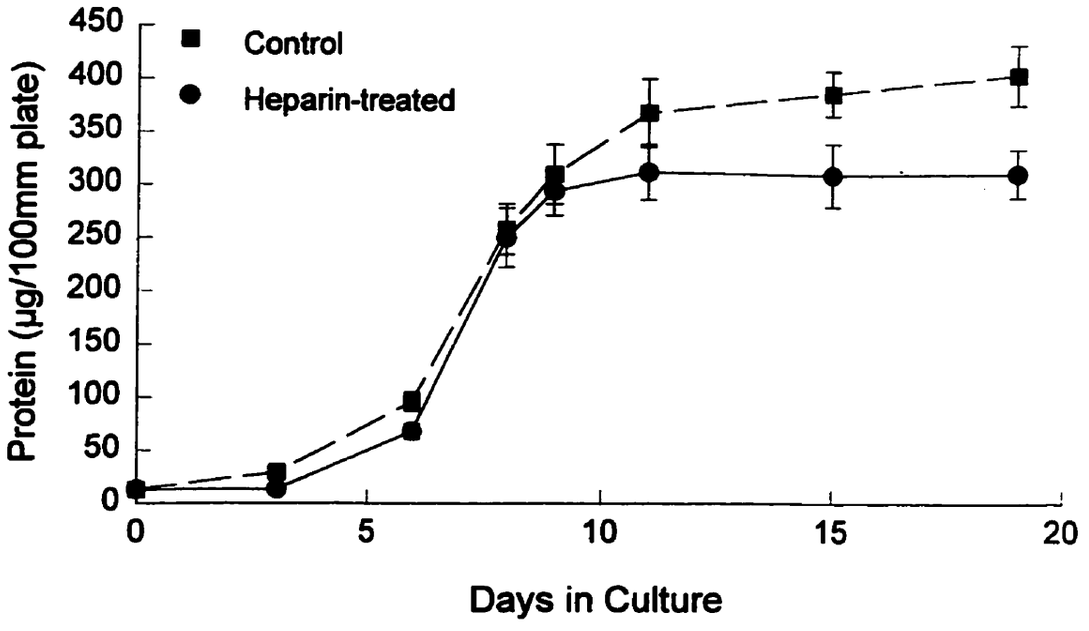


FIGURE 15. Effects of heparin on the temporal expression of phenotypic marker proteins in primary airway SMC cultures.

Protein contents were determined by protein electrophoresis, immunoblotting and laser densitometry. For all plates error bars represent standard error of the means obtained from four different experiments. Data on the ordinate in each plot are represented by arbitrary units obtained by normalizing laser densitometric data with total protein loaded per well on the gels and the number of cells on the plates from which the crude cell lysates were obtained.

A. and **B.** smMHC (200-204 kDa) and nmMHC (196-198 kDa) were separated using 4% SDS-PAGE. Total protein loaded onto each well was 7.5 μg.

C. and **E.** sm-α-Actin (43 kDa) and vimentin (57 kDa) were separated using 10% SDS-PAGE. Total protein loaded into each well was 5 μg for actin and 10 μg for vimentin.

D. MLCK (138 kDa) was separated using 7.5% SDS-PAGE . Total protein loaded into each well was 7.5 μg.

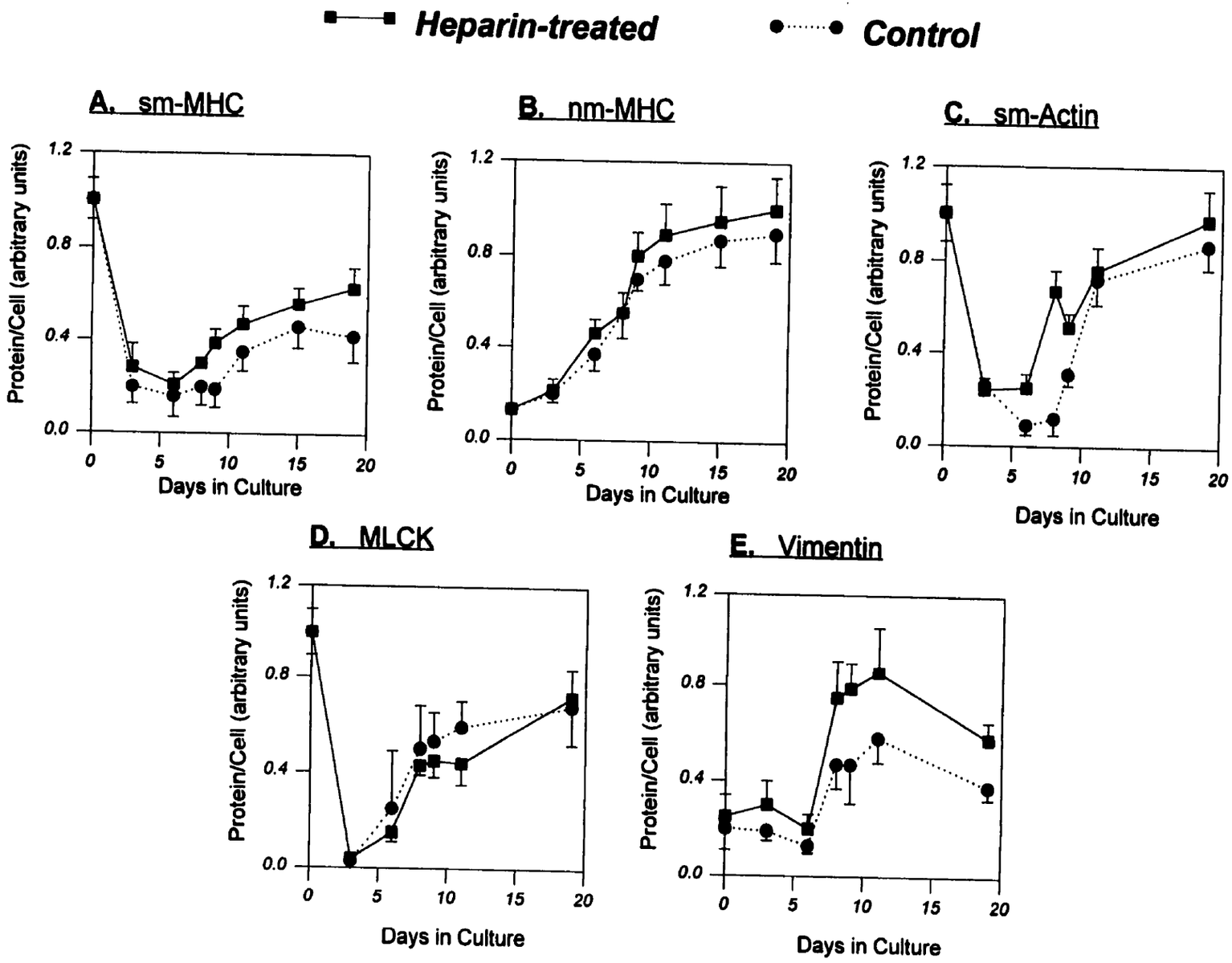
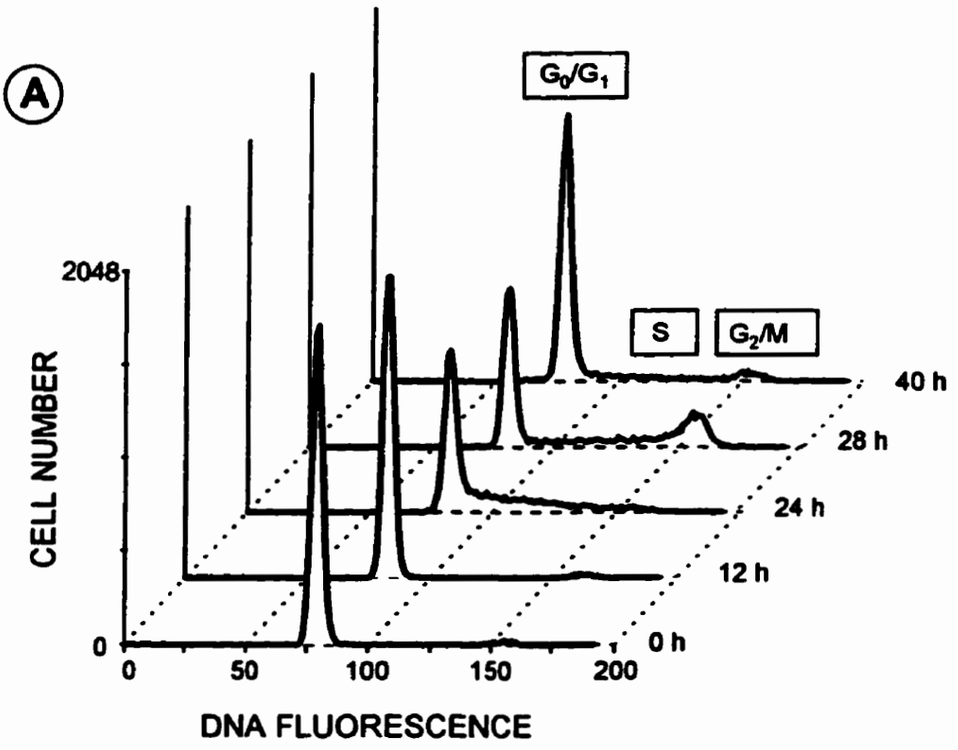

Heparin-treated
Control
A. sm-MHC
B. nm-MHC
C. sm-Actin
D. MLCK
E. Vimentin
Protein/Cell (arbitrary units)
Days in Culture
0.0
0.4
0.8
1.2
0
5
10
15
20

FIGURE 16. Cell cycle analysis of primary cultured tracheal SMC proliferation by flow cytometry.

Representative DNA histograms showing the distribution of propidium iodide-stained, airway SMCs throughout the cell cycle, at various times after growth arrested cells were stimulated with media containing 10% FBS (times indicated on the right side of each histogram). The histograms labelled as 0 h indicate the distribution of cells after 96 hours of growth arrest. PI fluorescence was captured and plotted on the abscissa using 256 channel resolution. The position of the cell populations recognized as being in either G_0/G_1, S, or G_2/M phases of the cell cycle by the PARA 1 software are indicated. Each histogram represents data obtained from 5000 cells.

A. Progression of control airway SMCs through the cell cycle after serum-stimulation.

B. Progression of airway SMCs through the cell cycle, in the presence of 100 μg/ml heparin, after serum stimulation.


A
G_0/G_1
S
G_2/M
2048
0
CELL NUMBER
40 h
28 h
24 h
12 h
0 h
0
50
100
150
200
DNA FLUORESCENCE

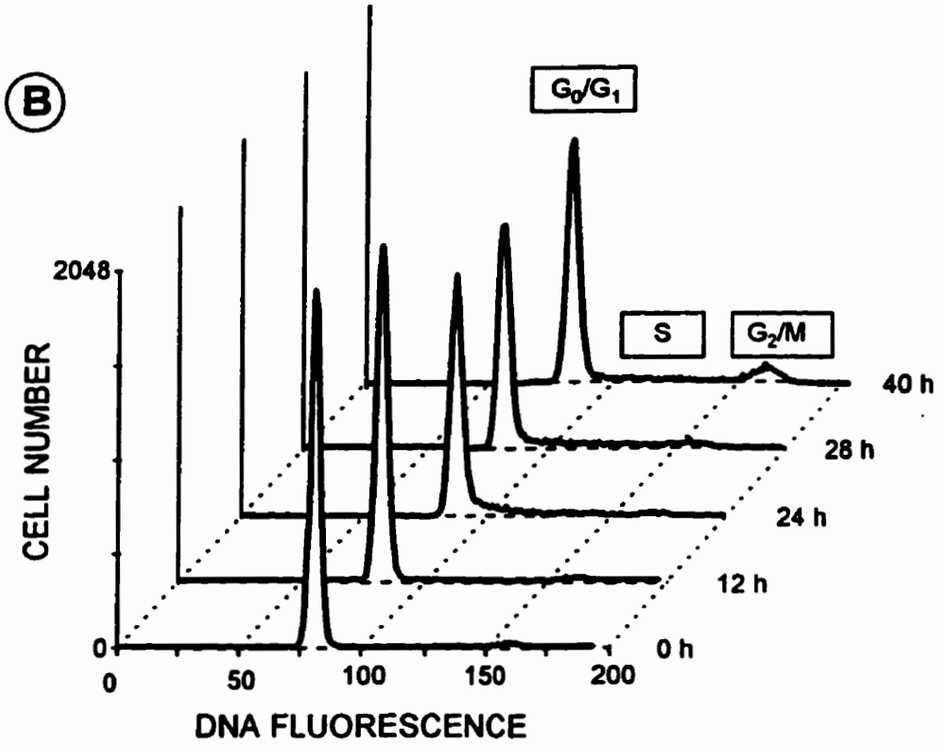

B
G_0/G_1
S
G_2/M
2048
0
CELL NUMBER
40 h
28 h
24 h
12 h
0 h
0
50
100
150
200
DNA FLUORESCENCE

FIGURE 17. Histogram depicting the fraction of arrested SMCs which entered the cell cycle from G_0/G_1 after serum-stimulation in the presence and absence of heparin.

The response of arrested SMCs to serum-stimulation (10% FBS) in the absence (Control) and presence of heparin - 100 µg/ml (Heparin Treated) is compared. Cell cycle entry was assessed determining the fraction of all cells analysed that were in either S- or G_2/M phase by flow cytometry and measuring propidium iodide staining for cellular DNA content. Bars represent the mean of three experiments done in triplicate; error bars represent standard error. (* - Determined to be significantly different from Control values by unpaired Student's t-test, $p<0.05$).

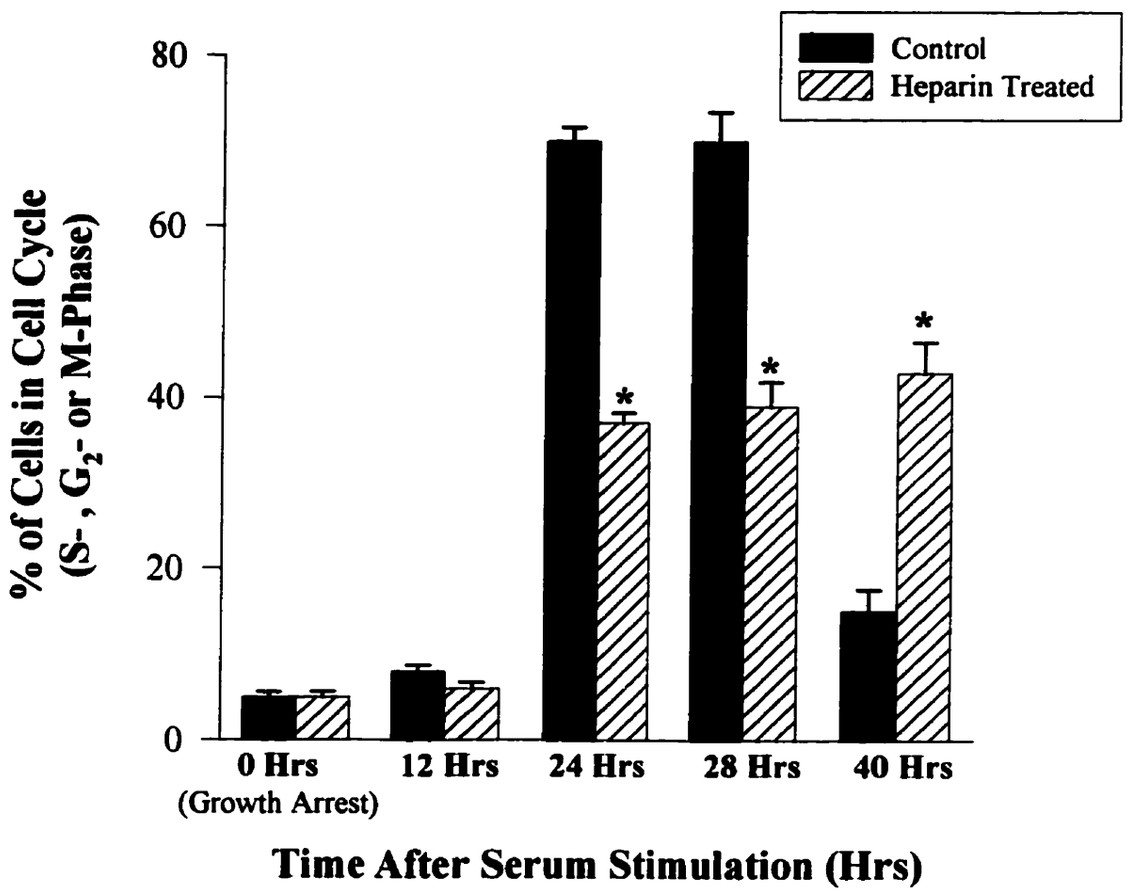
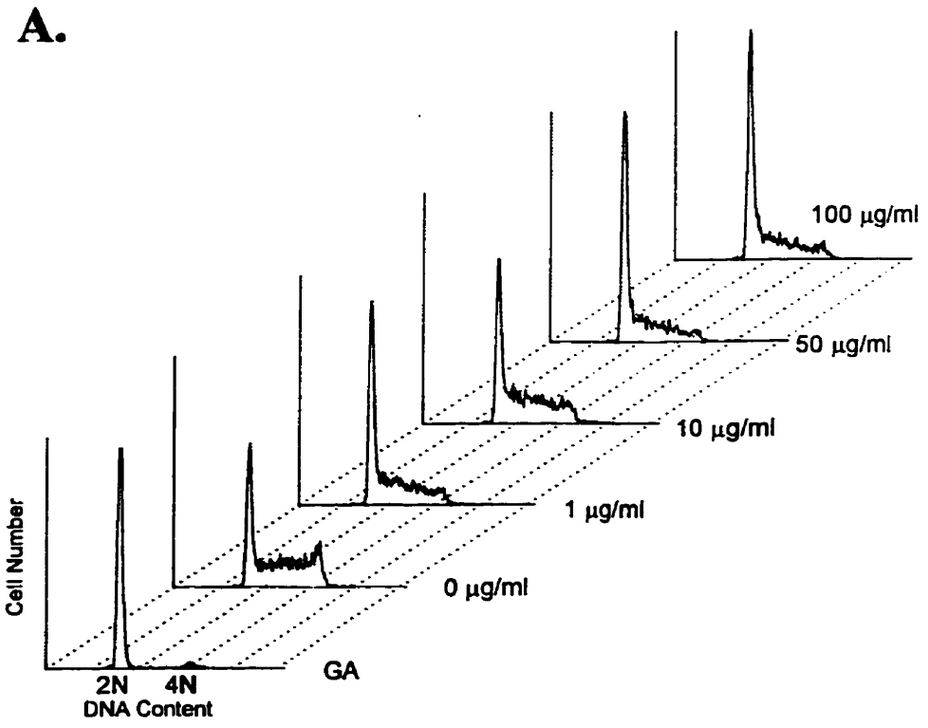

Control
Heparin Treated
80
60
40
20
0
% of Cells in Cell Cycle
(S- , G2- or M-Phase)
*
*
*
0 Hrs
(Growth Arrest)
12 Hrs
24 Hrs
28 Hrs
40 Hrs
Time After Serum Stimulation (Hrs)

FIGURE 18. Flow cytometric analysis of the concentration-response relationship of tracheal SMCs to heparin-induced inhibition of cell cycle entry.

The fraction of arrested tracheal SMCs that entered the cell cycle 28 hours after serum-stimulation in the presence of different concentrations of sodium heparin were compared using flow cytometry in conjunction with propidium iodide staining.

A. Representative PI-stained, DNA histograms obtained from cultured tracheal myocytes at growth arrest (GA) and 28 hours after stimulation with media containing 10% FBS and different concentrations of heparin (concentrations in μg/ml are shown to the right of each histogram). Each histogram represents data obtained from 5000 individual SMCs. The abscissa (DNA Content) represents propidium iodide fluorescence -- 2N and 4N refer to the position of cells in G_0/G_1 and G_2/M phases respectively.

B. Heparin concentration-response of SMCs. Bars represent the fraction of serum-responsive SMCs which were inhibited, in the presence of different concentrations of heparin, from entering the cell cycle after serum was added. Values are means of three experiments done in triplicate; error bars represent standard errors.


A.
100 µg/ml
50 µg/ml
10 µg/ml
1 µg/ml
0 µg/ml
GA
Cell Number
2N
4N
DNA Content

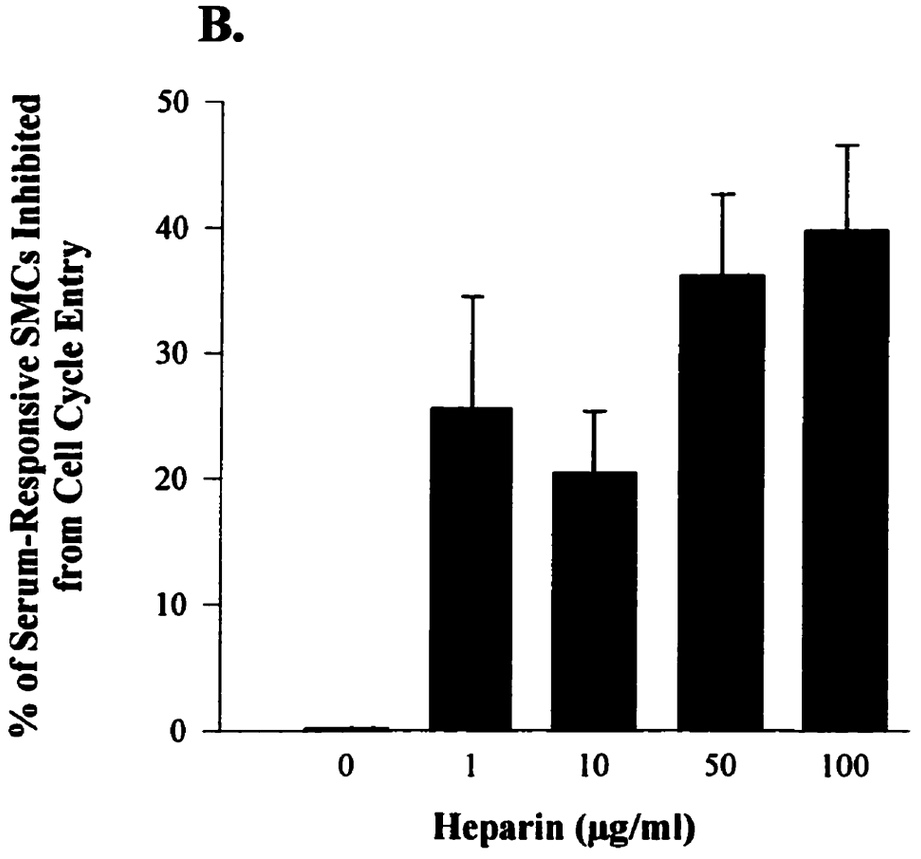

B.
% of Serum-Responsive SMCs Inhibited from Cell Cycle Entry
50
40
30
20
10
0
0
1
10
50
100
Heparin (µg/ml)

FIGURE 19. Bi-variate FACS analysis of the effects of heparin on the cell cycle distribution of serum-stimulated airway SMCs.

Growth arrested, cultured canine tracheal SMCs were stimulated to proliferative in media containing 10% FBS for 68 hours, then the cells were pulsed with BrdU (10μM) for 4 hours. Incorporation of BrdU into DNA-synthesizing cells and cell cycle distribution was assessed by FACS using a monoclonal FITC-anti-BrdU antibody and propidium iodide staining to measure cellular DNA content respectively.

Depicted are dot plots of the cell cycle distribution -- DNA content is represented by PI fluorescence along the abscissa. BrdU incorporation is represented by the log of FITC fluorescence on the Y-axis. Scale corresponds to the frequency distribution of cells in each plot. Each plot represents data obtained from 10,000 individual cells. Five different experiments were carried out.

A. A negative control plot of cells that were serum-stimulated, but were not pulsed with BrdU.

B. BrdU-pulsed, serum-stimulated cells in the absence of heparin.

C. BrdU-pulsed, serum-stimulated cells in the presence of 100 μg/ml sodium heparin.

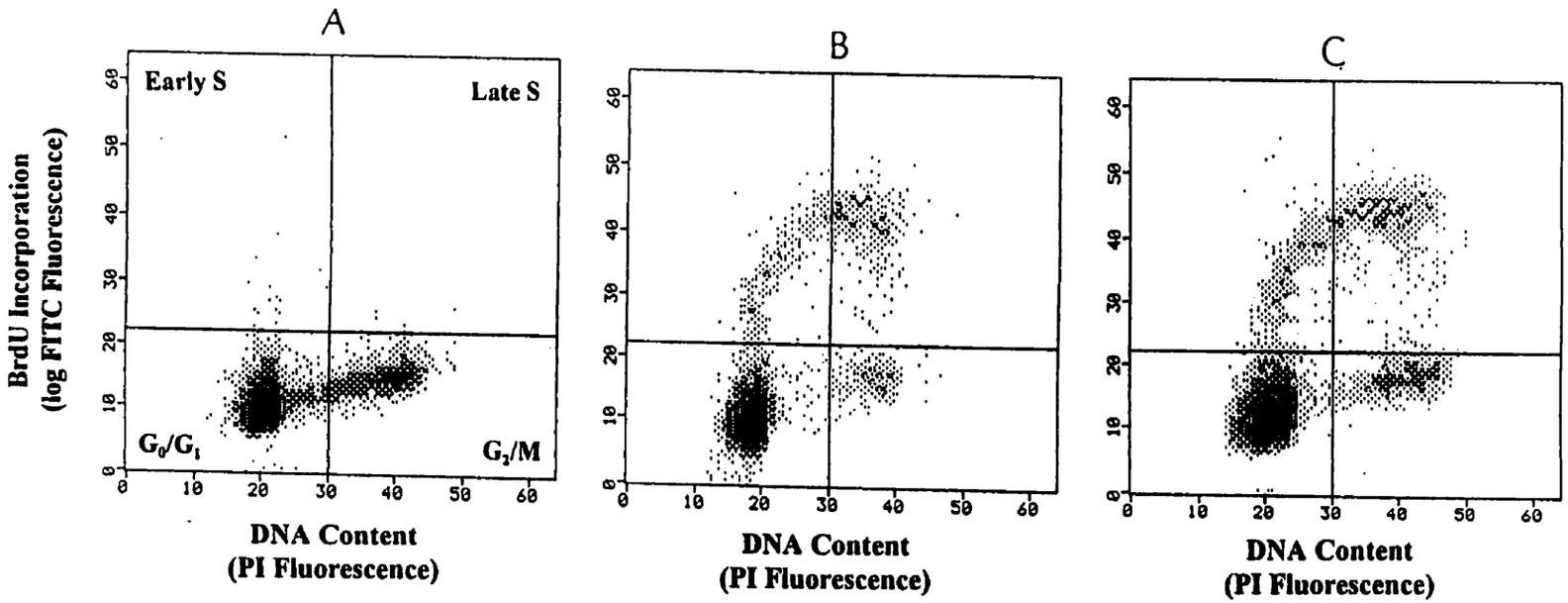

A
B
C
Early S
Late S
G_0/G_1
G_2/M
BrdU Incorporation
(log FITC Fluorescence)
DNA Content
(PI Fluorescence)
DNA Content
(PI Fluorescence)
DNA Content
(PI Fluorescence)

FIGURE 20. Distribution of airway srSMCs which entered the cell cycle in the presence and absence of heparin.

Distribution of cells was determined by bivariate FACS analysis of BrdU incorporation and DNA content. For control cultures 67.5% of all cells were in G_0/G_1 phase and whereas 78% of heparin-treated SMCs were in G_0/G_1 phase. The values in this figure represent the distribution of cells exclusive of those in G_0/G_1 phase. * - denotes data which is significantly different between control and heparin at a specific cell cycle phase ($p < 0.01$, $n = 5$).

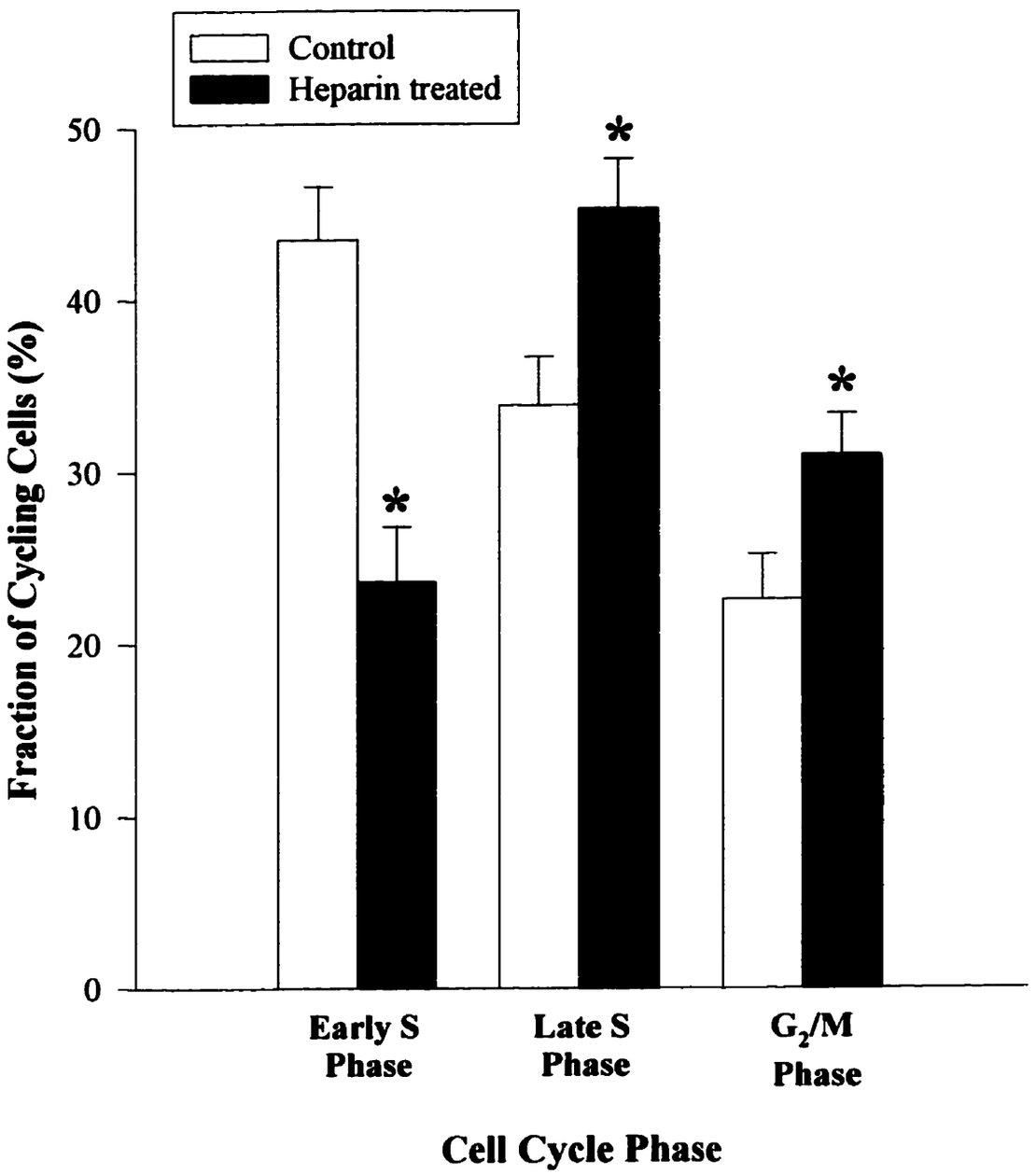

Control
Heparin treated
50
40
30
20
10
0
Fraction of Cycling Cells (%)
*
*
*
Early S Phase
Late S Phase
G2/M Phase
Cell Cycle Phase

FIGURE 21. Autoradiograms obtained using the in gel assay for MAP kinase activity in cultured tracheal myocytes in the presence and absence of heparin.

Growth arrested cells (0 minutes) were stimulated with serum with (+) or without (-) 100 μg/ml sodium heparin and cell lysates were obtained from 2 to 60 minutes thereafter to analyse MAP kinase activity using an in gel assay. Arrows indicate the position of the 42 kDa and 44 kDa MAP kinase bands (confirmed by Western blotting - data not shown). Bands seen on the autoradiograms indicate the position of proteins, fractioned by SDS-PAGE, that phosphorylated the gel-associated protein substrate (Major Basic Protein) during the enzyme assay. A negative control is also shown -- the panel shows the pattern seen, 20 minutes after serum addition, when no substrate was added to the gel.

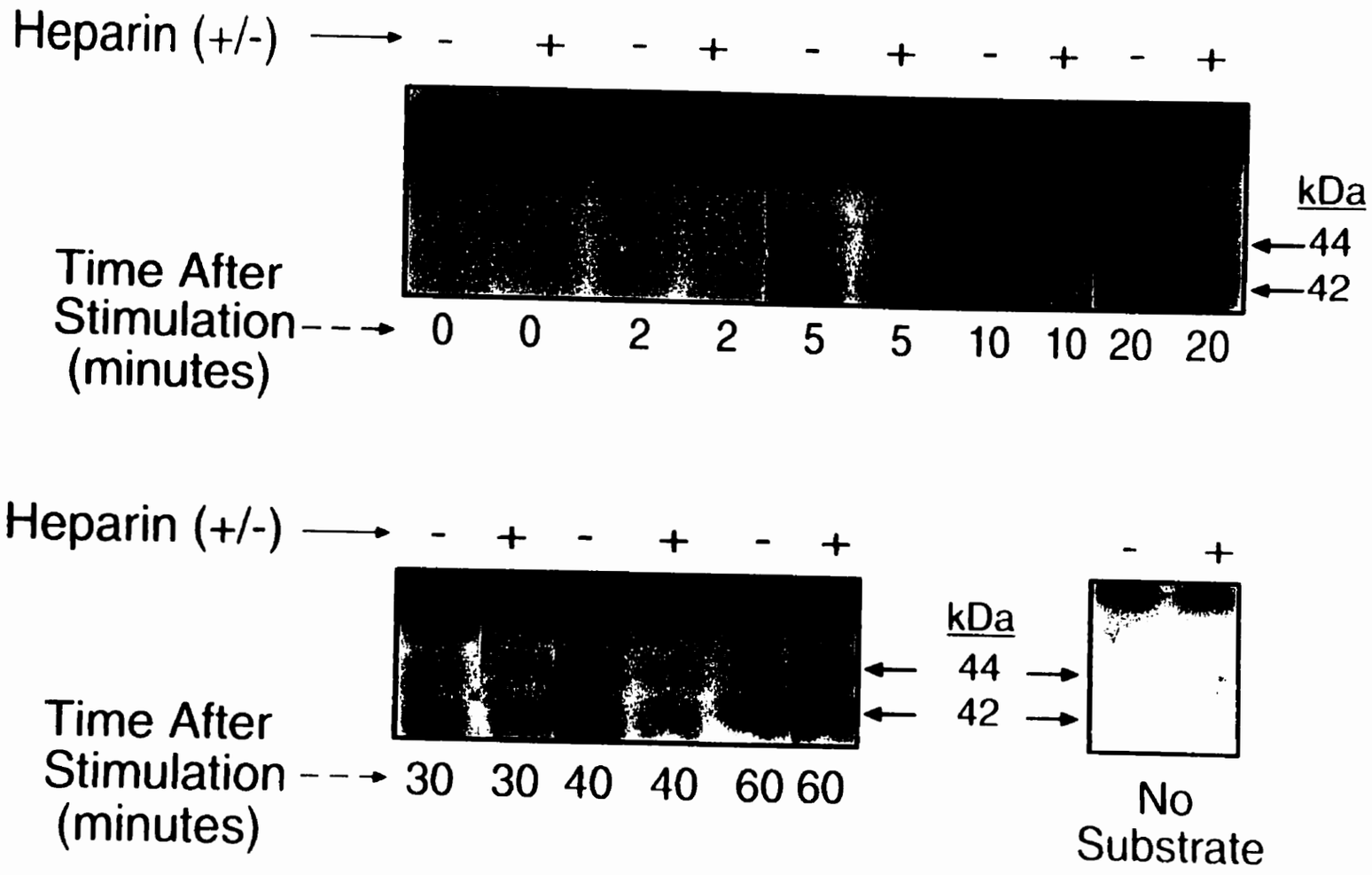

Heparin (+/-)
- + - + - + - + - +
kDa
44
42
Time After Stimulation (minutes)
0 0 2 2 5 5 10 10 20 20
Heparin (+/-)
- + - + - +
- +
kDa
44
42
Time After Stimulation (minutes)
30 30 40 40 60 60
No Substrate

FIGURE 22. Effects of heparin on temporal MAP kinase activation in cultured canine tracheal myocytes after serum-stimulation.

Temporal MAP kinase activity in control cells (●) and in heparin-treated (100 μg/ml) cells (■), determined by laser densitometry, obtained from autoradiograms using the in gel assay. Activity was normalized to the total protein loaded into each well lane. Error bars represent standard errors obtained from seven experiments. * - values that were significantly different ($p<0.01$) - ANOVA followed by Duncan's New Multiple Range Test

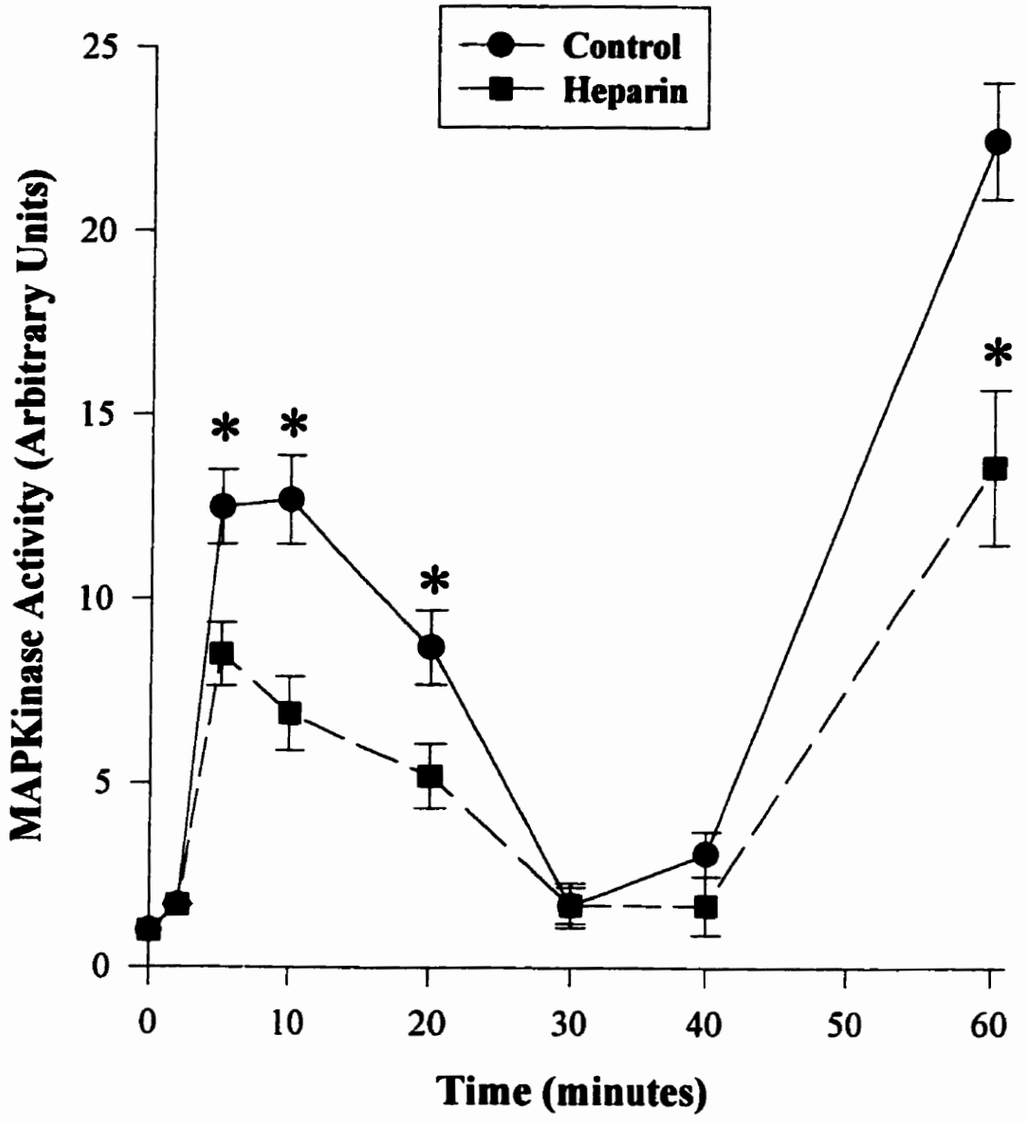
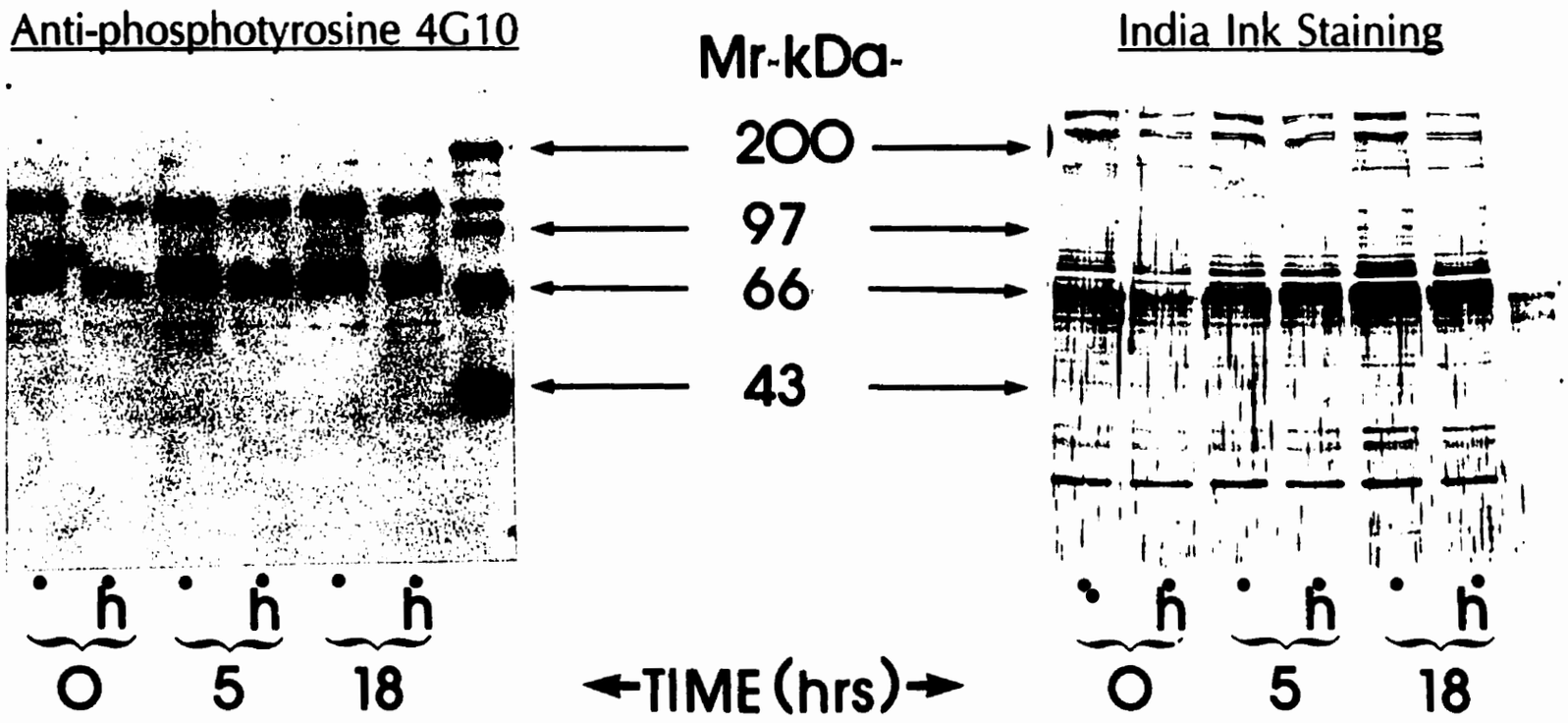

Control
Heparin
MAPKinase Activity (Arbitrary Units)
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (minutes)
*
*
*
*

FIGURE 23. Detection of tyrosine phosphorylation using an immunoblot assay in canine tracheal myocytes grown in the presence and absence of heparin.

Left panel: A chemilumigram obtained using monoclonal anti-phosphotyrosine antibody. For this assay, cell lysates were obtained 5 hours and 18 hours after serum stimulation of growth arrested cultures. Cultures treated with 100 μg/ml sodium heparin are denoted with an "h". Each well was loaded with 25 μg of crude cell lysate and proteins were fractionated by 10% SDS-PAGE.

Right panel: India Ink stain of the protein blot used to obtain the chemilumigram on the left - staining intensity demonstrates that equal protein loads were loaded in each lane.


Anti-phosphotyrosine 4G10
Mr-kDa-
India Ink Staining
200
97
66
43
h
h
h
h
h
h
0
5
18
TIME (hrs)
0
5
18

5.5 DISCUSSION

The results reported here confirm previous reports (Johnson et al., 1995; Kilfeather et al., 1995) that exogenous heparin possesses anti-proliferative activity for airway SMC primary cultures. In addition, data suggesting that subpopulations, differing in sensitivity to heparin, may comprise the overall population of airway SMCs in primary culture. With heparin treatment, cultured airway SMCs grew in a "cobblestone" pattern, and confluent cell density was approximately 30% lower than seen in control plates. Control cultures exhibited a typical "hill-and-valley" morphology, characterized by areas of focal overgrowth which were not evident in heparin-treated cultures. Interestingly, confluent cultures of heparin-resistant arterial SMCs have been reported to lack a "hill and valley" appearance and have lower cell densities than confluent control cultures (San Antonio et al., 1993; Caleb et al., 1996). This correlation which our observations supports the idea that heparin-insensitive airway SMCs contribute to the proliferation of primary cultures grown in the presence of heparin.

Based on electron microscopic analysis and immunocytochemical data, heparin-mediated inhibition of phenotypic modulation of arterial SMCs in culture has been reported (Chamley-Campbell and Campbell, 1981; Grainger et al., 1993). However, though exogenous heparins effectively inhibit SMC proliferation after balloon catheter-induced injury, a switch from α- to β-actin transcription in arterial SMCs immediately after injury is not affected (Clowes et al., 1988). Heparin treatment has been reported to induce reexpression of sm-α-actin and smMHC in vascular SMCs after arterial injury and in culture (Clowes et al., 1988; Desmoulière et al., 1991; Grainger et al., 1993). San

Antonio et al. (1993) reported that the percentage of heparin-resistant vascular SMCs that expressed sm-α-actin increased dramatically after exposure to heparin. It has been postulated that heparin maintains arterial SMCs in a quiescent state by controlling actin and protein expression (Desmoulière and Gabbiani, 1992).

The semi-quantitative immunoblot data reported in our studies indicate that spontaneous, phenotypic modulation of contractile airway SMCs in culture is not inhibited by heparin. Temporal expression patterns of sm-α-actin, smMHC, and MLCK suggest that, for airway SMCs, heparin does not effect reinduction of these mature-state phenotypic markers. Furthermore, heparin was not effective in inhibiting the accumulation of nmMHC, a putative marker for immature airway SMCs (Halayko et al., 1996a). Vimentin, an intermediate filament protein, was increased in post confluent, heparin-treated airway SMCs, the significance of this finding is unclear. Vimentin expression is associated with the immature SMC phenotype (Kocher et al., 1985) and the protein has been shown to be increased in content in hypertrophic SMCs (Turla et al., 1991; Malmqvist. 1994).

The findings reported in this study appear to contrast with some data obtained from arterial SMCs which indicate that the phenotypic state expressed by vascular SMCs is heparin-sensitive. Different smooth muscles differ widely in pharmacologic and contractile properties (Somlyo and Somlyo, 1994). Indeed, developmental paradigms indicate that arterial SMCs are segregated from airway SMCs on the basis of the site and source of progenitor cells (Le Lièvre et al., 1975; reviewed by Owens, 1995). Heterogeneity between different smooth muscles has also been observed at the molecular

level in which the content of marker proteins differ and smooth muscle-specific genes are under different transcriptional regulatory controls (Li et al., 1996b; Halayko et al., 1996a). Therefore it is, perhaps, not surprising that airway and arterial SMCs differ in their response to exogenous heparin with regard to expression of phenotypic state.

Heterogeneity in the responsiveness of vascular SMCs to a variety of potent mitogenic stimuli has been reported (Cook et al. 1994; Saltis et al. 1995; Majack et al. 1996). These observations are in concordance with the heterogeneous response seen for cultured airway SMCs to serum stimulation. Serum-stimulation of growth arrested airway SMC cultures consistently failed to provoke cell cycle entry in 30-40% of the SMCs present. It is not clear whether the heterogeneous response was due to the presence of stable serum insensitive and serum-responsive subpopulations. These experiments were carried out on 70% confluent cultures, hence, it is likely that cells in different areas of the plates differed in the degree of cell-to-cell contact present due to differences in cell density. Such artificial differences could account for the heterogeneous response we recorded during cell cycle analysis.

The data obtained during analysis of the effects of heparin on cell cycle progression suggest that divergent subpopulations of airway SMCs existed in growth arrested primary cultures. Figure 24 is a schematic representation of the hypothetical distribution of these different airway SMC subpopulations. The first major group identified in our studies was a heparin-sensitive, srSMC subtype, constituting approximately 40% of all srSMCs, which was inhibited from progressing to S phase after serum stimulation in the presence of heparin (labelled heparin-insensitive srSMC in

Figure 24). Due to the all-or-none nature of the heparin concentration-response characteristics of the airway srSMCs, this heparin-sensitive subgroup appears to constitute a distinct population of airway SMCs. Interestingly, Johnson et al. (1995) have shown that heparin inhibited DNA synthesis, measured by [^{3}H]thymidine uptake, by the same order of magnitude as observed in this investigation for inhibition of progression of srSMCs from G_0 to S phase.

Approximately 60% of the cells traversed the cell cycle in the presence of heparin. Hence, these cells constituted the second subpopulation of growth arrested, cultured airway srSMCs. This subpopulation appeared insensitive to early cell cycle event interference by heparin (labelled heparin-insensitive srSMC in Figure 24). These heparin-insensitive cells were not inhibited from G_0 to S phase transit; however, they appeared to be slowed during transit in latter cell cycle phases compared to srSMCs in the absence of heparin. The transit time for untreated srSMCs was about 38 hours whereas heparin-insensitive airway srSMCs required in excess of 44 hours to complete a single cell cycle in the presence of heparin. Bivariate cell cycle analysis of BrdU incorporation and DNA content revealed that heparin-treated cells accumulated in G_2/M phases indicating that the cells were being slowed during these phases and this could account for the prolonged cycle time of heparin-insensitive srSMCs in the presence of heparin. These data mimic those from similar experiments by Grainger et al. (1993) in which the heparin responsiveness of cultured arterial SMCs was studied. It is not clear whether a population of heparin-insensitive srSMCs might exist that are resistant to heparin effects in late cell cycle phases. The data obtained in our studies suggest that if a

population of heparin-resistant airway SMCs do exist, they would constitute a small fraction of all cells (Figure 24). It is not clear whether such a population may be the result of the culture process. Furthermore, the potential role of such a population *in vivo* is not obvious at this time.

Arterial smooth muscle is comprised of a heterogeneous population of SMCs which differ in morphology, electrophysiologic properties and growth characteristics in response to mitogenic stimuli *in vivo* and in culture (Lemire et al., 1994; Archer et al., 1996; Holifield et al., 1996; Majack et al., 1996). Numerous clonal cell lines have been isolated and characterized including several heparin-resistant cultures (San Antonio et al., 1993; Bârzu et al., 1996; Caleb et al., 1996). Recent work suggests that the key feature differentiating heparin-sensitive SMCs from their -resistant counterparts is the ability of the cells to express surface receptors for heparin which mediate endocytosis of the exogenous glycosaminoglycan (Letourneur et al., 1995). The findings presented in this investigation support a hypothesis that suggests airway smooth muscle is also composed of distinct, subpopulations of SMCs which may be important for subserving a variety of physiological functions. It appears that arterial SMCs of a specific phenotypic state may be responsible for intimal thickening and atherosclerotic plaque formation (Schwartz et al., 1986; Holifield et al., 1996). The possibility that different subtypes of airway SMCs may exist raises the possibility that different airway SMCs may contribute in specific ways to altered airway responsiveness, airways remodelling, medial thickening and fibrosis associated with the pathogenesis of bronchial asthma.

The biochemical and subcellular mechanisms by which heparin mediates

inhibition of SMC proliferation appears to be complex and to date remains unresolved. Heparin-sensitive and -insensitive pathways for arterial SMC proliferation may exist (Wright et al., 1989; Ottlinger et al., 1993; Geary et al., 1995). Therefore, a number of mechanisms, impacting at a different points in the cell cycle, have been postulated for heparin-induced inhibition of proliferation (Reilly et al., 1986 and 1987; Wright et al., 1989; Grainger et al., 1993; Ottlinger et al., 1993). Since the majority of these studies were carried out using vascular SMC cultures, the data obtained regarding gene expression or enzyme activity represented the average response of all cells on a culture plate. The finding that arterial SMCs are heterogeneous in their response to heparin, explains to some degree, the breadth and sometimes contradictory nature of the mechanisms postulated for heparin-mediated inhibition of SMC proliferation.

The MAP kinase family of serine and threonine specific kinases is activated in response to a wide range of mitogenic and other factors and is a key intermediate in cell signalling (Ruderman, 1993). MAP kinase activation has been shown to occur rapidly after mitogen stimulation, hence, it appears to be an important step in the regulation G_0/G_1 transition (Sturgill et al., 1991; Ruderman, 1993; Watson et al., 1993; Kelleher et al., 1995). In addition, sustained activation of the 42 and 44 kDa MAP kinase isoforms has been shown to be important in the signalling of a number of mitogenic factors for airway SMCs (Kelleher et al., 1995). In this investigation we have shown that heparin inhibited, by approximately 40%, both the activation of 42 and 44 kDa MAP kinase and the overall level of tyrosine phosphorylated proteins in serum-stimulated airway SMCs. Ottlinger et al. (1993) have previously reported that MAP kinase activation in response to

serum and phorbol ester stimulation was inhibited by heparin in arterial SMCs. It may be of note that the reduced levels of activated MAP kinase and tyrosine phosphorylation correlate with and are of the same order of magnitude as the reduction in the number of srSMCs that progress beyond G_0/G_1 in the presence of heparin. This correlation could indicate that sensitivity to heparin of signalling pathways involving MAP kinase may represent a point of distinction between heparin-sensitive and -insensitive/ -resistant airway SMC subtypes.

The analysis of tyrosine phosphorylated proteins has provided key insights into the identity of proteins involved in various signalling pathways (Sun and Tonks, 1994). Hirst et al. (1995) have demonstrated, using cultured rabbit tracheal myocytes, that a number of proteins become tyrosine phosphorylated within 5 minutes of serum stimulation. Antagonism of tyrosine phosphorylation using selective blockers inhibited airway SMC proliferation indicating that the activity of tyrosine kinases is a requisite component for mitogenic signalling in these cells (Hirst et al., 1995). The major tyrosine-phosphorylated proteins identified in our study were approximately 190, 116, 70-80, 58 and 53 kDa. It is not clear why tyrosine phosphorylated MAP kinase isoforms were not observed on the immunoblots; no samples were collected before 4 hours after serum-stimulation of arrested SMCs, hence, due to the rapid and transitory nature of MAP kinase activation, it is possible that peak phosphorylation may have occurred prior to sampling.

Green et al. (1993) demonstrated *in vitro*, that heparin released from mast cells in response to antigen challenge was inhibited by more than 70% in the presence of β_2-

agonists. Ahmed et al. (1992 and 1994), have demonstrated that heparin is an endogenous anti-inflammatory agent which can block allergen induced bronchoconstriction in sensitized sheep. Hence, the anti-proliferative effects of heparin on SMC growth (Johnson et al., 1995) has received interest from asthma investigators due to the high concentrations of it in airway mast cells and the ability of β_2-agonists, a common therapeutic intervention for asthma, to inhibit mast cell degranulation. We have found that airway SMCs appear to be comprised of heterogeneous subpopulations differing in response to exogenous heparin. This suggests that distinct subpopulation of airway SMCs may play critical roles in physiologic and pathophysiologic responses to different stimuli and may play distinct roles in alterations in airway responsiveness and structural remodelling. The elucidation of specific characteristics of airway SMC subpopulations and the factors which regulate them will be invaluable in determining future directions in the development of newer, more specific therapies for bronchial asthma.

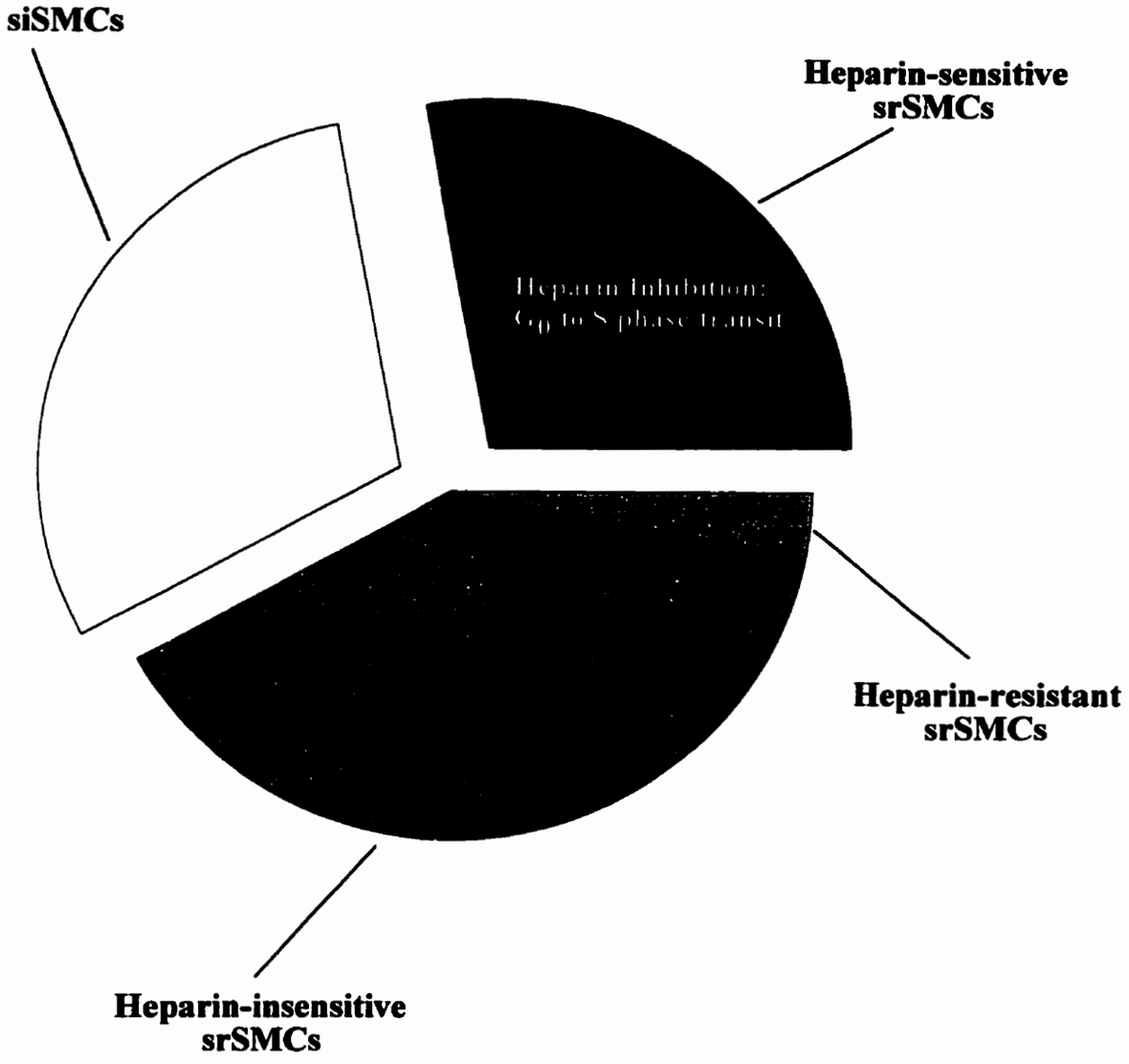
5.6 SUMMARY

Heparin and other mediators are released from pulmonary mast cells after antigen challenge. An endogenous suppressor of allergic inflammation, heparin also inhibits replication of cultured vascular and airway SMCs. The mechanism of this anti-mitotic effect is unclear. We compared the response of primary canine tracheal SMC cultures to foetal bovine serum in the presence of heparin with that of untreated control cultures. Heparin (100μg/mL) reduced tracheal SMC doubling rate by 40%. Temporal expression

of SMC phenotypic markers (myosin, actin, caldesmon, calponin and vimentin), assessed by immunoblotting and flow cytometry, was similar in heparin-treated and control SMC. Responses of quiescent cultured SMC were assessed by: 1) analysis of cell cycle progression using flow cytometry to measure DNA synthesis and content using bromodeoxyuridine incorporation and propidium iodide staining, and 2) measurement of MAP kinase activation by an in-gel assay and tyrosine kinase activation by immunoblotting; cells arrested in serum-free media were stimulated with heparin-enriched or control media containing serum. Cell cycle analysis revealed the fraction of SMCs which entered the cycle decreased in a dose dependent fashion when heparin was included (maximum decrease, 40% at 100μg heparin/ml). G_0 to G_1 transit time (16 hrs) was not different for the sub-population of SMCs which entered the cell cycle even in the presence of heparin. Temporal activation of MAP kinase in cultures was unaltered by heparin, but, peak activity and total activity were decreased by 50% and 20% overall respectively. Tyrosine kinase activity in response to serum stimulation was also inhibited in the presence of heparin. These data indicate that phenotypic modulation of cultured airway SMC is unaffected by heparin. However, the rate of increase in SMC in culture is inhibited by reduction of the number of cells responding to serum stimulation. Reduction in recruitment of tracheal SMC into the cell cycle by heparin may be related to inhibition of key signalling pathways important for regulation of proliferation in a specific sub-population of cells.

FIGURE 24. Hypothesized distribution of airway SMC subpopulations based on differences in response to serum stimulation and heparin-mediated inhibition of proliferation.

Based on cell cycle analysis studies, serum insensitive SMCs (siSMCs) constitute about 30% of growth arrested, cultured canine tracheal SMCs. The remaining serum-responsive SMCs (srSMCs) appear to be subdivided into two major groups, firstly, heparin-sensitive srSMCs and, secondly, heparin-insensitive srSMCs. These subpopulations differ in sensitivity to heparin-mediated inhibition of replication due to apparent differences in the site of action of heparin in the cell cycle. Heparin-resistant srSMCs may exist and have been included here, though it is not clear what fraction of the total population they might represent. These srSMCs are unaffected by heparin treatment.


siSMCs
Heparin-sensitive srSMCs
Heparin Inhibition: G0/G1 to S phase transit
Heparin-resistant srSMCs
Heparin-insensitive srSMCs

6. PHENOTYPIC HETEROGENEITY OF MYOCYTES FROM AIRWAY SMOOTH MUSCLE

6.1 BACKGROUND

Mature SMCs are phenotypically diverse mesenchymal cells capable of subserving multiple physiologically important functions, which include contraction, synthesis and deposition of extracellular matrix proteins, and proliferation (Campbell et al., 1988). Myocytes within the same smooth muscle and of different smooth muscles appear to be derived from multiple mesenchymal lineages (Le Lièvre et al., 1975; Frid et al, 1994; Topouzis and Majesky, 1996). Hence, the medial layers of the vasculature are comprised of phenotypically heterogeneous myocytes differing in regional and local distribution (Frid et al., 1994; Wohrley et al., 1995; Bochaton-Piallat et al., 1996; Holifield et al., 1996). For example, individual arterial myocytes differing in smMHC isoform expression (Meer and Eddinger, 1996) and in potassium channel composition (Archer et al., 1996) appear to be distributed heterogeneously in arteries. This could account for differences in regional reactivity to contractile agonists. Distinct subsets of arterial SMCs, which retain the capacity rapidly revert to an immature state, appear to subserve restenotic and atherogenic lesion formation in endothelium-injured blood vessels (Schwartz et al., 1986; Aikawa et al., 1993; Bochaton-Piallat et al., 1996; Holifield et al., 1996). Hence, characterization of the distribution of phenotypically divergent myocytes populations within a smooth muscle is an important requirement for elucidation of regulation of normal physiological and pathophysiological phenomenon.

Airway smooth muscle plays a critical role in determining airflow resistance. It is the acute spasm of the bronchi that manifests exacerbations of asthma. Airway smooth muscle has been shown to possess regionally heterogeneous contractile and

pharmacologic properties (Chitano et al., 1993 and 1996; Ma et al., 1996). Chronic bronchial asthma is associated with airways remodelling which is often associated with thickening of medial smooth muscle layers (Ebina et al., 1993). Interestingly, regional differences in the contribution of SMC hyperplasia and/or hypertrophy to increased airway smooth muscle mass appear to exist (Ebina et al., 1993). These data indicate that bronchial myocytes from distinct airways may respond in a dissimilar fashion to mitogenic, inflammatory mediators. It has been suggested that pathogenesis of airways remodelling may parallel the processes occurring in focal atherosclerotic lesion formation (Halayko and Stephens, 1994); however, investigations elucidating the contributions of specific subsets of airway SMCs to the pathogenesis of asthma have not been reported. Airway smooth muscle is typically thought to be composed of a homogeneous myocyte populations and no studies have examined the distribution of SMC phenotype in different airways.

Given the multi-functionality of SMCs and the phenotypic plasticity they retain in the mature state it is critical to be able to identify the phenotypic state of SMCs in order to assess their contribution to normal and pathophysiological events in different organs. Investigators have used several criteria to identify SMC phenotype, including morphology and expression of specific marker proteins (reviewed by Sartore et al., 1994, and; Owens 1995). We have characterized the phenotype marker protein expression patterns in pulmonary SMCs of divergent phenotype (Halayko et al., 1996). In these studies we demonstrated that differences in cellular smMHC, nmMHC, sm-α-actin, calponin and *h*-caldesmon contents were associated with different contractile properties

of pulmonary smooth muscle.

The information presented heretofore leads to the evolution of a specific hypothesis:

SPECIFIC HYPOTHESIS -- Airway smooth muscles from different locations in the lung are composed of heterogeneous myocytes which can be distinguished on the basis of molecular phenotypic marker content.

In this regard, heterogeneity in ploidy and cellular content of phenotypic marker proteins in freshly dispersed normal, mature canine tracheal and bronchial SMCs were examined, using flow cytometry. The data obtained will provide information concerning the breadth, multitude and distribution of myocytes in different airway smooth muscles. This information will be of benefit in understanding the contribution of airway myocytes to the normal physiology and pathophysiology of the airways.

6.2 SPECIFIC AIMS

1. To assess, using indirect-immunofluorescence and FACS analysis, the cellular content of phenotypic marker proteins in individual SMCs dispersed from freshly obtained canine tracheal and bronchial smooth muscle.

2. To measure the cellular DNA content distribution of individual SMCs dispersed from freshly obtained canine tracheal and bronchial smooth muscle.

6.3 EXPERIMENTAL DESIGN

Canine tracheas and lungs were obtained from freshly sacrificed animals and airway smooth muscle samples were prepared by careful dissection on ice. Cervical tracheal segments were used to obtain trachealis -- muscle was cleaned extensively of epithelia, serosa and adventitia using fine dissecting instruments. Cleaned bronchial smooth muscle samples were obtained from 3rd and 4th generation segmental mainstem bronchi. The bronchial muscle was carefully cleaned of epithelia, submucosa and adventitia by dissection. Airway myocytes were dispersed enzymatically and then were fixed and stained for smMHC, sm-α-actin, calponin or *h*-caldesmon using indirect immunofluorescent techniques (Section 3.4.1.1 and 3.4.1.3). Cells were also stained for DNA using propidium iodide (Section 3.4.1.3). Cell staining was measured by FACS and the distribution of staining obtained analysed as described in Section 3.4.1.4.

6.4 RESULTS

6.4.1 Airway Myocyte Ploidy

FACS analysis of PI-stained canine tracheal SMCs (n = 6) revealed that 87.7 ± 6 % of the cells possessed a diploid (2N) complement of DNA and the remaining cells (12.3 ± 5 %) possessed a tetraploid (4N) complement of DNA (Figure 25A). A similar distribution of diploid (91.8 ± 7 %) and tetraploid (8.2 ± 5 %) cells was measured in bronchial myocyte preparations (n = 4) (Figure 25B). Virtually no tracheal or bronchial SMCs were detected which a DNA content intermediate to 2N and 4N indicating that few cells were in S-phase of the cell cycle and, hence, were not proliferating.

6.4.2 Smooth Muscle MHC in Airway Myocytes

Bivariate FACS analysis of cellular smMHC and DNA revealed that SMCs exhibiting a wide range in contractile protein content exist in canine airway smooth muscle (Figure 26). For both tracheal and bronchial myocytes, greater than 95% of all cells stained positively for smMHC indicating that few non muscle cell types were present in the smooth muscle preparations. The distribution of smMHC staining was similar in diploid and tetraploid cells from both tracheal and bronchial smooth muscle. Bivariate dot plots revealed the presence of at least two clusters of diploid tracheal myocytes, differing in staining intensity for smMHC (Figure 26B). A similar pattern of staining distribution appeared to be exhibited by bronchial myocytes.

Representative histograms of the frequency distribution of indirect immunofluorescent smMHC-staining for tracheal and bronchial myocytes are shown in Figure 27. These plots revealed the presence of two prominent peaks of immunofluorescence in myocytes obtained from both tissues. The fluorescence intensity (channel number) of the two peaks seen in tracheal preparations, 27.4 ± 4.1 and 49.7 ± 5.6 respectively, were significantly different ($n = 6$, $p < 0.01$) suggesting that they represented SMCs groups of relatively high and low smMHC content. The fraction of cells that fell into the high and low immunofluorescnce groups were 65 ± 7 % and 35 ± 5 % respectively. The fluorescent intensity of the two peaks seen in bronchial preparations, 25.1 ± 6.7 and 42.3 ± 6.3 respectively, were also significantly different ($n = 4$, $p < 0.05$) as determined by Student's *t*-test. The mean fluorescent intensity of the low intensity peak was similar in tracheal and bronchial preparations, however, the mean

channel number for the high intensity peak for tracheal myocytes was approximately 17% greater than the high intensity peak from bronchial myocyte preparations. The distribution of bronchial myocytes in the high and low immunofluorescence groups was about 40 ± 6 % and 60 ± 8 % respectively. Interestingly, this ratio was nearly opposite to that seen for tracheal preparations.

6.4.3 Smooth Muscle α-Actin in Airway Myocytes

For sm-α-actin, FACS analysis discriminated positive staining of both diploid and tetraploid airway myocytes (Figure 28). Negative controls showed a sharp peak of uniform, dull staining (Figures 28 and 29). The fraction of cells that stained brighter than the negative control exceeded 85% for both tracheal and bronchial preparations. A population of sm-α-actin-positive myocytes appeared as a broad shoulder of fluorescence near the sharp peak of negatively stained cells (Figure 29). The mean fluorescent intensity (channel number) of these groups were estimated to be 11.3 ± 4.1, and 9.1 ± 3.4 for tracheal and bronchial myocytes respectively. A second population of cells appeared as a prominent cluster of brighter sm-α-actin immunofluorescence in tracheal and bronchial myocytes (Figures 28 and 29). Mean fluorescence intensity (channel number) of the high intensity populations were 40.8 ± 5.9, and 32.6 ± 6.2 in tracheal and bronchial preparations respectively. The mean intensity of the high and low intensity populations were different in both tracheal ($n = 6$, $p < 0.01$) and bronchial ($n = 4$, $p < 0.05$) preparations. These data indicate that two groups of airway myocytes, possessing different contents of sm-α-actin, were present in cells obtained from airway smooth

muscle. The distribution of high and low sm-α-actin immunofluorescence in tracheal preparations was 63 ± 6 % high- and 37 ± 4 % low-intensity, whereas in bronchial preparations 42 ± 7 % high and 58 ● 6 % low staining cells were seen.

6.4.4 Calponin and *h*-Caldesmon in Tracheal SMCs

Bivariate dot plots of tracheal SMCs demonstrating calponin immunofluorescnce in PI-stained cells indicated that 83 ± 3.5 % (n = 4) of cells exhibited positive staining (Figure 30). A single, broad peak of immunofluorescnce (mean channel position) was detected for diploid cells (30.5 ± 4.9) and tetraploid cells (33.6 ± 5.1%) in all preparations suggesting that a continuum in calponin content was present. Diploid and tetraploid cells did not appear to differ in calponin content as staining intensity was the same in both subpopulations (n = 4, p >0.05).

Indirect immunofluorescent staining for *h*-caldesmon was seen in 92 ± 7.1 % (n = 4) of tracheal myocytes. Similar to the staining pattern seen for calponin, positive *h*-caldesmon immunoflourescnce (mean channel position) appeared as a single broad peak for both diploid (41.5 ± 4.3) and tetraploid populations (42.0 ± 5.8). Staining was not different between diploid and tetraploid subpopulations (n = 4, p > 0.05), however, in all experiments, approximately 5% of diploid myocytes appeared as a shoulder of dull fluorescence associated with the major peak of fluorescence.

FIGURE 25. FACS analysis of ploidy in canine tracheal and bronchial SMCs.

SMCs enzymatically dispersed from tracheal (**Panel A**) and 3rd generation mainstem bronchial smooth muscle (**Panel B**) were stained for cellular DNA content using propidium iodide and the distribution of diploid (2N) and tetraploid (4N) cells was determined by FACS analysis. The lower panels of each histogram are inserts displaying raw data collected from representative samples (in the lower panels the ordinate scale is a measure of autofluorescence detected using a 540 ± 10 nm band pass filter). Cell distribution was determined from 6 different animals; 5000 different cells were analysed for each histogram.

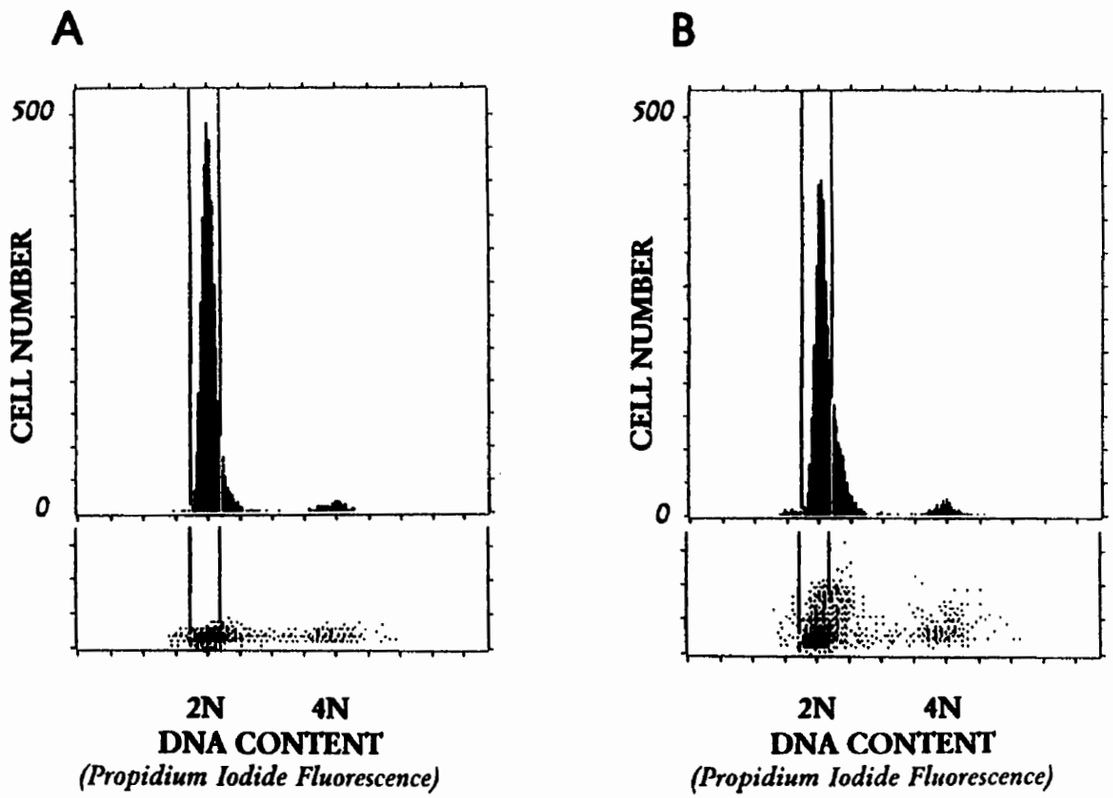

A
500
CELL NUMBER
0
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)
B
500
CELL NUMBER
0
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)

FIGURE 26. Bivariate FACS analysis of smMHC content and ploidy of canine tracheal and bronchial SMCs.

Representative dot plots of SMCs showing smMHC content by indirect FITC-immunofluorescence and DNA content by propidium iodide staining. Each axis has 64-channel resolution. The abscissa is linear scale. The ordinate represents data obtained using a three decade log amplifier, hence, channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. The SMCs were dispersed from cleaned dissected tissues and then stained as described in Methods. Each plot presents data collected from 5000 individual cells.

A. Negative control (tracheal SMCs).

B. Cervical tracheal SMCs.

C. 3rd generation mainstem bronchial SMCs.

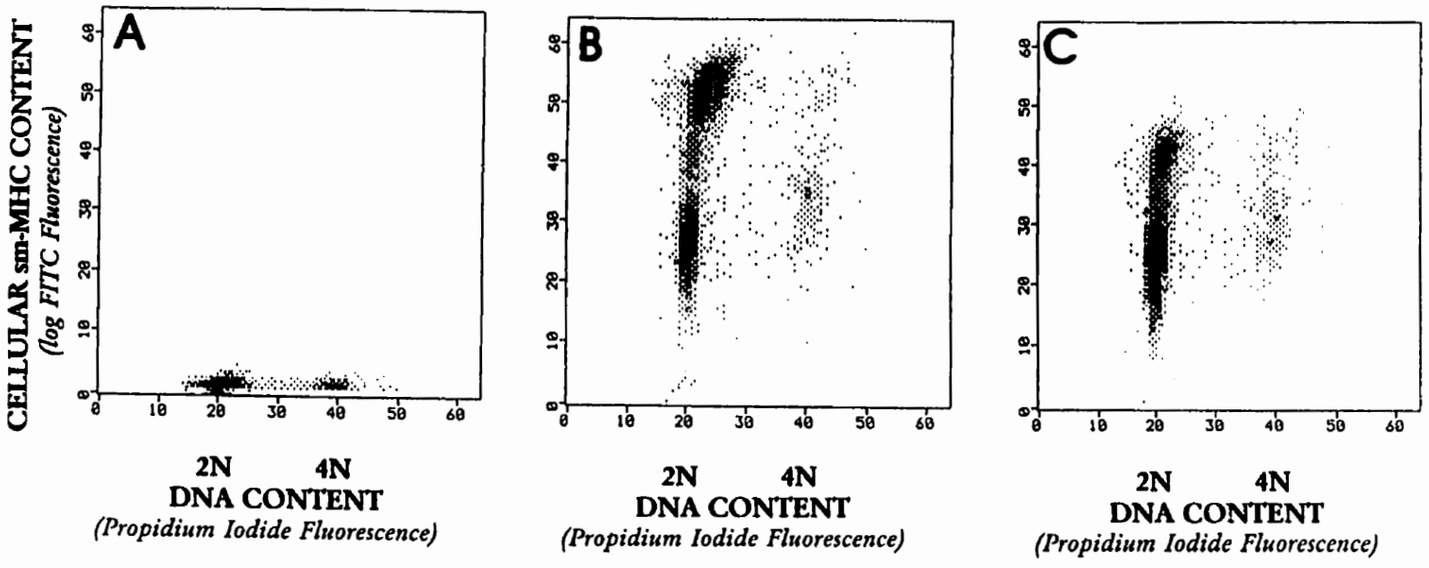

A
B
C
CELLULAR sm-MHC CONTENT
(log FITC Fluorescence)
0
10
20
30
40
50
60
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)

FIGURE 27. FACS analysis of the distribution of indirect-immunofluorescence of smMHC in freshly dispersed canine tracheal and bronchial SMCs.

Representative histograms are shown. The abscissa is a 3 decade log scale of 64-channel resolution -- channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. SMCs were isolated and stained as described in Methods. Each histogram presents data from 5000 different cells.

A. Negative control (tracheal SMCs).

B. Cervical tracheal SMCs.

C. 3rd generation mainstem bronchial SMCs.

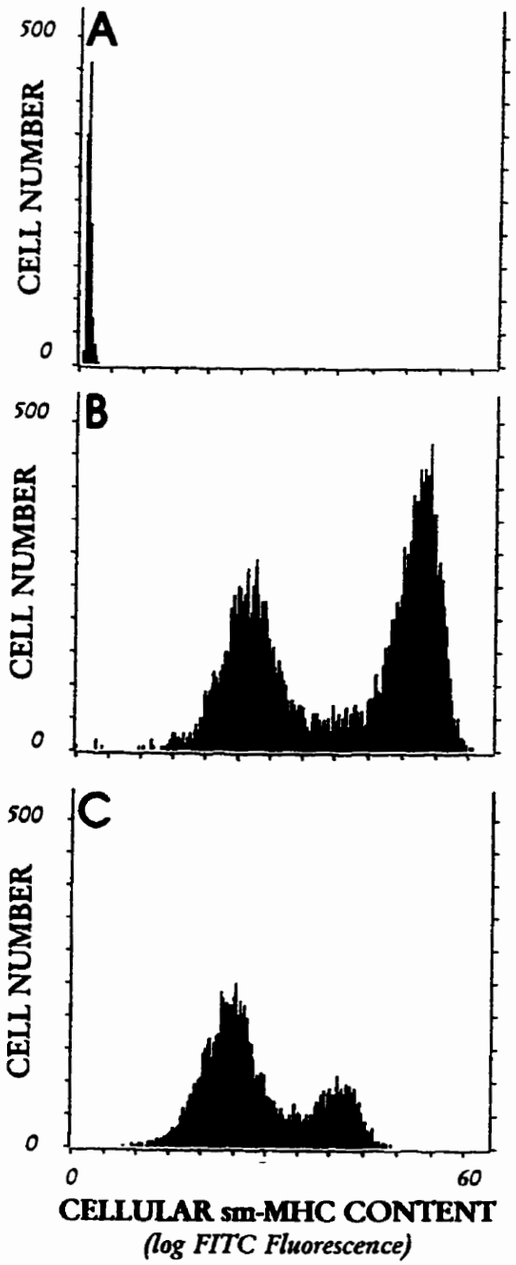

A
500
CELL NUMBER
0
B
500
CELL NUMBER
0
C
500
CELL NUMBER
0
0
60
CELLULAR sm-MHC CONTENT
(log FITC Fluorescence)

FIGURE 28. Bivariate FACS analysis of sm-α-actin content and ploidy of freshly dissociated canine tracheal SMCs.

Representative dot plots of SMCs showing sm-α-actin content by indirect FITC-immunofluorescence and DNA content by propidium iodide staining. Each axis has 64-channel resolution. The abscissa is linear scale. The ordinate represents data obtained using a three decade log amplifier, hence, channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. The SMCs were dispersed from cleaned dissected tissues and then stained as described in Methods. Each plot represents data obtained from 5000 individual cells.

A. Negative control (tracheal SMCs).

B. Cervical tracheal SMCs.

C. 3rd generation mainstem bronchial SMCs.

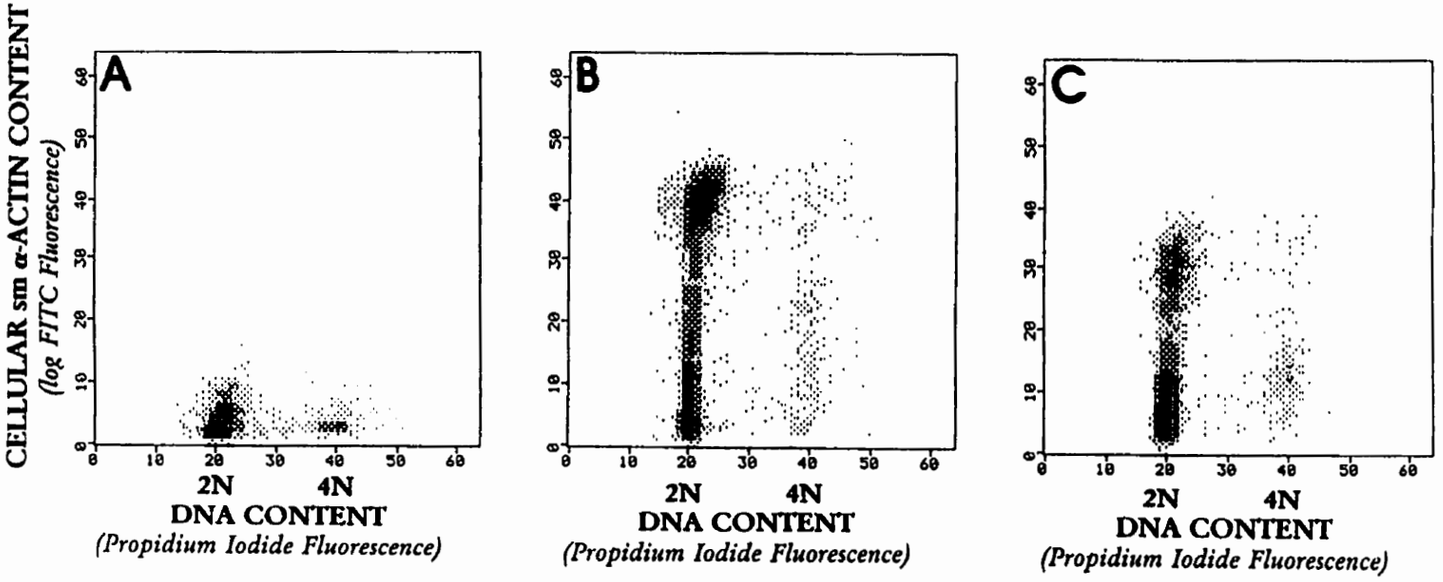

CELLULAR sm α-ACTIN CONTENT
(log FITC Fluorescence)
A
B
C
0
10
20
30
40
50
60
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)

FIGURE 29. FACS analysis of the distribution of indirect-immunofluorescence of sm-α-actin in freshly dissociated canine tracheal and bronchial SMCs.

Representative histograms are shown. The abscissa is a 3 decade log scale of 64-channel resolution -- channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. SMCs were isolated and stained as described in Methods. Each histogram depicts data from 5000 cells.

A. Negative control (tracheal SMCs).

B. Cervical tracheal SMCs.

C. 3rd generation mainstem bronchial SMCs.

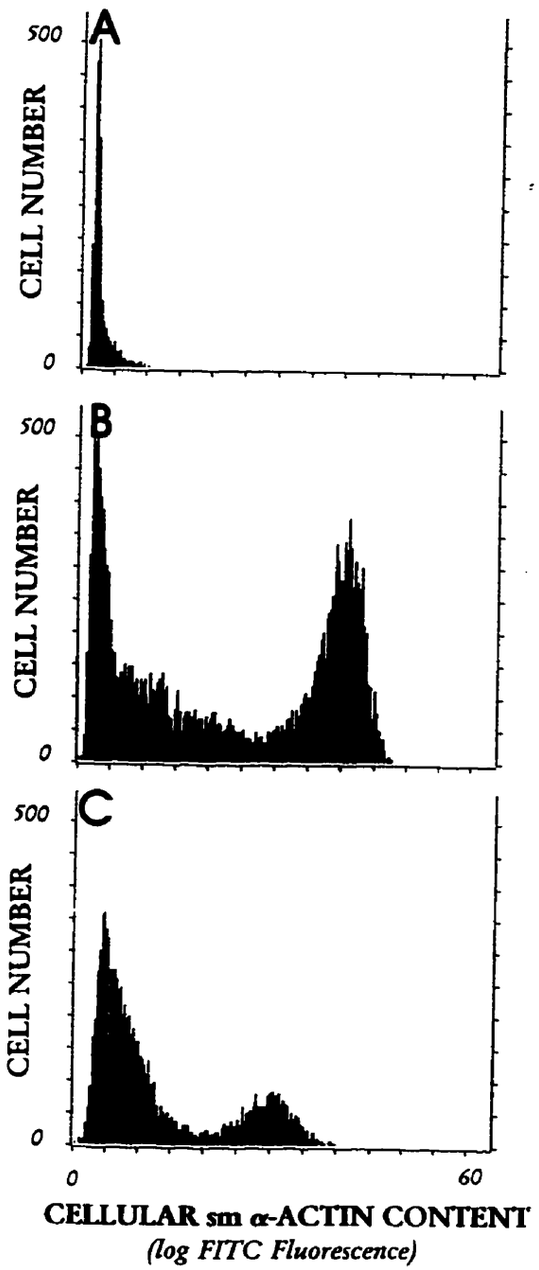

A
500
CELL NUMBER
0
B
500
CELL NUMBER
0
C
500
CELL NUMBER
0
0
60
CELLULAR sm α-ACTIN CONTENT
(log FITC Fluorescence)

FIGURE 30. Bivariate FACS analysis of calponin content and ploidy of dissociated canine tracheal SMCs.

Representative dot plots of SMCs showing calponin content by indirect FITC-immunofluorescence and DNA content by propidium iodide staining. Each axis has 64-channel resolution. The abscissa is linear scale. The ordinate represents data obtained using a three decade log amplifier, hence, channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. The SMCs were dispersed from cleaned dissected tissues and then stained as described in Methods.

A. Negative control.

B. Cervical tracheal SMCs.

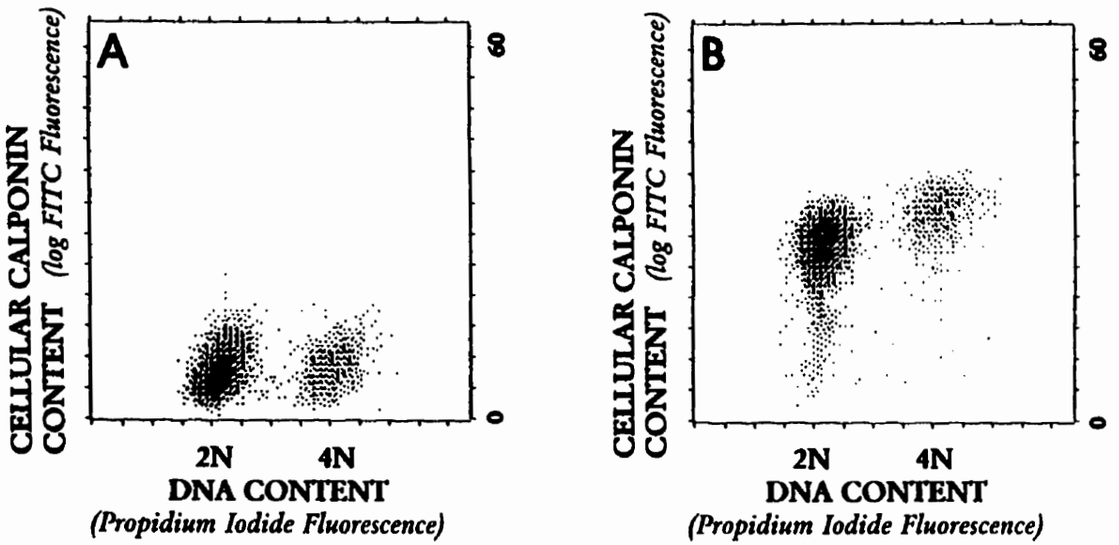
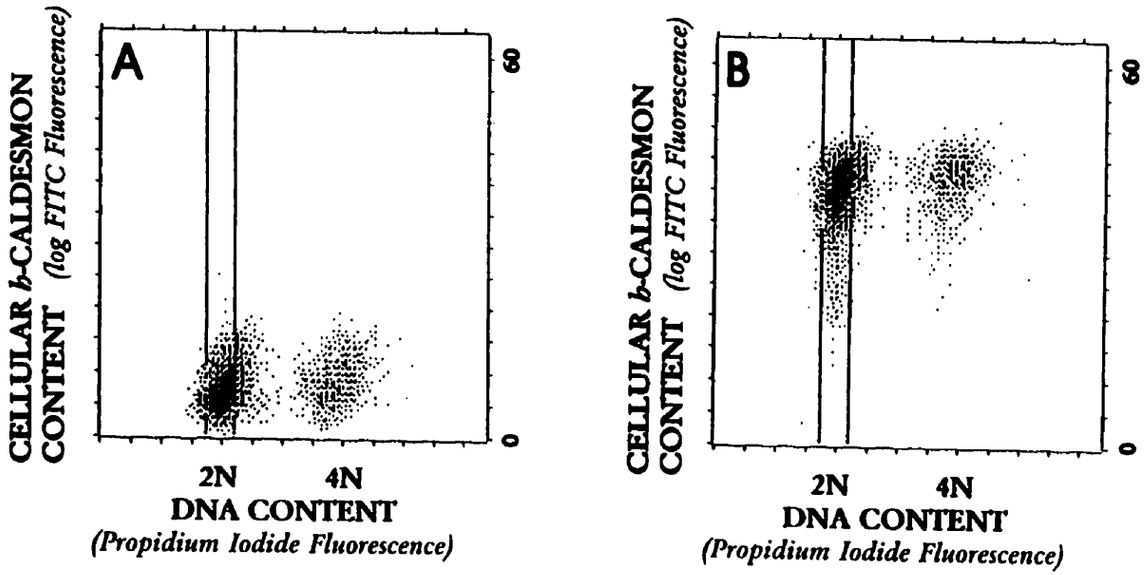

A
B
CELLULAR CALPONIN CONTENT (log FITC Fluorescence)
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)
0

FIGURE 31. Bivariate FACS analysis of *h*-caldesmon content and ploidy of freshly dispersed canine tracheal SMCs.

Representative dot plots of SMCs showing caldesmon content by indirect FITC-immunofluorescence and DNA content by propidium iodide staining. Each axis has 64-channel resolution. The abscissa is linear scale. The ordinate represents data obtained using a three decade log amplifier, hence, channel numbers 0, 20, 40, and 60 on this axis correspond to 10^0, 10^1, 10^2 and 10^3 on a log scale. The SMCs were dispersed from cleaned dissected tissues and then stained as described in Methods. Data from 5000 cells are depicted in each plot.

A. Negative control.

B. Cervical tracheal SMCs.


A
B
CELLULAR b-CALDESMON CONTENT (log FITC Fluorescence)
60
0
2N
4N
DNA CONTENT
(Propidium Iodide Fluorescence)

6.5 DISCUSSION

Vascular SMCs have been known to exist as stable diploid and polyploid cells in arterial smooth muscle for some time (Goldberg et al. 1984; Owens 1989). The frequency with which polyploidy has been observed in arteries appears to be associated with hypertrophic growth as the result of aging and/or duration of a hypertensive state (Owens 1989; Dominiczak et al. 1996). It is thought that the accumulation of tetraploid cells with aging and in disease may be the result of incomplete cell division during the course of replication or may be associated with hypertrophic growth of SMCs in response to specific chemical and physical stimuli (Owens, 1989). Owens (1989) postulated that hypertrophic growth in response to excessive workload may be an adaptational advantage, as it allows for an increase in muscle mass without sacrificing contractile potential as would be the case if SMCs replicated completely. Also of note are the observations of Goldberg et al. (1984) which indicated that in primary culture, diploid aortic rat myocytes proliferated significantly more rapidly than did tetraploid myocytes from the same animals.

The intensity of cellular smMHC and sm-α-actin immunofluorescence measured in our studies for tetraploid canine tracheal and bronchial SMCs was equal to that of diploid cells. This observation suggests that the former cells are likely stable, distinct populations of mature contractile cells which normally exist in adult airway smooth muscle. This observation is consistent with those reported for sorted diploid and tetraploid rat aortic SMCs (Goldberg et al., 1984). Clonal, tetraploid aortic myocyte cultures were established and which maintained a stable tetraploid state over numerous

generations. The distribution of myocytes with different DNA content was the same in the tracheal and bronchial airways assayed. These data suggest that replicative activity of myocytes in different sized airways may be similar. It would be of interest in the future to monitor the occurrence of polyploidy and hypertrophy of airway SMCs with aging and in association with the pathogenesis of chronic asthma, a disease in which airway SMC hyperplasia and hypertrophy is a hallmark pathological feature.

Antibodies with high antigenic specificity appear to be to be well suited for immunofluorescence analysis of intracellular proteins in combination with flow cytometry (Bauer and Jacobberger, 1994). Detection of intracellular proteins requires fixation and permeablization of cells to permit free diffusion of antibody into the cells. Cold alcohol fixation has been shown to be useful for the detection of numerous intracellular proteins, including nuclear antigens, by flow cytometry (Kastan et al., 1989; Glade et al., 1995). Several methodological considerations for optimizing immunofluorescent analysis of intracellular proteins by flow cytometry must be addressed in order to ensure reliable measurements. Significant factors include, the titre of primary and secondary antibodies used, the extent of non-specific interactions of the antibodies used and the degree of background autofluorescence (Bauer and Jacobberger, 1994).

For the studies described herein secondary antibodies were used at concentrations stipulated by the manufacturer. To ensure that optimal primary antibody titres were used, in preliminary experiments, cells were incubated in a wide range of antibody concentrations. Analysis of immunofluorescence by flow cytometry showed that if

antibody concentrations were too low or if they were too high, immunofluorescent intensity of most stained cells was reduced. Hence, primary antibody concentrations were considered to be optimal at the lowest titre that achieved maximal immunofluorescence of antigen-positive cells. No detectable autofluorescence was detected in unstained cells, hence, this parameter was not considered problematic. To account for nonspecific affinity of primary and secondary antibodies, negative controls were measured for each experiment. Negative control cells were incubated with non-immune sera from the same species from which primary antibodies were obtained and then were incubated in the same secondary antibody solution that was used for labelling cells incubated in primary antibody. Non-specific immunofluorescnce was generally low and rarely reached levels greater than 10 % of the brightest immunofluorescence seen for primary antibody-incubated cells labelled cells.

In these studies we have examined the intracellular content of a variety of smooth muscle proteins using indirect immunofluorescent methods. The antibodies for smMHC, sm-α-actin, and calponin used have been used previously in immunoblot assays for semi-quantitative analysis of protein markers of airway SMC phenotype (Halayko et al., 1996a; refer to Chapter 4 in this thesis). In each case, the antibodies showed strong specificity for the antigens of interest. The monoclonal antibody used for the detection of *h*-caldesmon in these studies has low affinity for the nonmuscle *l*-isoform The *l*-caldesmon isoform has been shown to be present only in low abundance in a number of mature SMC types, including airway myocytes (Ueki et al., 1987; Owens, 1995; Halayko et al., 1996a). This suggests that staining of ethanol-fixed mature airway myocytes with

this antibody should be quite specific for the *h*-isoform, though, a small contribution from the *l*-isoform to total caldesmon-immunofluorescence cannot be ruled out conclusively.

Tracheal myocytes did not appear to be distributed into distinct cell clusters based on calponin or *h*-caldesmon immunofluorescence. Calponin and *h*-caldesmon are actin filament-associated proteins thought to play a role in regulating smooth muscle contraction (Walsh, 1994). The expression of both proteins is correlated with late maturation of vascular myocytes during development and are considered to be reliable markers for mature vascular and airway SMCs (Owens, 1995; Halayko et al., 1996a). There was evidence for a small subpopulation of tracheal myocytes with low immunofluorescence for caldesmon. However, fewer than 5% of all cells fell in this area, hence, the cells could represent nonmuscle cells that expressed *l*-caldesmon. Nonetheless, a relatively broad range of immunofluorescence, which spanned more than one decade on a logarithmic scale, was seen suggesting that not all airway myocytes express these phenotype marker proteins homogeneously.

The presence of two distinct clusters of immunofluorescnce for sm-α-actin and smMHC was observed in airway myocytes. This observation suggests that at least two subpopulations of mature canine SMCs may exist which can be discriminated on the basis of contractile protein content. To the best of our knowledge this is the first reported observation of its kind for airway smooth muscle. Distinct subpopulations of SMCs have been identified in other smooth muscles based on contractile and cytoskeletal protein content. Holifield et al (1996) recently reported the existence of two distinct populations

of cells in canine carotid artery based on *in situ* characterization of cellular phenotype using antibodies and cDNA probes for smMHC and sm-α-actin. Frid et al. (1994) identified multiple populations of SMCs in the bovine main pulmonary artery based on heterogeneous expression of a variety of smooth muscle-specific cytoskeletal proteins. Two types of cultured SMCs from rabbit urinary bladder, which differ in size, morphology and content of smMHC, sm-α-actin, *h*-caldesmon and vimentin have also been identified (Lau and Chacko 1996).

Since dual labelling of isolated SMCs was not performed in our studies it is not clear whether the same myocytes that had high smMHC content also had higher sm-α-actin content. It is, however, inviting to speculate as to the functional relevance of these data since it has been shown that smooth muscles known to differ in contractile properties have been distinguished on the basis of the content and isoform stoichiometry of contractile and cytoskeletal proteins (Fatigati and Murphy, 1984; Woodcock-Mitchell et al., 1993; Miano et al., 1994; McHugh, 1995; Halayko et al. 1996a). The presence of distinct airway myocyte subpopulations differing in contractile protein content suggest that airway smooth muscle is composed of a phenotypically heterogeneous population of myocytes which differ in contractile properties.

We observed that the distribution of high:low smMHC and sm-α-actin-containing myocytes was greater in tracheal preparations compared to bronchial smooth muscle preparations. Furthermore, the intensity of immunofluorescence in the high-staining tracheal group was nearly 20% greater than that seen for the high-staining bronchial group. Recently, the contractile properties of airway smooth muscle from large extra-

pulmonary and smaller intra-pulmonary airways were compared *in vitro* (Ma et al., 1996). These experiments demonstrated that extra-pulmonary smooth muscle preparations are characterized by higher maximum shortening capacity and faster velocity of shortening. In addition, heterogeneous sensitivity of airways of different sizes to pharmacologic and antigenic compounds have been described (Chitano et al., 1993; Sigurdsson et al., 1995). The data obtained in our studies, which show clearly that tracheal and bronchial smooth muscles are composed of different distributions of heterogeneous myocyte subpopulations, correlate well with previously observed heterogeneity in contractile and pharmacologic properties between large and small airways.

Elucidation of the heterogeneous nature of airway smooth muscle myocytes may have important implications in our understanding of the normal physiologic and pathophysiologic roles played by these cells. Their role will only be clarified if the functional aspects of myocyte heterogeneity are established in future. To this end determining the origin of airway myocyte heterogeneity and the developmental and molecular mechanisms that regulate it should be important future research goals.

6.6 SUMMARY

The medial layer of the airways has been thought to be composed of a phenotypically homogeneous smooth muscle cell population. However, inter-airways differences in contractile properties and pharmacological responsiveness are known to exist. Also, airway smooth muscle thickening in asthma, is manifest by distinct patterns

of hyperplastic and hypertrophic myocyte growth in different sized airways. Using flow cytometry we examined inter- and intra-airway heterogeneity of canine SMCs. SMCs dissociated from the trachea or 3rd-4th order mainstem bronchi were fixed and stained for DNA, using propidium iodide, and for specific protein markers of ASM phenotype by indirect immunofluorescence. Distinct populations of diploid and tetraploid cells were seen in tracheal and bronchial samples; tetraploid cells accounted for 12.3±5% and 8.2±5% of tracheal and bronchial myocytes respectively. Over 95% of tracheal SMCs and 90% of bronchial SMCs expressed contractile SMC markers (*i.e.* smMHC, sm-α-actin, calponin and *h*-caldesmon). Diploid and tetraploid cells demonstrated broad heterogeneity in marker protein content. Indeed, cells containing relatively "high" and "low" amounts of smooth muscle myosin and actin could be discerned. The latter group was more abundant in bronchial smooth muscle than in tracheal smooth muscle. These data demonstrate that airway smooth muscle is not composed of a homogeneous SMC population. Inter- and intra-airway heterogeneity in distribution of SMC phenotypes exist. Different sub-populations of airway myocytes may be important for subserving different functions including contraction and proliferative responses to pathological stimuli.

7. CHARACTERIZATION OF ISOLATED AIRWAY SMOOTH MUSCLE CELL SUBPOPULATIONS

7.1 BACKGROUND

Medial smooth muscle layers of arteries are composed of heterogeneous populations of phenotypically divergent myocytes (Frid et al., 1994; Neylon et al., 1994; Archer et al., 1996; Topouzis and Majesky, 1996; Holifield et al., 1996). The members of these subpopulations vary in a number of physiological properties which include responses to mitogenic and antiproliferative stimuli (San Antonio et al., 1993; Lindner et al., 1995; Wohrley et al., 1995; Topouzis and Majesky, 1996), and to contractile stimuli (Archer et al., 1996). Furthermore, the cells exhibit dissimilar biochemical properties, differing in contractile and cytoskeletal protein content (Frid et al., 1994; Meer and Eddinger , 1996), membrane ion channel protein composition (Archer et al., 1996), expression of growth factors and requisite receptors (Majesky et al., 1988; Lindner et al., 1995) and synthesis and metabolism of extracellular matrix components (Giachelli et al., 1991; Letourneur et al., 1995b Lemire et al., 1996).

It is clear that vascular SMCs from a particular source and from different sources exhibit heterogeneity. Recently, the specific contribution of different vascular myocyte subpopulations to pathogenesis of diseases such as atherosclerosis and hypertension, in which vascular myocytes are thought to orchestrate vascular remodelling, has been addressed (Wohrley, et al., 1995; Holifield et al., 1996; Topouzis and Majesky, 1996). It now appears that in response to injury or pathophysiologic insult, proliferative responses in arteries may be subserved by distinct cell populations which differ from mature, contractile cells comprising the arterial media, in morphologic and biochemical characteristics (Wohrley et al., 1995; Holifield et al., 1996).

Several types of arterial SMC populations, differing in biochemical, morphological and growth characteristics, have been established and maintained in culture (San Antonio et al., 1993; Weissberg et al., 1993; Lemire et al., 1994; Benzakour et al., 1996; Bochaton-Piallat et al., 1996; Holifield et al., 1996). It has been noted that these cell populations can differ drastically in the capacity to attach to cell culture plates, and in rate of replication after cell attachment (Holifield et al., 1996; Lau and Chacko, 1996). Lau and Chacko (1996) described the existence of at least two populations of SMCs in cultures of myocytes from rabbit urinary bladder. Studies of this type have been vital in extending knowledge of the multifunctional nature of SMCs and in clarification of the potential contribution of myocytes to disease pathogenesis. The potential significance of airway SMC heterogeneity to the pathogenesis of bronchial asthma, a disease characterized by airway remodelling and medial thickening, has been addressed (Halayko and Stephens, 1994). To date, however, no investigations of airway myocyte heterogeneity have been reported.

Primary cultures of airway SMCs are commonly used to study mechanisms that regulate proliferation and contraction (reviewed by Hall and Kotlikoff, 1995). Little is known, however, about heterogeneity of the myocytes obtained from airways. Indeed, it is not known definitively if cells grown in primary airway SMC culture are representative of all the cells dispersed from intact tissue or whether cultured myocytes are a select group of cells with superior capacity for attachment to culture plates and for replication. Hence due to reports of vascular SMC heterogeneity, and the lack of systematic analysis of same for airway myocytes, we endeavoured to identify and sort morphologically

dissimilar airway myocytes using flow cytometry, and to assess their properties in primary culture. The experiments were performed under the umbrella of the following specific hypothesis:

SPECIFIC HYPOTHESIS - Airway smooth muscle is composed of phenotypically dissimilar myocytes which exhibit diverse morphological, biochemical and growth characteristics in primary culture.

The results obtained in these studies will provide new insights into the nature of airways myocytes maintained in primary culture. Characterization of the properties of phenotypically dissimilar airway SMCs will also allow for elucidation of the role of the airway myocyte in physiologic and pathophysiologic pulmonary phenomena .

7.2 SPECIFIC AIMS

1. To assess the size and shape of enzymatically dissociated, living airway SMCs using flow cytometry and to isolate, by cell sorting, populations of myocytes of dissimilar morphology.

2. To characterize, using immunocytochemical methods, the morphological and phenotypic characteristics of airway SMC subpopulations isolated by cell sorting.

3. To establish primary cultures of the airway SMC subpopulations isolated by cell sorting.

4. To assess the characteristics of cultured airway SMC subpopulations, *i.e.* attachment efficiency, proliferative activity and, using immunocytochemical methods, phenotypic marker protein content.

7.3 Experimental Design

Canine tracheas were obtained from sacrificed animals and smooth muscle samples were prepared by careful dissection on ice, in Ca^{2+}-free Krebs Henseleit. Cervical tracheal segments were used to obtain trachealis muscle. The muscle was cleaned extensively of epithelia, serosa and adventitia using fine dissecting instruments. Cells were dissociated using collagenase, elastase and Nagarse protease as described (Section 3.4.3) and were subsequently resuspended in DMEM / 10% FBS / 10 mM taurine and stored on ice. Cell size and shape was assessed on the basis of light scatter patterns obtained by flow cytometry. Cell populations that could be distinguished on the basis of light scatter pattern were isolated by cell sorting using flow cytometry (Section 3.4.3).

In some experiments, isolated cell populations were pelleted onto microscope slides by cytospin and the phenotype of the cells was subsequently assessed using immunocytochemistry, by staining for phenotype marker proteins (Section 3.3.8). In some experiments sorted cells were seeded into cell culture plates and the attachment efficiency and proliferative activity were measured (Section 3.4.3 and 3.2.1). Four cell culture experiments, using a different animal in each experiment, were performed. Where possible measurements of cell number on culture dishes were performed in

triplicate. Measurements of cell size in culture were made by manual methods from phase contrast micrographs of the cultured cells; micrographs were calibrated from pictures taken of a micrometer grid at the same magnification used to capture cell images. In some cultures the cells were grown on glass coverslips and the phenotype of the myocytes was characterized using immunocytochemical staining for phenotype marker proteins (Section 3.3.7).

7.4 RESULTS

7.4.1 Isolated Tracheal SMC Morphology: Flow Cytometry Light Scatter Pattern and Cell Sorting

Cells isolated from tracheal smooth muscle by enzymatic digestion, for flow cytometry, exhibited multiple morphologic states in cell suspensions (Figure 32A). Two cell shapes appeared to be the most prominent; about half of all cells were spheroid (mean diameter 29 ● 5 μm, n = 15) whereas the remainder were elongate, ranging between 200 - 300 μm in length immediately after cell dispersal (mean cell length = 247 ± 42 μm, n = 15). For easy reference, spheroid and elongate cell populations were called Type A and Type B and will be referred to as such hereafter. Elongate, Type B cells appeared to contract with time in suspension; mean cell length decreased to 140 ± 20 μm after 4 hours of storage in culture media on ice. A similar change in cell size was seen in Type B cells if the cell suspensions were used for seeding primary cell cultures (Figure 32B).

The forward light scatter *versus* 90° light scatter pattern observed for the tracheal

myocyte suspensions revealed the presence of two prominent clusters of cells (Figure 33). Cell sorting revealed that the clusters were predominantly comprised of spheroid and elongate cells of approximately equal size dimensions as those measured for Type A and Type B cells immediately after enzymatic dispersal. The mean channel positions of 90° and forward light scatter for Type A cells were 19.6 ± 5.1 (n = 6) and 11.2 ± 3.1 (n = 6) respectively. The mean channel position for 90° light scatter was quite narrow for Type B cells (33.3 ± 1.9, n = 6) whereas a characteristically broad forward light scatter character was observed (44.2 ± 5.6). The position of the light scatter clusters for Type A and Type B cells was quite consistent between experiments, varying by less than 5% along the abscissa or ordinate.

After cell sorting, Type B cells appeared to be somewhat contracted as their mean cell length was 165 ± 20 μm (n = 15) (Figure 33). A significant number of cells exhibited light scatter characteristics which were intermediate to those designated as Type A and Type B cells. The resolution afforded by the light scatter dot plots was not sufficient to determine whether the intermediate cells constituted a distinct subgroup of cells or if they were Type A or Type B cells exhibiting spurious light scatter. For this reason during cell sorting procedures, the electronic gates that determined whether a cell would be identified as a member of the Type A or Type B population, were set such that few, if any, intermediate cells were included in either cell cluster. The gates were set manually after inspection of bivariate light scatter dot plots to ensure that each encompassed on the central portion of the cell clusters observed. Casual observation during the course of cell sorting experiments suggested that greater than 95% of all the

cells sorted into a tube as a discrete population, were composed of the cell type expected.

7.4.2 Cytospin - Immunocytochemistry of Acutely Dissociated Tracheal Myocytes

Enzymatically dispersed tracheal SMC suspensions were cytospun onto glass microscope slides, fixed, and subsequently immunostained. Antibodies that recognized a number of known markers of airway SMC phenotypic state were used to assess the cytodifferentiation of Type A and Type B cells. Figures 34 through 37 demonstrate the staining patterns seen using monoclonal antibodies for sm-α-actin, smMHC, desmin, and vimentin respectively. Type B cells exhibited heterogeneous staining for sm-α-actin and for smMHC (Figures 34 and 35) . Positive staining of the majority of the cells was strong whereas the remaining, positively stained Type B cells appeared to be more weakly labelled. Greater than 90% or all Type B cells were sm-α-actin and smMHC-positive. These data appear to correlate well with the distribution of tracheal myocytes detected by FACS analysis of cellular sm-α-actin and smMHC content in the previous chapter (see Figures 26 to 29). Positive staining was observed in only 50% of Type A cells and the intensity of positive staining for sm-α-actin and smMHC appeared weak in comparison to Type B cells.

Figures 36 and 37 demonstrate the distribution of staining for the intermediate filament proteins, desmin and vimentin, that was observed in ctyospun tracheal myocytes. Type A cells did not appear to express desmin protein; cell staining using anti-desmin antibodies was negligible. Conversely, desmin-positive myocytes accounted for approximately 50% of all Type B cells (Figure 36). The majority of Type A and

Type B cells were vimentin-positive (Figure 37). Cell-to-cell heterogeneity, similar to that seen for sm-α-actin and smMHC staining, in the intensity of vimentin staining was observed for Type B cells suggesting that vimentin may be differentially expressed. Few Type A cells stained weakly for vimentin suggesting that this intermediate filament protein is expressed at a homogeneous level in most Type A cells.

7.4.3 Characteristics of Type A and Type B Tracheal Cells in Primary Culture

Sorted Type A and Type B tracheal cells exhibited dramatically different characteristics in cell culture. Cell viability, assessed by Trypan blue exclusion after cell sorting, was 85 ● 7 % and 72 ± 10 % (n = 4) for Type A cells and Type B cells respectively. Type A cells seeded at a density 5 x 10^3 cells / cm^2 began attaching to culture plate substrata within one hour of plating and first showed evidence of cell spreading 6 hours after seeding. More than 60% of the Type A cells seeded attached to the culture plates and became proliferative when maintained in media supplemented with 10% FBS. Increasing the concentration of FBS in the media did not increase the efficiency of cell attachment. In contrast, Type B cells appeared resistant to cell culture as only a few cells, less than 10% of those seeded, were attached to the culture plates 48 hours after initial seeding. Increasing the concentration of serum to 20% had no effect and increasing seeding cell density to 25 x 10^3 cells / cm^2 failed to improve the fraction of cells that attached to culture plates. Figure 32B depicts the difference in morphology of Type A and Type B cells seeded for 4 hours in primary culture. Type A cells attached to the culture plate whereas most Type B cells remained floating and became contracted.

Type A cells exhibited growth characteristics typical of airway myocyte primary cultures prepared from unsorted, enzymatically dispersed SMCs (Figure 38, see also Figure 4). Cell number began increasing approximately 5 days after cell seeding. The proliferative phase of the growth curve spanned 3-4 days and at confluence cell density was $96.1 \times 10^3 \pm 4.5 \times 10^3$ cells / cm^2 and a "hill-and-valley" cell pattern was seen. Type A cells continued to proliferate readily even in 5th passage. The growth curve for Type B cells demonstrates that cell number does not increase significantly over 14 days in primary culture (Figure 38). Type B cells that attached to culture dishes were sparsely seeded, and only after two weeks in culture did a few isolated patches of cells appear. Cultures maintained for up to 40 days in culture did not become confluent, instead when the patches of cells became more densely populated, and cell-to-cell contacts were made, proliferation was inhibited entirely.

Figure 39 shows phase contrast micrographs of Type A and Type B cells in clonal cell culture. Type A cells commonly grew in a parallel orientation over 30-50% of the culture plate surface, forming end-to-end cell contacts with each other. Cells appeared to be relatively homogeneous in size (approximately 145.8 ± 40 μm long and 17.2 ± 4.1 μm wide) and were in general, spindle-shaped. The cells were characterized by the presence of dark, perinuclear granules surrounding a central, oval-shaped nucleus. A few larger cells which were often multi-nucleate were also apparent in the Type A cell cultures, however, they constituted < 5% of the total cell population. Type A cells in other areas of the culture plate grew in less organized patterns.

Figure 39 also demonstrates two morphologic cell types, intermediate and large,

seen growing on Type B cell-seeded plates. The large cell type constituted approximately 70% of all Type B cells in culture. Intermediate cells were typically triangular in shape and were measured 87.3 ± 10.2 μm along the longest axis. These cells contained substantial numbers of perinuclear granules, were rarely multi-nucleate, and each possessed a large centrally located, circular nucleus. Large Type B cells in culture measured 203.1 ● 15.2 μm along their long axis and 89.3 ± 9.6 μm in width. Large cells were frequently multi-nucleate and contained both dark and light granular structures in perinuclear regions. The cytoplasm was characterized by phase dark, longitudinally oriented "stripes" which may have corresponded to folds in the plasma membrane. Large cells sometimes formed broad, end-to-end cellular contacts, however, in general, cell orientation did not appear to be organized in specific patterns.

Figure 40 shows representative fluorescent immunocytochemical staining of Type A cells and large Type B cells in primary culture. These cells were seen in a Type B cell sorted culture, 5 days after seeding. Usually, sorted Type B cell cultures were populated with 5 - 7 % of contaminating Type A cells. Type A and Type B cells stained well for the mature SMC phenotype markers, sm-α-actin and smMHC. Large Type B cells exhibited large numbers of longitudinally oriented actin and myosin-positive filaments. Staining in large Type B cells extended to the cell periphery and showed evidence of increased staining in perinuclear domains. Type A cells demonstrated a more diffuse staining pattern in which distinct, stained filaments were not obvious. Peripheral cytoplasmic domains of Type A cells were often weakly stained for sm-α-actin. Unlike the immunostaining patterns observed in ctyospun, acutely dissociated Type A and Type

B cells, immunostaining of cultured cells appeared homogeneous, with most cells exhibiting approximately similar staining intensities.

FIGURE 32. Phase contrast micrograph of enzymatically dispersed canine tracheal SMCs.

Cells were dissociated using collagenase, elastase and Nagarse protease as described in Section 3.2.1. Both pictures were taken at the same magnification. Legend: 1 cm = 65 μm.

A. Acutely dissociated tracheal myocytes just seeded for cell culture. The picture was taken about 5 minutes after the cell slurry was added to a 100 mm culture plate (cell density was 4×10^4 cells/ml). Most cells in this plate were still unattached at the time the picture was taken. Note that two distinct cell shapes can be seen, elongate cells between 225-350 μm in length (lower left) and spheroid cells between 25 and 40 μm in diameter (top left).

B. Dissociated tracheal SMCs 4 hours after seeding for cell culture. Numerous spheroid cells have settled on the bottom of the plate and are attached -- these appear as dark, round shapes in this picture (the cells are slightly out of the plane of focus). Elongate cells still appear to be floating (see centre of plate) and remain unattached to the culture substrata. Note that the elongate cells appear to be contracted relative to the elongate cells seen just after plating (Panel A above). Elongate cells were typically 120-165 μm in length at this time.

A

B

FIGURE 33. Flow cytometry light scatter pattern obtained for freshly dissociated tracheal SMCs.

Cells were dissociated using collagenase, elastase and Nagarse protease as described in Section 3.2.1. Forward light scatter (an index of cell size) and 90° light scatter (an index of cellular granularity and cell size) were collected on 64 channel linear and logarithmic (3 decade) scales respectively.

Top Panel. Representative bivariate dot plot of the light scatter of cells dissociated from canine tracheal smooth muscle. The two clusters of cells seen were designated as being either Type A (relatively low forward and 90° light scatter) or Type B (relatively high forward and 90° light scatter). The areas delineated by circles represent the gates used for sorting the cell populations using flow cytometry.

Bottom Panel. Representative histogram showing the distribution of Type A and Type B cells as determined from forward light scatter measures. The ratio noted represents that which was obtained for the specific experiment shown - the ratio obtained for an experiment was variable and depended on the precise size and position of the electronic sorting gates used. The cells pictured in the inserts represent the morphology of the cells after sorting. Magnification scale for the inserts is 1 cm = 100 μm.

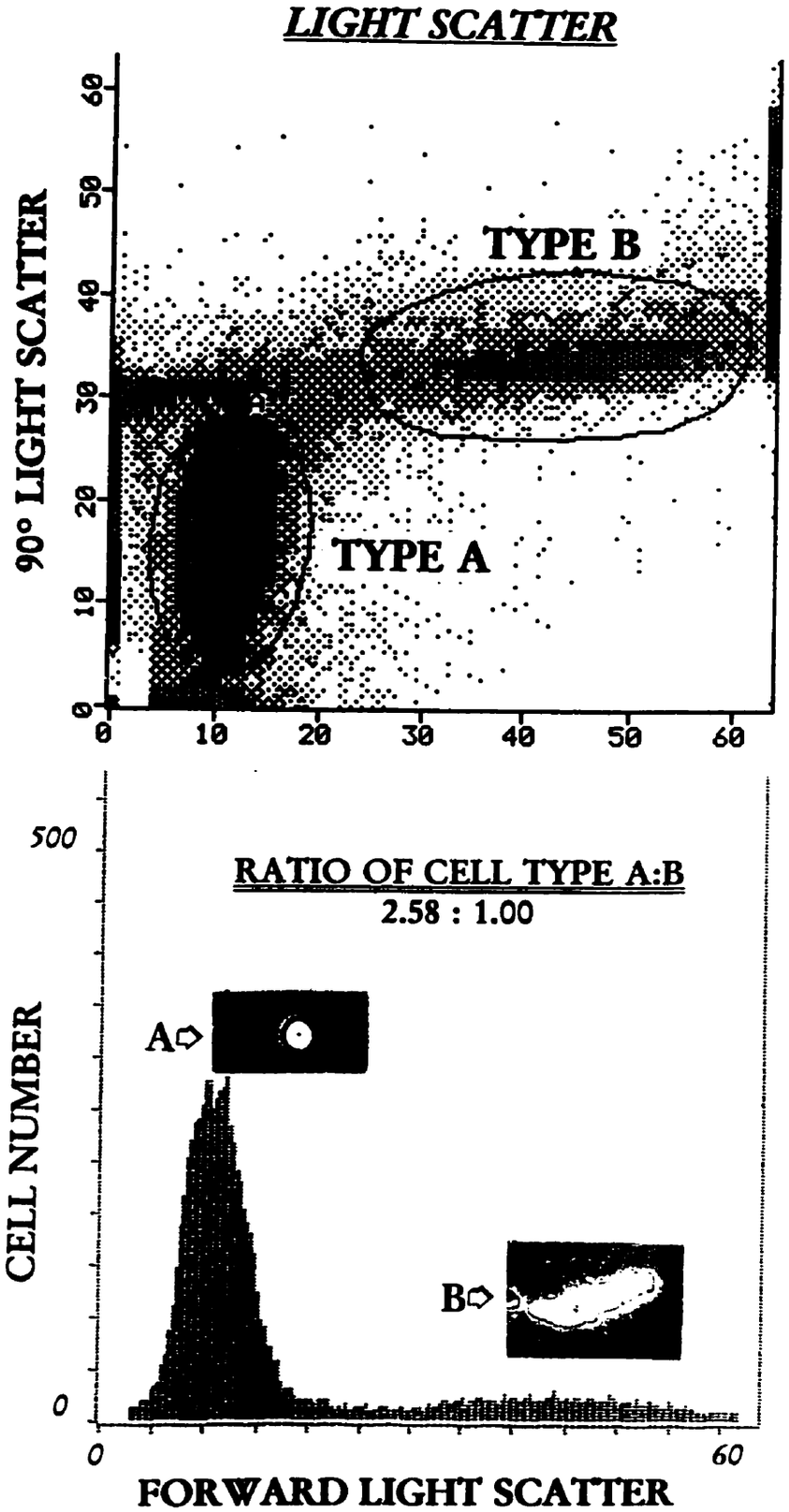

LIGHT SCATTER
90° LIGHT SCATTER
TYPE B
TYPE A
0
10
20
30
40
50
60
500
RATIO OF CELL TYPE A:B
2.58 : 1.00
A
B
CELL NUMBER
FORWARD LIGHT SCATTER

FIGURE 34. Cytospin of acutely dissociated tracheal SMCs stained for sm-α-actin using peroxidase-anti-peroxidase.

Diaminobenzidine was used as a substrate (refer to Section 3.3.8 for staining protocol). All cell preparations were also counterstained with Harris' haematoxylin to visualize nuclei.

A. Low power light micrograph of enzymatically dispersed canine tracheal SMCs stained for sm-α-actin. Scale legend: 1 mm = 15 μm. Note the presence of elongate cells of varying degrees of staining.

B. Higher power light micrograph of enzymatically dispersed SMCs stained for sm-α-actin. Scale legend: 1 mm = 27 μm.

C. Negative control. High power micrograph of enzymatically dispersed tracheal SMCs. Scale legend: 1 mm = 27 μm.

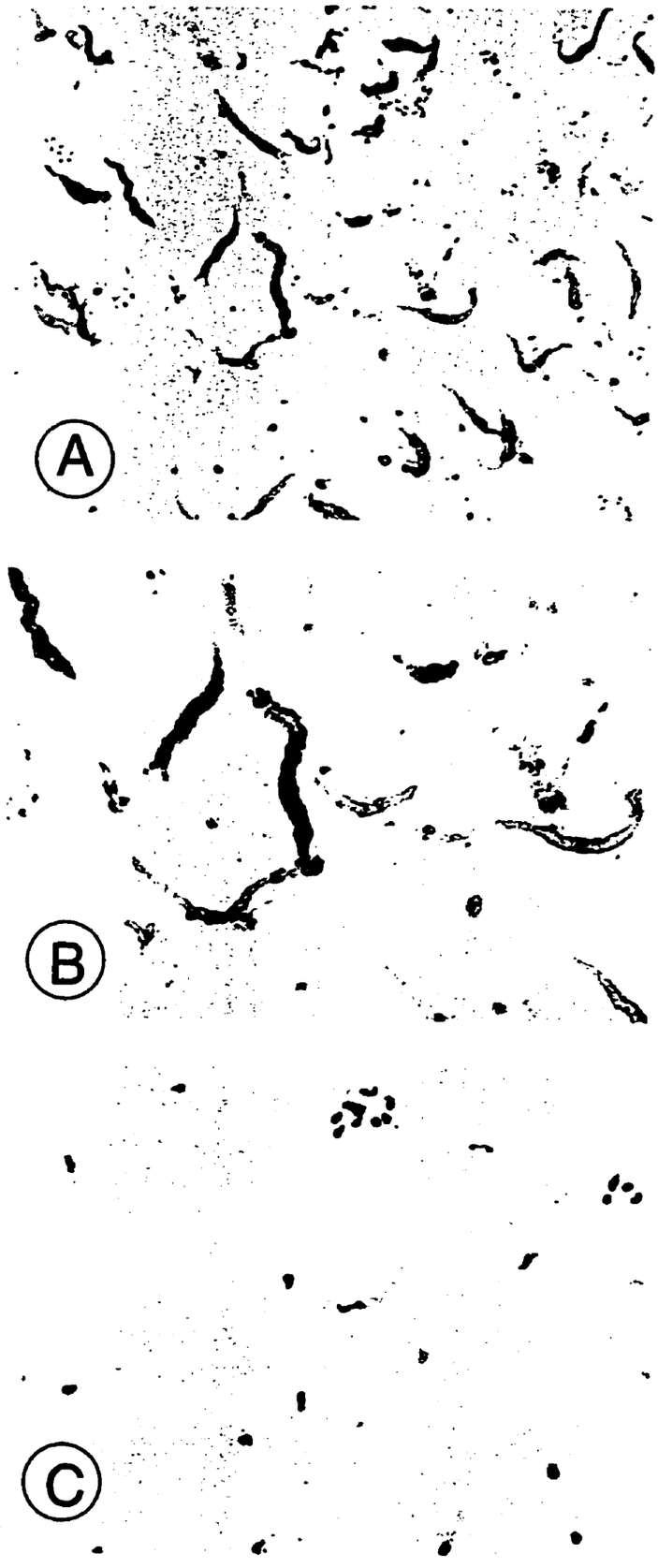
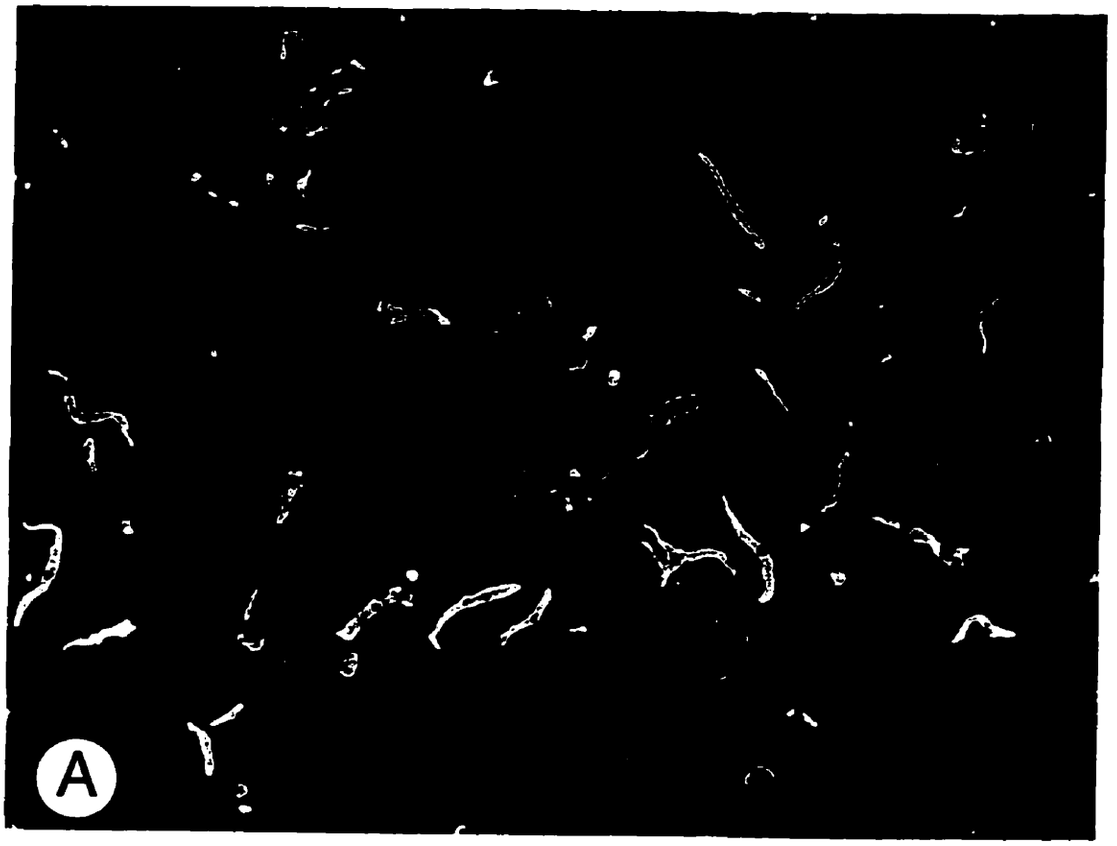

A
B
C

FIGURE 35. Immunofluorescence staining for smMHC in cytospins of acutely dissociated canine tracheal SMCs.

Cells were stained by indirect methods using Cy3 conjugated secondary antibodies as described in Sections 3.3.7.2 and 3.3.8.

A. Low power fluorescent micrograph of cytospun tracheal myocytes previously dispersed by enzymatic digestion. Note the presence of brightly stained (appears yellow in picture) Type A and Type B cells, however, the frequency of brightly stained Type A cells is lower than that seen for Type B cells. Moderately stained cells are also apparent, these appear as red cells with flecks of yellow in them. Scale legend: 1 mm = 10 μm.

B. Higher power fluorescent micrograph of a different field from the same SMC preparation shown in Panel A. Scale legend: 1 mm = 25 μm.


A


B

FIGURE 36. Cytospin of acutely dissociated tracheal SMCs stained for desmin using peroxidase-anti-peroxidase.

Diaminobenzidine was used as a substrate (refer to Section 3.3.8 for staining protocol). All cell preparations were also counterstained with Harris' haematoxylin to visualize nuclei.

A. Low power light micrograph of cytospun tracheal myocytes stained for desmin. Note the lack of Type A cell staining. Approximately 40 % of Type B cells stained positively. Scale legend: 1 mm = 20 μm.

B. Higher power light micrograph of cytospun myocytes. Scale legend: 1 mm = 10 μm.

C. High power micrograph of negative control SMC preparation. Scale legend : 1 mm = 10 μm.

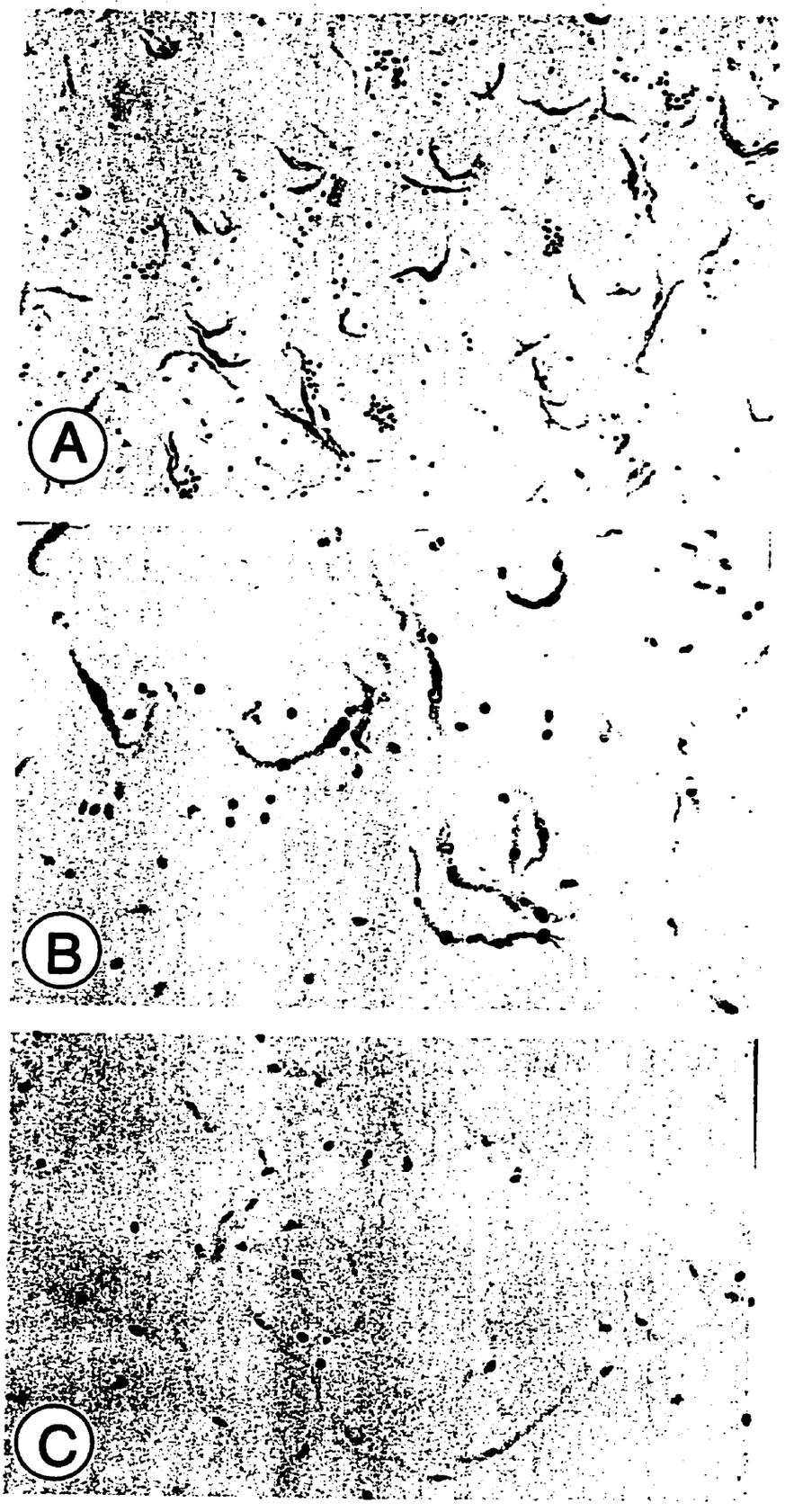

A
B
C

FIGURE 37. Immunofluorescence staining for vimentin in cytospins of acutely dissociated canine tracheal SMCs.

Cells were stained by indirect methods using Cy3-3 conjugated secondary antibodies as described in Sections 3.3.7.2 and 3.3.8.

A. Low power fluorescent micrograph of cytospun tracheal myocytes previously dispersed by enzymatic digestion. Brightly stained (appear yellow) Type A and Type B cells are present. Moderately stained cells are also apparent, these appear as red cells with flecks of yellow in them, however, staining appears to be more homogeneous than that seen for smMHC, desmin and sm-α-actin. Scale legend: 1 mm = 10 μm.

B. Higher power fluorescent micrograph of a tracheal SMC preparation. Scale legend: 1 mm = 25 μm.


A


B

FIGURE 38. Growth characteristics of sorted Type A and Type B tracheal myocytes in primary culture.

Cells were first sorted by flow cytometry, on the basis of light scatter properties then were cultured in DMEM / 10% FBS. Plating density for both cell populations was 5 x 10^3 cells/cm^2. The growth curves represent means of three different experiments done in duplicate. Vertical bars, where present, represent standard error.

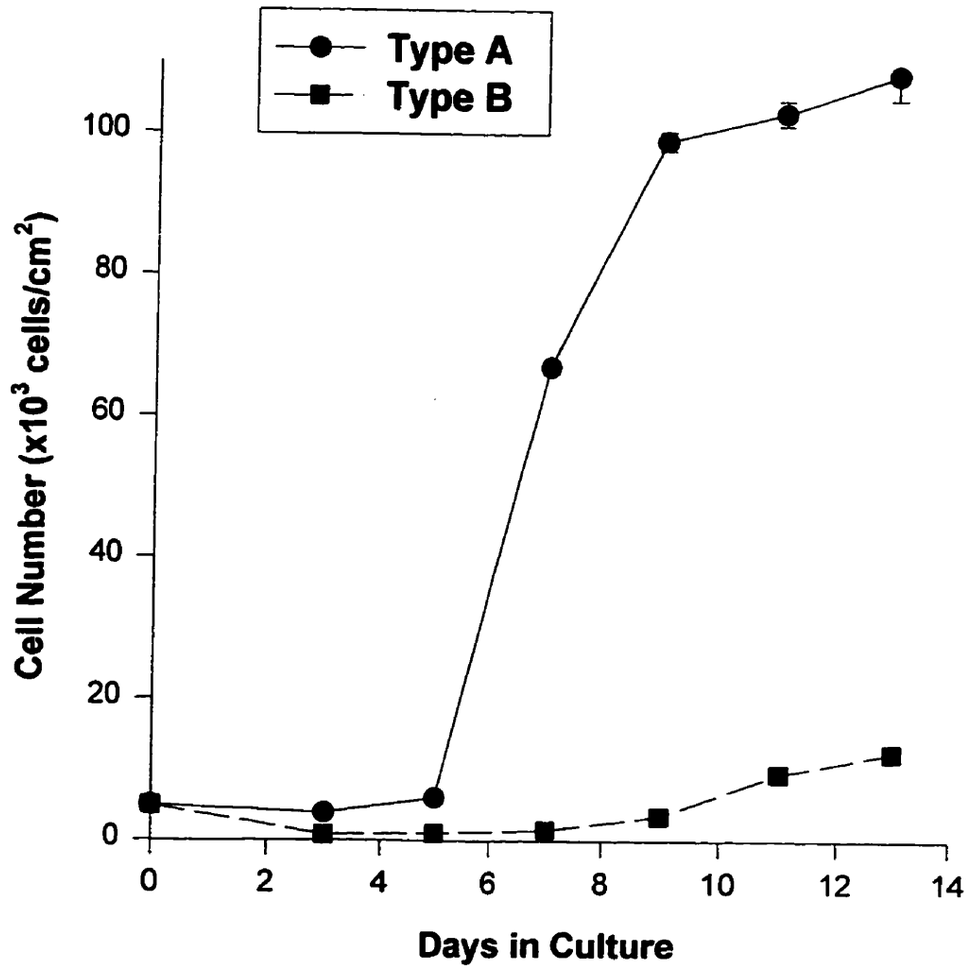

Type A
Type B
Cell Number (x10^3 cells/cm^2)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days in Culture

FIGURE 39. Morphological diversity of sorted Type A and Type B canine tracheal myocytes in culture.

Phase contrast micrographs showing typical morphologies of growth arrested, cultured cells. All pictures are of cells from cultures which had been growth arrested for 96 hours.

Type A picture was taken 11 days after cell seeding. Hence cultures were approximately 70% confluent and had been cultures in the presence of 10% FBS for 7 days prior to growth arrest. This picture shows an area where myocytes had aligned themselves in a parallel manner. Areas like this constituted approximately 50% of the entire culture. In the remaining areas cells grew in a less organized pattern. At confluence these cultures resembled the "hill-and-valley" pattern typical of airway myocyte primary cultures.

Two cell morphologies, "Intermediate" and "Large", were evident in Type B cultures. Pictures were taken of cultures 18 days after initial seeding, hence, cells were maintained in the presence of serum for 2 weeks prior to 96 hours of growth arrest. These cells were seen only in focal regions on the plates and at the time the pictures were taken the plates were less that 20% confluent. Original seeding density of the culture plates shown was 20×10^3 cells / cm^2.

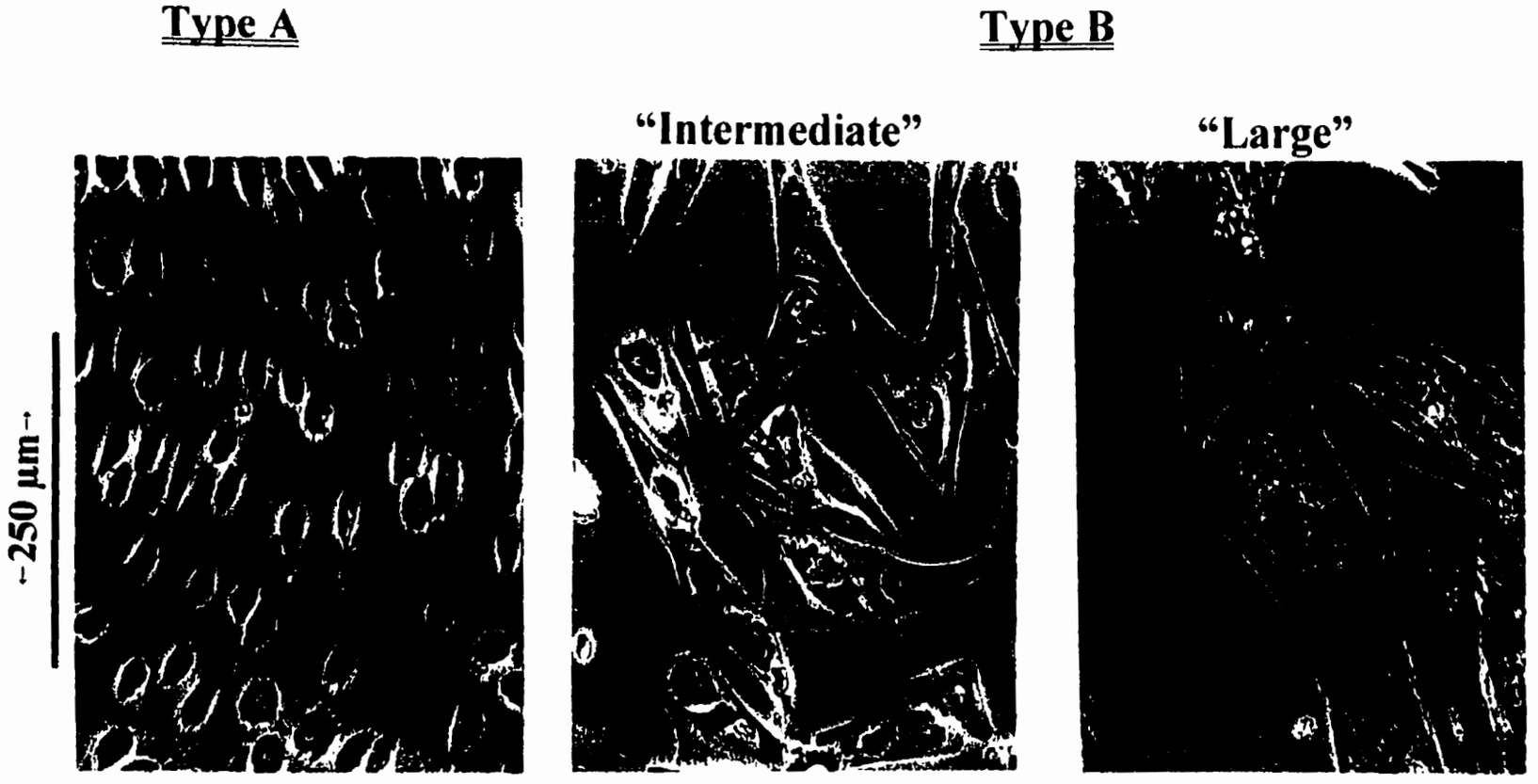

Type A
Type B
"Intermediate"
"Large"
250 µm

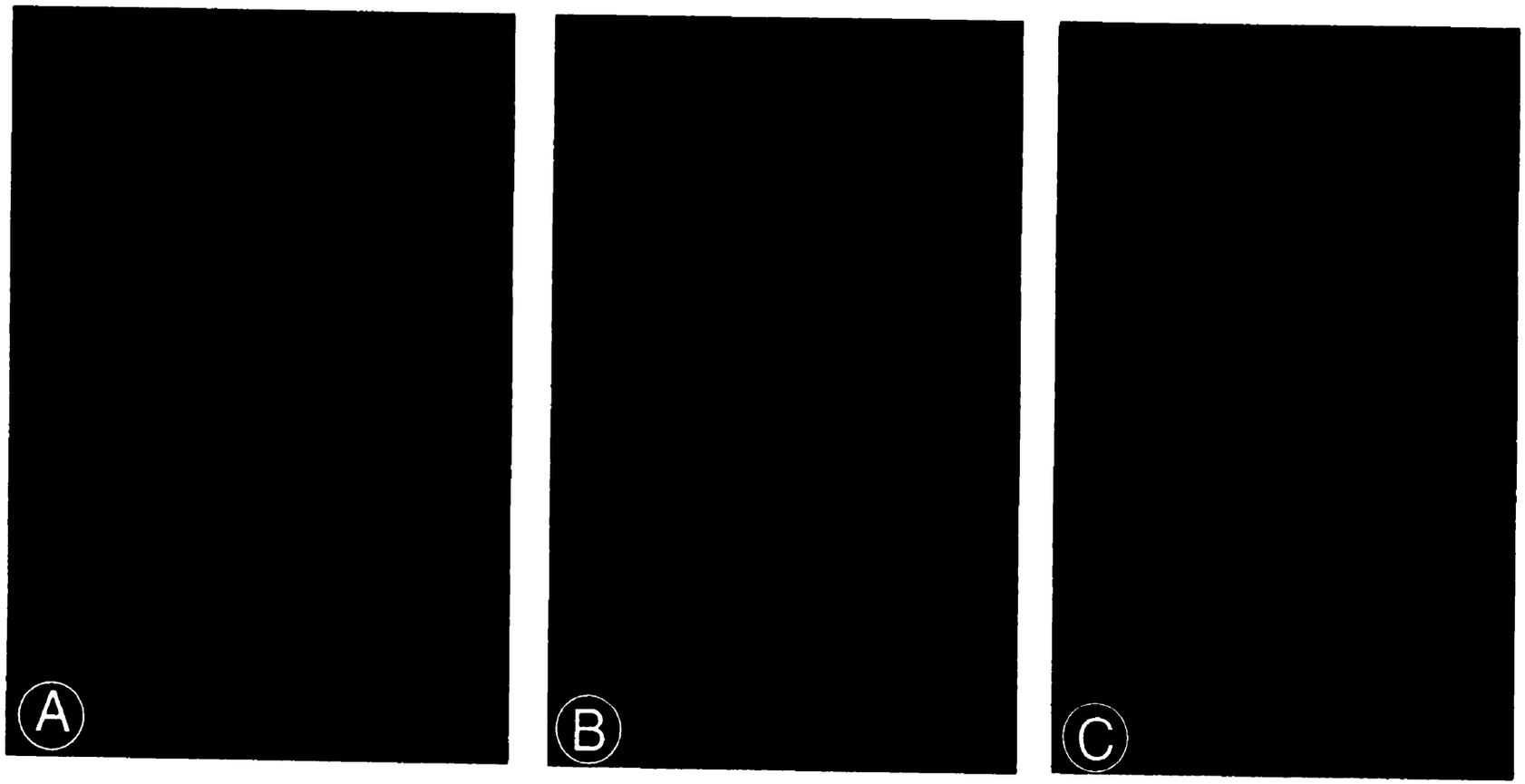
FIGURE 40. Fluorescent immunocytochemical staining for sm-α-actin, smMHC and DNA of Type A and Type B airway SMCs in primary culture.

The micrographs shown were obtained from the same field of sorted Type B SMCs grown in culture for 5 days. In this field a Type A cell is evident (upper cell in each panel) -- this cell had been coincidentally included in the sorted Type B cell population. Scale legend : 1 mm = 0.6 μm.

A. Cells stained for sm-α-actin. Secondary antibody used was conjugated with Cy3-3. Note the absence of staining in peripheral regions of the smaller, Type A cell. In addition, staining appears to be more diffuse and less intense, with fewer distinct microfilaments in the Type A cell.

B. Cells stained for smMHC. Secondary antibody used was conjugated with FITC. Both the Type A and Type B cell stain strongly for smMHC. Distinct microfilaments can be seen in the Type B cell. Staining in the Type A cell was more diffuse than that in the Type B cell.

C. Cells stained with the DNA specific bisbenzimide dye, Hoechst 33342. Note that the Type B cell was binucleate. The frequency of binucleation in Type B cells was approximately 25 %. Binucleation was seen in Type A SMC cultures but the frequency was less than 10 %.


A
B
C

7.5 Discussion

Based on morphological and immunocytochemical characterization of canine tracheal cells, at least two distinct subpopulations of cells were identified. The first population, called Type A cells, were relatively small and spheroid. They stained weakly for the SMC protein markers smMHC and sm-α-actin and appeared to be immunocytochemically negative for desmin, an intermediate filament protein that appears to be a reliable marker of smooth muscle cell maturation (Kocher et al., 1985). Type A cells did stain for vimentin, an intermediate filament protein expressed in mature SMCs and at high levels in immature SMCs (Kocher et al., 1985; Rubbia and Gabbiani, 1989). Type A cells attached well to plates when placed in primary culture and replicated readily in media supplemented with serum.

A second tracheal cell population, called Type B cells, were relatively large, elongate, and fusiform when dispersed from canine trachealis. They showed a tendency to contract when kept in cell suspension for several hours or when plated in culture, and they often decreased to as little as 40% of the length seen immediately after isolation. Type B cells were immunopositive for both smMHC and sm-α-actin, however, individual cell staining was heterogeneous and positive cells were seen to be either strongly or moderately stained. A similar pattern of staining for vimentin was observed. Approximately 50% of Type B cells were also desmin-positive. Type B cells did not attach well to culture plates; those that did attach grew slowly and only in isolated patches.

Both cell types were immunopositive for smMHC and sm-α-actin, though to

different degrees, hence, Type A and Type B cells appear to be of smooth muscle lineage. However, based on the differential expression of the smooth muscle-specific marker proteins, morphology, and pattern of replication in culture, Type A and Type B cells appear to be of divergent phenotype. This interpretation correlates well with observations that two broad subpopulations of cells / SMCs are present in the walls of human and animal arteries (Walker et al., 1986; Frid et al., 1994; Neylon et al., 1994; Ehler et al., 1995; Mironov et al., 1995; Wohrley et al., 1995; Holifield et al., 1996; Topouzis and Majesky, 1996). The majority of arterial cells are medially located, relatively large and fusiform shaped, and express smMHC, sm-α-actin. Furthermore these cells appear to express late-differentiation markers of maturation, including desmin and meta-vinculin, in abundance (Frid et al., 1994). The second major group of cells described in the arterial wall are located in intimal, intra-lamellar and adventitial layers. They are generally small, spheroid or stellate cells that stain positively for sm-α-actin and non muscle myosin, but stain weakly or are negative with respect to smMHC and other markers of late maturation (Sartore et al., 1994; Mironov et al., 1995). Clearly, the two subpopulations we described in airway smooth muscle cell preparations appear to be segregated in a manner similar to that described for arterial smooth muscle.

Our observation that Type B cells attached poorly to culture plates and failed to proliferate suggests that they may represent a terminally differentiated, subpopulation of mature, contractile SMCs. The viability of Type B cells after cell dispersion was equal to that of Type A cells, which attached well to culture plates and replicated readily. Therefore, it is unlikely that poor cell attachment was due to excessive cell damage. The

fact that Type B cells shortened during storage in suspension, and after seeding for culture suggests that these cells retained the ability to contract. Neylon et al. (1994) reported that the resting membrane potentials of large, fusiform cells from rat aortae were less negative than that of small, stellate cells indicating that the former might be more sensitive to contractile agonism. Similar observations concerning cell attachment, proliferation and contraction during storage have been reported for elongate cells isolated from canine carotid media and rabbit urinary bladder (Lau and Chacko, 1996; Holifield et al., 1996). Collectively, these data indicate that all mature SMCs may not retain the capacity for phenotypic modulation. This possibility requires further investigation of the molecular aspects that govern airway smooth muscle differentiation and maturation.

The heterogeneous immunostaining pattern observed in Type B cells for smMHC and sm-α-actin mimicked our earlier observations that canine airway myocytes can be discriminated into at least two subpopulations on the basis of contractile protein content (refer to Figures 26-29 in Chapter 6). It is also of note that Type B cells exhibited two distinct morphologies, intermediate and large, in cell culture. However, immunocytochemical analysis was not performed to assess the phenotypic state of each of these morphologic variants. Furthermore, since Type A cells stained relatively weakly for these marker proteins, the subpopulations discriminated using immunofluorescence and flow cytometry may correspond in a qualitative manner to the Type A and Type B cells identified in this study. In cell culture, few multi-nucleate Type A cells were observed, whereas multi-nucleate cells were often seen in Type B cells. This may indicate that the tetraploid SMC populations seen in the flow cytometry studies reported

earlier (Figure 25) may have been comprised mainly of Type B cells.

Clonal cell cultures of putative myoblastic or immature vascular SMCs have been established and described (Walker et al., 1986; Cook et al., 1994; Lemire et al., 1994; Bochaton-Piallat et al., 1996). These cells are generally small and epitheloid-like, they divide frequently, apparently capable of autocrine-driven replication, and secrete high levels of matrix proteins. The morphologic and immunocytochemical characteristics of canine tracheal Type A cells resemble those of the cells described above, and Type A cells replicate readily in culture. However, we did not observe continued proliferation of Type A cells when the cells were maintained in serum-free culture media, thus, indicating that they were not capable of autocrine mitogenic stimulation in the conditions we employed. Since acutely dissociated Type A cells contained low levels of smMHC and desmin they would appear to be of a more-or-less immature, differentiated SMC phenotype which is capable of phenotypic modulation and proliferation.

Primary cultures of airway SMCs from a number of sources are in routine use in several laboratories (Panettieri et al., 1989 and 1990; Hirst et al., 1992; Hall and Kotlikoff, 1995; Halayko et al., 1996a). Cultures have been used to investigate the mitogenic potential of exogenous compounds, signalling pathways that regulate mitogenesis and contraction and regulation of expression of smooth muscle specific proteins (reviewed by Hall and Kotlikoff, 1995; Halayko et al., 1996a; Solway et al., 1996). The results of our current investigation suggest that methods used in establishing primary airway SMC cultures likely select for myocytes with Type A cell characteristics. That is, enzymatically dispersed Type A airway myocytes likely adhere preferentially to

culture plates and subsequently become the dominant cell type in cultures by outgrowing poorly replicating Type B cell populations. This artificial cell selection has until now, not been a recognized feature of airway myocyte primary cell cultures.

Morphological and immunocytochemical observations indicate that arterial SMCs present in neointimal atherosclerotic lesions are of an immature phenotype (Zanellato et al., 1990; Sartore et al., 1994; Holifield et al., 1996). Identification of which cells in the normal arterial wall that are precursors for SMCs in these lesions is a topic of considerable debate, however, recent investigations demonstrated that small, spheroid cells, called Type 2 cells, from the intima appear to populate sites of injury and proliferate (Holifield et al., 1996). These cells appear to be synonymous with a population of meta-vinculin-negative cells, called L3i, in the pulmonary artery (Frid et al., 1994). Wohrley et al. (1995), have reported that pulmonary arterial thickening in response to hypoxic insult is subserved by this meta-vinculin-negative, medial SMC subpopulation. We observed that the canine airway media contains Type A cells, a subpopulation that is morphologically similar to the previously described, arterial Type 2 and L3i cells (Frid et al., 1994; Holifield et al., 1996). Furthermore, Type A airway cells appear to be readily capable of proliferation. Therefore, it is intriguing to speculate that these cells could have a pathogenic role in airways remodelling associated with chronic asthma in a manner similar to that of Type 2 and L3i arterial cells in vascular remodelling associated with injury and in response to hypoxia.

We have observed that airway myocytes differing in phenotype and proliferative characteristics are present in canine tracheal smooth muscle. This finding suggests that

phenotypically diverse airway myocytes may contribute in a dissimilar fashion to airway smooth muscle function in normal and pathologic airways. The developmental origin and molecular factors regulating the expression of different phenotypes are not known. Elucidation of the pathogenesis of diseases such as bronchial asthma may hinge on clarification of these mechanisms, hence, future studies aimed in this area are warranted.

7.6 SUMMARY

Contractile and proliferative properties of airway smooth muscle are determinants of airway hyperresponsiveness and remodelling in asthma. Smooth muscle is composed of heterogeneous subtypes of smooth muscle cells that subserve distinct functions and determine tissue properties. Using enzymatic digestion, we dissociated canine tracheal SMCs to assess diversity in cellular properties. Flow cytometry revealed two SMC clusters differing in cell size. Smaller cells (~25 μm diameter), Type A, were spheroid whereas larger cells (>125 μm long), Type B, were elongate. Cells were isolated by cell sorting then cytospun and immunostained for α-actin, smMHC, desmin and vimentin. Type B cells showed either "high" or "low" staining. Type A cells stained less intensely for smMHC and α-actin than did Type B cells. About 50 % of Type B cells and <10% of Type A cells were desmin positive. Vimentin staining was heterogeneous in Type B cells. Sorted cells were also seeded in primary culture. Attachment efficiency of Type B to plastic dishes was <10% whereas >60% of Type A cells attached to culture substrata. Type A cells proliferated readily when grown in 10% serum whereas Type B cells appeared to be resistant to replication in culture. These data confirm the existence of

heterogeneous subsets of airway SMCs differing in mitogenic activity, morphology, and immunobiochemical and molecular attributes. Elucidation of mechanisms regulating expression of SMC phenotype may be critical in understanding the contribution of distinct SMC subtypes to airway hyperresponsiveness.

8. CONCLUSIONS AND SIGNIFICANCE

The principal conclusion which can be drawn from the studies described herein is that airway smooth muscle is composed of heterogeneous groups of myocytes. The results of several studies, which indicate that heterogeneity exists at several levels, have been presented:

1) Type A and Type B tracheal myocytes differ in attachment efficiency and proliferative frequency when seeded in primary culture. Hence, it would appear that normal airway SMC primary cultures are populated chiefly by Type A cells. This observation could have an important impact on the interpretation of experimental results obtained in this project and by other investigators. Results here (Chapter 4) showed that contractile protein content diminished within several days of primary culture. The content of contractile proteins in acutely dissociated Type B cells was much greater than in Type A cells. Collectively, these findings might be interpreted to indicate that the loss of contractile protein content seen in primary culture was due to the loss of Type B cells from the total cell population. This revelation calls into question the interpretation that all airway SMCs in primary culture may modulate phenotypically. Though this conclusion cannot be dismissed on this basis it clearly cannot be the only explanation for the data presented in Chapter 4.

2) Dominance of the Type A airway myocyte phenotype in primary cell cultures -- this contention suggests that studies which have been designed to assess the proliferation of airway SMCs in primary culture have likely focussed on the cell type that contributes

most significantly to replication. It cannot be stated with certainty that cells that are proliferative in culture are also proliferative *in vivo*, however it seems reasonable to assume that Type A cells may contribute significantly to airway smooth muscle thickening in chronic asthma. Pertaining to the results obtained from studies described herein, it appears that the serum insensitive- and serum responsive-SMC identified in culture, were likely members of the Type A subpopulation. Indeed, not only did cultured cells differ in serum responsiveness, but they exhibited distinct sensitivity to the antiproliferative action of heparin. This indicates that the broad classification of airway myocytes as being either Type A or Type B oversimplifies the heterogeneous nature of airway SMCs and clearly, subclasses of these cell type must exist.

3) Distinct subsets of airway myocytes can be discriminated based on the content of contractile protein content. Studies by ourselves (Halayko et al., 1996a; Chapter 4) and others (Fatigati and Murphy, 1984) indicate that contractile properties of smooth muscle can be predicted on the basis of contractile protein content. Data obtained from flow cytometry studies indicated that tracheal and bronchial smooth muscle were composed of distinct distributions of myocytes differing in contractile protein content. Tracheal smooth muscle had a greater proportion of SMCs with high smMHC and sm-α-actin content, and this correlated well with previous studies that indicated tracheal smooth muscle shortens more and shortens faster than bronchial smooth muscle (Ma et al., 1996). Collectively these observations indicate that the distribution of myocyte subpopulations in different airways plays a role in determining the physiologic

properties of that tissue.

This conclusion has important implications regarding the role of the SMC in the pathogenesis of bronchial asthma. Though it is a matter of considerable debate, some investigators believe that changes in the intrinsic properties of airway smooth muscle are related to the manifestation of asthma. If this is the case, then factors that may lead to alterations in the distribution of specific subtypes of airway myocytes may lead to changes in smooth muscle properties that could correlate with the onset of asthma-like symptoms. This is, of course highly speculative, however, the data obtained in this project stress the need to examine phenotypic heterogeneity of the myocytes in specimens obtained from normal and asthmatic human airways.

4) If the properties of airway smooth muscle are determined by the distribution of heterogeneous myocytes that comprise the tissue, then studies aimed at elucidating mechanisms underlying different characteristics need to be carried out using single cell preparations. Typically, pharmacological properties of airway smooth muscle have been studied using muscle strip preparations. Several studies have shown the contractile properties and pharmacologic responsiveness to be altered in muscles obtained from human asthmatic or from animal models of bronchial hyperresponsiveness (Schellenberg and Foster, 1984; de Jongste et al., 1987; Stephens et al., 1991 and 1993; Bramley et al., 1993). Similar investigations using single airway myocyte preparations would be most elucidating in understanding the potential role of the phenotype distribution of myocytes in determining airways hyperresponsiveness in human asthma and animal models of the

disease.

5) Contributions from vascular biology far exceed those from other areas, however, evidence is now emerging suggesting that predictable heterogeneity exists in airway smooth muscle and other non-vascular smooth muscle types. The developmental programs described for vascular SMCs and the pathogenic programs characterized for atherosclerosis and hypertension may parallel events occurring in other smooth muscle types during development and/or disease pathogenesis. Indeed, investigators wishing to pursue studies aimed at clarifying the contribution of SMC populations to the pathology of diseases such as asthma, where SMC proliferation has been identified, should follow the numerous leads presented from studies of vascular SMCs.

9. FUTURE PROSPECTS

The results presented in this dissertation describe the properties of phenotypically diverse airway smooth muscle cell subpopulations. Undoubtedly, discrimination of phenotypic variants is dependent upon the cellular and molecular characteristics and properties that are assayed. At this time, a new fundamental concept of airway smooth muscle cell biology has emerged as the major contribution of this project. That is, airway smooth muscle cells differ in ways that may have important implications to our understanding of how these cells participate in normal airway physiology and in pathophysiology of airways diseases. This is an accepted notion regarding vascular smooth muscle cells and the role they play in the pathogenesis of atherosclerosis and hypertension (Ross, 1994; Owens, 1995; Katoh and Periasamy, 1996). It is, however a new approach which is only now being considered by investigators interested in the ontogeny of airway smooth muscle and the role of airway myocytes in the pathogenesis of bronchial asthma.

Inasmuch as airway smooth muscle is thought to be an important component of the pathology of asthma, its contribution is usually only considered in the context of the acute exacerbation of the disease. Thickening of the bronchial wall, which includes sub-epithelial fibrosis, oedema and smooth muscle hypertrophy, are accepted features which contributes to bronchial hyperresponsiveness. The pathological responses to induced injury of the vascular wall are now a fairly well characterized sequence of events which includes a role for smooth muscle cells in the deposition of tissue matrix and in intimal thickening through cellular hyperplasia which is promoted in an autocrine fashion. The vascular smooth muscle cell is capable of participating in this manner because of its

ability to express a range of phenotypic states in response to changes in environment. Thus far, asthma researchers have not exploited this concept and, hence, have not included the functional heterogeneity of airway smooth muscle in schemes describing the pathogenesis of chronic asthma. There is, in fact, good evidence that the requisite factors to promote the phenotypic plasticity of smooth muscle are present in airways of asthmatic individuals. Markers of the phenotypic diversity of smooth muscle cells have been identified and many more will certainly be identified in the future. If these markers were to be used as tools to assess the differentiated state of airway smooth muscle cells in asthmatic airways, perhaps a clearer role for phenotypic modulation of bronchial myocytes in the pathogenesis of chronic asthma might become apparent.

In skeletal muscle, the *MyoD* family of genes determine commitment of embryonic precursors to myogenesis and regulate transcription of muscle specific genes (Olson, 1990). Recent studies in the field of vascular cell and molecular biology have begun to identify transcription factors in smooth muscle cells which may determine the phenotypic state that different cells express (Patel et al., 1992; Gorski et al., 1993; Blank et al., 1995; Jain et al., 1996; Miano et al., 1996). Elucidation of the molecular mechanisms which determine the phenotypic state of smooth muscle cells will open new possibilities for genetic approaches in the ablation of vascular pathologies such as atherosclerosis. Similarly, information of this nature will be vital in suppression of the contribution of airway smooth muscle cells to the disease state. Recent work with transgenic mouse models suggests that molecular regulation of smooth muscle-specific gene expression is quite different between smooth muscles of vascular and visceral origin

(Li et al., 1996; Moessler et al., 1996). This is unfortunate as it may limit the potential for direct application of some of the fundamental information that has been obtained from vascular smooth muscle cells.

Future work needs to address the specific developmental and molecular mechanisms that control segregation of mesenchymal cells to the smooth muscle lineage and that regulate phenotypic expression in differentiated airway myocytes. This will involve identification, cloning and characterization of the factors that regulate phenotype- and lineage-specific transcription factors in airway smooth muscle cells. The studies presented in this report establish the existence of specific phenotypic subpopulations of airway SMCs. These populations could be used immediately to search for state-specific transcription factors and to identify important regulatory mechanisms controlling phenotypic modulation and commitment to a terminally differentiated state. Clearly the future of airway smooth muscle cell and molecular biology is an area with potential for significant advances.

10. BIBLIOGRAPHY

Abraham, W.M., Abraham, M.K., and Ahmed, T. 1996. Protective effect of heparin onimmunologically induced tracheal smooth muscle contraction in vitro. *Int. Arch. Allergy Immunol.* **110**:79-84.

Ahmed, T., Syriste, T., Mendelssohn, R., Sorace, D., Mansour, E., Lansing, M., Abraham,W.M., and Robinson, M.J. 1994. Heparin prevents antigen-induced airway hyperresponsiveness: interference with IP_3-mediated mast cell degranulation? *J. Appl. Physiol.* **76**:893-901.

Aikawa, M., Sivam, P.N., Kuro-o, M., Kimura, K., Nakahara, K-i., Takewaki, S-i., Ueda, M., Yamaguchi, H., Yazaki, Y., Periasamy, M. and Nagai, R. 1993. Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. *Circ. Res.* **73**:1000-1012.

Andrea, J.E. and Walsh, M.P. 1992. Protein kinase C of smooth muscle. *Hypertension* **20**:585-595.

Antonissen, L.A., Mitchell, R.W., Kroeger, E.A., Kepron, W., Tse, K.S. and Stephens, N.L.. 1979. Mechanical alterations of airway smooth muscle in a canine asthmatic model. *J. Appl. Physiol.* **46**:681-687.

Applegate, D., Feng, W., Green, R.S., and Taubman, M.B. 1994. Cloning and expression

of a novel acidic calponin isoform from rat aortic vascular smooth muscle. *J. Biol. Chem.* **269**:10683-10690.

Archer, S.L., Huang, J.M.C., Reeve, H.L., Hampl, V., Tolarová, S., Michelakis, E., and Weir, E.K. 1996. Differential distribution of electrophysiologically distinct myocytes in conduit and resistance arteries determines their response to nitric oxide and hypoxia. *Circ. Res.* **78**:431-442.

Assender, J.W., Kontny, E., and Fredholm, B.B. 1994. Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation. *FEBS Lett.* **342**:76-80.

Au, Y.P.T., Dubrowolska, G., Morris, D.R., and Clowes, A.W. 1994. Heparin decreases activator protein-1 binding to DNA in part by posttranslational modification of Jun B. *Circ. Res.* **75**:15-22.

Au, Y.P.T., Montgomery, K.F., and Clowes, A.W. 1992. Heparin inhibits collagenase gene expression mediated by phorbol ester-responsive element in primate arterial smooth muscle cells. *Circ. Res.* **70**:1062-1069.

Avner, B.P., Delongo, J., Wilson, S., and Ladman, A.J. 1981. A method for culturing canine tracheal smooth muscle cells in vitro: morphologic and pharmacologic

observations. *Anat. Rec.* **200**:357-370.

Ayala, L.E., and Ahmed, T. 1991. Is there a loss of a protective muscarinic receptor mechanism in asthma? *Chest* **96**:1285-1291.

Babaev, V.R., Bobryshev, Y.V., Stenina, O.V., Tararak, E.M., and Gabbiani, G. 1990. Heterogeneity of smooth muscle cells in atheromatous plaque of human aorta. *Am. J. Pathol.* **136**:1031-1042.

Babij, P., Zhao, J., White, S., Woodcock-Mithchell, J., Mithcell, J., Absher, M., Baldor, L., Periasamy, M. and Low, R.B. 1993. Smooth muscle myosin regulation by serum and cell density in cultured rat lung connective tissue cells. *Am. J. Physiol.(Lung Cell. Mol. Physiol.).* **265**:L127-L132.

Babij, P., and Periasmy, M. 1989. Myosin heavy chain isoform diversity in smooth muscle is produced by differential RNA processing. *J. Mol. Biol.* **210**:673-679.

Bader, B.L., Jahn, L., and Franke, W.W. 1988. Low level expression of cytokeratins 8, 18 and 19 in vascular smooth muscle cells of the human umbilical cord and in cultured cells derived therefrom, with an analysis of the chromosomal locus containing the cytokeratin 19 gene. *Eur. J. Cell Biol.* **47**:300-319.

Bagby, R.M. 1990. Ultrastructure, cytochemistry, and organization of myofilaments in vertebrate smooth muscle cells. *In* Ultrastructure of Smooth Muscle. *Edited by* P.M. Motta. Kluwer Academic Publishers, Boston. pp. 23-61.

Bai, T.R. 1990. Abnormalities in airway smooth muscle in fatal asthma. *Am. Rev. Respir. Dis.* **141**:552-557.

Bai, T.R., Mak, J.C.W., and Barnes, P.J. 1994. A comparison of beta-adrenergic receptors and in vitro relaxant responses to isoproterenol in asthmatic airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* **6**:647-651.

Barnes, P.J. 1993. Inflammation. *In* Bronchial Asthma, Mechanisms and Therapeutics. *Edited by* E.B. Weiss and M. Stein. Little, Brown and Company Inc., Boston, Mass. pp 80-94.

Barnes, P.J., Rodger, I.W., and Thomson, N.C. 1988. Pathogenesis of asthma. *In* Asthma:Basic Mechanisms and Clinical Management. *Edited by* P.J. Barnes, I.W. Rodger, and N.C. Thomson, Academic Press Ltd., London. pp 415-444.

Bârzu, T., Pascal, M., Maman, M., Roque, C., Lafont, F., and Rousselet, A. 1996. Entry and distribution of fluorescent antiproliferative heparin derivatives into rat vascular smooth muscle cells: Comparison between heparin-sensitive and heparin-resistant

cultures. *J. Cell. Physiol.* **167**:8-21.

Bauer, K.D., and Jacobberger, J.W.1994. Analysis of intracellular proteins. *Meth. Cell Biol.* **41(A)**:351-376.

Beasley, R., Roche, W.R., Roberts, J.A., and Holgate, S.T. 1989. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* **139**:806-817.

Beasley, R., Burgess, C., Crane, J., Pearce, N., and Roche, W. 1993. Pathology of asthma and its clinical implications. *J. Allergy Clin. Immunol.* **92**:148-154.

Belkin, A.M., Ornatsky, O.I., Kabokov, A.E., Glukhova, M.A. and Koteliansky, V.E. 1988. Diversity of vinculin/meta-vinculin in human tissues and cultivated cells: expression of muscle specific variants of vinculin in human aorta smooth muscle cells. *J. Biol. Chem.* **111**:2159-2170.

Belknap, J.K., Grieshaber, N.A., Schwartz, P.E., Orton, E.C., Reidy, M.A., and Majack, R.A. 1996. Tropoelastin gene expression in individual vascular smooth muscle cells: Relationship to DNA synthesis during vascular development and after arterial injury. *Circ. Res.* **78**:388-394.

Benditt,E.P., and Benditt, J.M. 1973. Evidence for the monclonal origin of human atherscleroticplaques. *Proc. Natl. Acad. USA* **70**:1753-1756.

Birukov, K.G., Stepanova, G.O.V., Nanaev, A.K., and Shirinsky, V.P. 1991. Expression of calponin in rabbit and human aortic smooth muscle cells. *Cell Tissue Res.* **266**:579-584.

Ben-Jebria, A., Marthan, R., Rossetti, M., and Savineau, J.P. 1993. Effect of passive sensitization on the mechanical activity of human isolated bronchial smooth muscle induced by substance P, neurokinin A and VIP. *Br. J. Pharmacol.* **109**:131-136.

Benzakour, O., Kanthou, C., Kanse, S.M., Scully, M.F., Kakkar, V.V., and Cooper, D.N. 1996. Evidence for cultured human vascular smooth muscle cell heterogeneity: Isolation of clonal cells and study of their growth characteristics. *Thromb. Haemo.* **75**:854-858.

Björk, T., Gustafsson, L.E., and Dahlén, S. 1992. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am. Rev. Respir. Dis.* **145**:1087-1091.

Black, J.L. 1996. Role of airway smooth muscle cells. *Am. J. Resp. Crit. Care Med.* **153**(Suppl.):S2-S4.

Black, J.L., Marthan, R., Armour, C.L., and Johnson, P.R.A. 1989. Sensitization alters contractile responses and calcium influx in human airway smooth muscle. *J. Allergy Clin. Immunol.* **84**:440-447.

Blaes, N., Bourdillon, M.-C., Daniel-Lamaziere, J.-M., Michaille, J.-J., Adujar, M., and Covacho, C. 1991. Isolation of two morphologically distinct cell lines from arterial smooth muscle cells expressing high tumorigenic potentials. *In Vitro Cell Dev. Biol.* **27A**:725-734.

Blank, R.S., McQuinn, T.C., Yin, K.C., Thompson, M., Takeyasu, K., Schwartz, R.J., and Owens, G.K. 1992. Elements of the smooth muscle alpha-actin promoter required in *cis* for transcriptional activation in smooth muscle. Evidence for cell-type specific regulation. *J. Biol. Chem.* **267**:984-989.

Blank, R.S., Swartz, E.A., Thompson, M.M., Olson, E.N., and Owens, G.K. 1995. A retinoic acid-induced clonal cell line derived from P19 embryonal carcinoma cells expresses smooth muscle characteristics. *Circ. Res.* **76**:742-749.

Blank, R.S., Thompson, M.M., and Owens, G.K. 1988. Cell cycle versus density dependence of smooth muscle alpha actin expression in cultured rat aortic smooth muscle cells. *J. Cell. Biol.* **107**:299-306.

Blennerhassett, M.G., Kannan, M.S., and Garfield, R.E. 1987. Functional characterization of cell-to-cell coupling in cultured rat aortic smooth muscle *Am. J. Physiol.(Cell Physiol.)* **252**:C555-C569.

Bobik, A., Grooms, A., Millar, J.A., Mitchell, A., and Grinspukel, S. 1990. Growth factor activity of endothelin on vascular smooth muscle. *Am. J. Physiol. (Cell Physiol.)* **248**:C408-C415.

Bochaton-Piallat, M.L., Ropraz, P., Gabbiani, F., and Gabbiani, G. 1996. Phenotypic heterogeneity of rat arterial smooth muscle cell clones. Implications for the development of experimental intimal thickening. *Arterioscler. Thromb. Vasc. Biol.* **16**:815-820.

Bodner, M., Castrillo, J.L., Theill, L.E., Deerinck, T., Ellisman, M. and Karin, M. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55**:505-518.

Bowers, C.W., and Dahm, L.M. 1993. Maintenance of contractility in dissociated smooth muscle: low density cultures in a defined medium. *Am. J. Physiol. (Cell Physiol)* **264**:C229-C236.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of dye-binding. *Anal. Biochem.* **72**:248-254.

Bradley, B.L., Azzawi, M., Jacobson, M., Assoufi, B., Collins, J.V., Irani, A.-M.A., Schwarts, L.B., Durham, S.R., Jeffery, P.K., and Kay, A.B. 1991. Eosinophils, T-lymphocytes, mast cells, neutrophils, macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* **88**:661-674.

Bramley, A.M., Thomson, R.J., Roberts, C.R., and Schellenberg, R.R. 1993. Hypothesis: excessivebronchoconstriction in asthma is due to decreased airway elastance. *Eur. Respir. J.* 7:337-341.

Bretscher, A., and Lynch, W. 1985. Identification and localization of immunoreactive forms of caldesmon in smooth muscle and nonmuscle cells: A comparison with the distributions of tropomyosin and α-actinin. *J. Cell Biol.* **100**:1656-1663.

Brewster, C.E.P., Howarth, P.H., Djukanovic, R., Wilson, J., Holgate, S.T., and Roche, W.R. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* **3**:507-511.

Busch, S.J., Martin, G.A., Barnhart, R.L., Masayuki, M, Cardin, A.D., and Jackson, R.L. 1992. Trans-repressor activity of nuclear glycosamino glycans on fos and jun/AP-1 oncoprotein mediated transcription. *J. Cell. Biol.* **116**:31-42.

Busse, W.W., Calhoun, W.F., and Sedgwick, J.D. 1993. Mechanisms of airway inflammation in asthma. *Am. Rev. Respir. Dis.* **147**:S20-S24.

Byrne, B.J., Kaczorowski, Y.J., Coutu, M.D., and Craig, S.W. 1992. Chicken vinculin and metavinculin are derived from a single gene by alternative splicing of a 207-base pair exon uniqhe to meta vinculin. *J. Biol. Chem.* **267**:12845-12850.

Caleb, B.L., Hardenbrook, M., Cherington, V., and Castellot, J.J.,Jr. 1996. Isolation of vascular smooth muscle cell cultures with altered responsiveness to the antiproliferative effect of heparin. *J.Cell. Physiol.* **167**:185-1951.

Campbell, G.R., and Campbell, J.H. 1985. Smooth muscle phenotypic changes in arterial wall homeostasis Implications for the pathogenesis of atherosclerosis. *Expt. Mol. Path.* **42**:139-162.

Campbell, J.H., and Campbell, G.R. 1987. Chemical stimuli of the hypertrophic response in smooth muscle. *In* Hypertrophic Response in Smooth Muscle. *Edited by* C.L. Siedel. CRC Press, Boca Raton. FL. pp. 153-192.

Campbell, G.R., Campbell, J.H., Manderson, J.A., Horrigan, S., and Rennick, R.E. 1988. Arterial smooth muscle. A multifunctional mesenchymal cell. *Arch. Pathol. Lab. Med.* **112**:977-986.

Campbell, J.H., Rennick, R.E., Kalevitch, S.G., and Campbell, G.R. 1992. Heparin sulfate-degrading enzymes induce modulation of smooth muscle phenotype. *Exp. Cell Res.* **200**:156-167.

Campbell, J.H., Tachas, G., Black, M.J., Cockerill, G., and Campbell, G.R. 1991. Molecular biology of vascular hypertrophy. *Basic Res. Cardiol.* **86**(Suppl 1):3-11.

Cardoso, W.V. 1995. Transcription factors and pattern formation in the developing lung. *Am. J. Physiol.* **269**:L429-L442.

Castellot, J.J., Jr., Favreau, L.V., Karnovsky, M.J., and Rosenberg, R.D. 1982. Inhibition of vascular smooth muscle cell growth by endothelial-derived heparin. Possible role of a platelet endoglycosidase. *J. Biol. Chem.* **257**:11256-11260.

Castellot, JJ., Jr., Wong, K., Herman B., Hoover, R.L., Albertini, D.F., Wright, T.C., Caleb, B.L.,and Karnovsky, M.J. 1985. Binding and internalization of heparin by vascular smooth muscle cells. *J. Cell. Physiol.* **124**:13-20.

Castellot, J.J., Jr., Wright, T.C., and Karnovsky, M.J. 1987. Regulation of vascular smooth muscle cell growth by heparin and heparin sulfates. *Semin. Thromb. Hemost.* **13**:489-503.

Chamley-Campbell, J.H., and Campbell, G.R. 1981. What controls smooth muscle phenotype? *Atherosclersosis* **40**:347-357.

Chamley-Campbell, J.H., Campbell, G.R., and Ross, R. 1979. The smooth muscle cell in culture. *Physiol. Rev.* **59**:1-61.

Chitano, P., Sigurdsson, S.B., Halayko, A.J., and Stephens, N.L. 1993. Relevance of classification by size to topographical differences in bronchial smooth muscle response. *J. App. Physiol.* **75**:2013-2021.

Chitano, P., Sigurdsson, S.B., Stephens, A.M., Becker, A.B., Kepron, W., and Stephens, N.L. 1996. Increased response to antigen and histamine release in smaller sensitized canine bronchi. *Resp. Physiol.* **103**:253-261.

Chomczynski, P., and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.

Chopra, L.C., Twort, C.H.C., Cameron, I.R., and Ward, J.P.T. 1991. Inositol (1,4,5) triphosphate and guanosine 5'-*O*-(3-thiotriphosphate) induced Ca^{2+} release in cultures airway smooth muscle. *Br. J. Pharmacol.* **104**:901-906.

Chou, R-G. R., Stromer, M.H., Robson, R.M., and Huiatt, T.W. 1992. Assembly of

contractile and cytoskeletal elements in developing smooth muscle cells. *Dev. Biol.* **149**:339-348.

Clark, T., Ngai, P.K., Sutherland, C., Gröschel-Stewart, U. and M.P. Walsh. 1986. Vascualr smooth muscle caldesmon. *J. Biol. Chem.* **261**:8028-8035.

Clowes, A.W., Clowes, M.M., Kocher, O., Ropraz, P., Chaponnier, C., and Gabbiani, G. 1988. Arterial smooth muscle cells in vivo: relationship between actin isoform expression and mitogenesis and their modulation by heparin. *J. Cell. Biol.* **107**:1939-1945.

Clowes. A.W., Clowes, M.M., Au, Y.P.T., Reidy, M.A., and Belin, D. 1990. Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ. Res.* **67**:61-67.

Clowes, A.W., and Karnovsky, M.J. 1977. Suppression by heparin of smooth muscle cells proliferation in injured arteries. *Nature* **265**:625-626.

Clyman, R.I., McDonald, K.A., and Kramer, R.H. 1990. Integrin receptors on aortic smooth muscle cells mediate adhesion to fibronectin, laminin, and collagen. *Circ. Res.* **67**:175-186.

Cobb, J.L.S., and Bennett, T. 1970. An ultrastructural study of mitotic division in differentiated gastric smooth muscle cells. *Z. Zellforsch.* **103**:177-189.

Cohen, D.M., and Murphy, R.A. 1978. Differences in cellular contractile protein contents among porcine smooth muscles. *J. Gen. Physiol.* **72**:369-380.

Colbert, M.C., Kirby, M.L., and Robbins, J. 1996. Endogenous retinoic acid signalling colocalizes with advanced expression of the adult smooth muscle myosin heavy chain isoform during development of the ductus arteriosus. *Circ. Res.* **78**:790-798.

Cook, C.L., Weiser, M.C.M., Schwartz, P.E., Jones, C.L., and Majack, R.A. 1994. Developmentally timed expression of an embryonic growth phenotype in vascular smooth muscle cells. *Circ. Res.* **74**:189-196.

Cutz, E., Levison, H., and Cooper, D.M. 1978. Ultrastucture of airways in children with asthma. *Histopathol.* **2**:407-421.

Czmeci-Smith, G., Stahl, R., Showalter, L., and Carey, D. 1993. Differential expression of transmembrane proteoglycans in vascular smooth muscle. *J. Biol. Chem.* **268**:18740-18747.

Daemen, M.J.A.P., and De Mey, J.G.R. 1995. Regional heterogeneity of arterial

structural changes. *Hypertension* **25** (part 1):464-473.

Daemen, M.J., Lombardi, D.M., Bosman, F.T., and Schwartz, S.M. 1991. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ. Res.* **68**:450-456.

Darby, I., Skalli, O., and Gabbiani, G. 1990. α-smooth muscle actin is transiently expressed by myofibroblasts during experimantal wound healing. *Lab. Invest.* **63**:21-29.

Daykin, K.S., Widdop, S., and Hall, I.P. 1993. Control of histamine induced inositol phospholipid hydrolysis in cultured human tracheal smooth muscle cells. *Eur J Pharmacol Mol Pharmacol Sect* **246**:135-140.

De, S., Zelazny, E.T., Souhrada, J.F., and Souhrada, M. 1993. Interleukin-1β stimulates the proliferation of cultured airway smooth muscle cells via platelet derived growth factor. *Am. J. Respir. Cell Mol. Biol.* **9**:645-651.

de Jongste, J.C., Mons, H., Bonta, I.L., and Kerrebijn, K.F. 1987. Human asthmatic airway responses in vitro - a case report. *Eur. J. Respir. Dis.* **70**:23-29.

DeLuca, L.M. 1991. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J.* **5**:2924-2933.

Desmoulière, and Gabbiani, G. 1992. The role of arterial smooth muscle cells in the pathogenesis of atherosclerosis. *Cerebrovasc. Dis.* **2**:63-71.

Desmoulière, A., Rubbia-Brandt, L., and Gabbiani, G. 1991. Modulation of actin isoform expression in cultures arterial smooth muscle cells by heparin and culture conditions. *Arteriosclerosis Thromb* **11**:244-253.

Devine, C.E., Simpson, F.O., and Bertaud, W.S. 1971. Surface features of smooth muscle cells from the mesenteric artery and vas deferens. *J. Cell. Sci.* **8**:427-443.

Djukanovic, R., Roche, W.R., Wilson, J.W., Beasley, C.R.W., Twentyman, O.P., Howarth, P.H., and Holgate, S.T. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* **142**:434-457.

Dolowitz, D.A. and Dougherty, T.F. 1960. The use of heparin as an anti-inflammatory agent. *The Laryngoscope* **LXX**:873-884.

Dominiczak, A.F., Devlin, A.M., Lee, W.K., Anderson, N.H., Bohr, D.F., and Reid, J.L. 1996. Vascular smooth muscle polyploidy and cardiac hypertrophy in genetic hypertension. *Hypertension* **27**:752-759.

Draeger, A., Gimona, M., Stuckert, A., Celis, J.E., and Small, J.V. 1991. Calponin.

Developmental isoforms and a low molecular weight variant. *FEBS Lett.* **291**:24-28.

Duband, J.-L., Gimona, M., Scatena, M., Sartore, S., and Small, J.V. 1993. Calponin and SM22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* **55**:1-11.

Dunnill, M.S. 1960. The pathology of asthma with special reference to the changes in the bronchial mucosa. *J. Clin. Pathol.* **13**:27-33.

Dunnill, M.S., Masserella, G.R., and Anderson, J.A. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis and in emphysema. *Thorax.* **24**:176-179.

Ebina, M., Takahashi, T., Chiba, T., Motomiya, M. 1993. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Respir. Dis.* **148**:720-726.

Ebina, M., Yaegashi, H., Chibo, R., Takahashi, T., Motomiya, M., and Tanemura, M. 1990. Hyperreactive site in the airway tree of asthmatic patients revealed by thickening of bronchial muscles. *Am. Rev. Respir. Dis.* **141**:1327-1332.

Eddinger, T.J., and Murphy, R.A. 1991. Developmental changes in actin and myosin

heavy chain isoform expression in smooth muscle. *Arch. Biochem. Biophys.* **284**:232-237.

Egashira, K., Tomoike, H., Yamamoto, Y., Yamada, A., Hayashi, Y., and Nakamura,M. 1993. Histamine-induced coronary spasm in regions of intimal thickening in miniature pigs: Role of serum cholesterol and spontaneous or induced intimal thickening. *Circulation* **74**:826-837.

Ehler, E., Jat, P.S., Noble, M.D., Citi, S., and Draeger, A. 1996. Vascular smooth muscle cells of H-2K^b-tsA58 transgenic mice. Characterization of cell lines with distinct properties. *Circulation* **92**:3289-3296.

Ellwart, J., and Dormer, P. 1985. Effect of 5-fluoro-2'-deoxyuridine (FdUrd) on 5-bromo-2'-deoxyuridine (BrdUrd) incorporation into DNA measured with a monoclonal BrdUrd antibody and by the BrdUrd/Hoechst quenching effect. *Cytometry* **6**:513-520.

Erjefält, J.S., Erjefält, I., Sundler, F., and Persson, C.G.A. 1994. Micro-circulation derived factors in airways epithelial repair in vivo. *Microvasc. Res.* **48**:161-178.

Erjefält, J.S., Erjefält, I., Sundler, F., and Persson, C.G.A. 1995. In vivo restitution of airway epithelium. *Cell Tissue Res.* **281**:305-316.

Fager, G., Camejo, G., and Bondjers, G. 1992. Heparin-like glycosaminoglycans influence growth and phenotype of human arterial smooth muscle cells in vitro. I. Evidence for reversible binding and interaction of the platelet-derived growth factor by heparin. *In Vitro Cell. Dev. Biol.* **28a**:168-175.

Fan, T., Yang, M., Halayko, A.J., Mohapatra, S.S., and Stephens, N.L. 1996. Two inbred strains of mouse of disparate IgE and IL-4 production, differ in tracheal smooth muscle responsiveness. *Am. J. Respir. Cell Mol. Biol.*, In press.

Farmer, S.G., Ensore, J.E., and Burch, R.M. 1991. Evidence that cultured airway smooth muscle cells contain bradykinin B_2 and B_3 receptors. *J. Am. Respir. Cell. Mol. Biol.* **4**:273-277.

Fatigati, V., and Murphy, R.A. 1984. Actin and tropomyosin variants in smooth muscles. Dependence on tissue type. *J. Biol. Chem.* **259**:14383-14388.

Firulli, A.B., Miano, J.M., Bi, W.Z., Johnson, D., Casscells, W., Olson, E.N., and Schwarz, J.J. 1996. Myocyte enhancer binding factor-2 expression and activity in vascular smooth muscle cells. Association with the activated phenotype. *Circ. Res.* **78**:196-204.

Fleisch, J.H. 1980. Pharmacological aspects of airway smooth muscle. *Lung Biol. Health*

Dis. **15**:191-216.

Florio, C., Martin, J.G., Styhler, A. and Heisler, S. 1994. Antiproliferative effect of prostaglandin E_2 in cultures guinea pig tracheal smooth muscle cells. *Am. J. Physiol. (Lung Cell Physiol.)* **266**:L131-L137.

Frid, M.G., Shekhonin, B.V., Koteliansky, V.E., and Glukhova, M.A. 1992. Phenotypic changes in human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev. Biol.* **153**:185-193.

Frid, M.G., Moiseeva, E.P., and Stenmark, K.R. 1994. Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. Circ. Res. **75**:669-681.

Frid, M.G., Printesva, O.Y., Chiavegato, A., Faggin, E., Scatena, M., Koteliansky, V.E., Pauletto, P., Glukhova, M.A., and Soartore, S. 1993. Myosin heavy-chain isoform composition and distribution in developing and adult human aortic smooth muscle. *J. Vasc. Res.* **30**:279-292.

Gabbiani, G., Schmid, E., Winter, S., Chaponnier, C., De Chastonay, C., Vandekerckhove, J., Weber, K., and Franke, W. 1981. Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a

specific-type actin. *Proc. Natl. Acad. Sci. USA* **78**:298-300.

Gabella, G. 1984 Structural apparatus for force transmission in smooth muscles. *Physiol.Rev.* **64**:455-477.

Gabella, G. 1990. General aspects of the fine structure of smooth muscles. *In* Ultrasctructure of Smooth Muscle. *Edited* by P.M. Motta. Kluwer Academic Publishers, Boston, MA. pp.1-22.

Garfinkel, L.I., Periasamy, M., and Nadal-Ginard, B. 1982. Cloning and characterization of cDNA sequences corresponding to myosin light chains 1, 2, and 3, troponin-C, troponin-T, alpha-tropomyosin and alpha-actin. *J. Biol. Chem.* **257**:11078-11086.

Garg, H.G., and Lyon, N.B. 1991. Structure of collagen fibril-associated, small proteoglycans of mammalian origin. *Adv. Carbohydr. Chem. Biochem.* **49**:239-261.

Gaylinn, B.D., Eddinger, T.J., Martino, P.A., Monical, P.L., Hunt, D.F., and Murphy, R.A. 1989. Expression of nonmuscle myosin heavy and light chains in smooth muscle. *Am. J. Physiol. (Cell Physiol.)* **257**:C997-C1004.

Geary, R.L., Koyama, N., Wang, T.W., Vergel, S., and Clowes, A.W. 1995. Failure of heparin to inhibit intimal hyperplasia in injured baboon arteries. The role of heparin-

sensitive and -insensitive pathways in the stimulation of smooth muscle cell migration and proliferation. *Circulation* **91**:2972-2981.

Geiger, B., Tokuyasu, K.T., Dutton, A.H., and Singer, S.J. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA* **77**:4127-4131.

Giachelli, C., Bae, N., Lombardi, D., Majesky, M., and Schwartz, S.M. 1991. Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes in vitro and is identical to osteopontin (secreted phosphoprotein 1,2aR). *Biochem. Biophys. Res. Commun.* **177**:867-873.

Gimona, M., Herzog, M., Vandekerckhove, J., and Small, J.V. 1990. Smooth muscle specific expression of calponin. *FEBS Lett.* **274**:159-162.

Gimona, M., Sparrow, M.P., Strasser, P., Herzog, M., and Small, J.V. 1992. Calponin and SM22 isoforms in avian and mammalian smooth muscle: absence of phosphorylation in vivo. *Eur. J. Biochem.* **205**:1067-1075.

Giuriato, L., Chiavegato, A., Pauletto, P., and Sartore, S. 1995. Correlation between thepresence of an immature smooth muscle cell population in tunica media and the development of atherosclerotic lesion. A study on different-sized rabbit arteries from

cholesterol-fed and Watanabe heritable hyperlipemic rabbits. *Atherosclerosis* **116**:77-92.

Giuriato, L., Scatena, M., Chiavegato, A., Tonello, M., Scannapieco, G., Pauletto, P., and Sartore, S. 1992. Non-muscle myosin isoforms and cell heterogeneity in developing rabbit vascular smooth muscle. *J. Cell Sci.* **101**:233-246.

Glade, C.P., Botermans, R.J., van Erp, P.E., and van de Kerkhof, P.C. 1995. The dynamics of the response of normal skin to single and multiple epicutaneos leukotriene B4 applications analyzed by three-color flow cytometry. *Acta. Derm. Venereol.* **75**:437-441.

Glukhova, M., Frid, M., Shekhonin, B., Balabanov, Y., and Koteliansky, V. 1990. Expression of fibronectin variants in vascular and visceral smooth muscle cells in development. *Dev. Biol.* **141**:193-202.

Glukhova, M.A., Kabakov, A.E., Belkin, A.M., Frid, M.G., Ornatsky, O.I., Zhidkhova, N.I., and Koteliansky,V.E. 1986. Metavinculin distribution in adult human tissues and cultured cells. *FEBS Lett.* **207**:139-141.

Glukhova, M.A., Kabakov,A.E., Frid, M.G., Ornatsky, O.I., Belkin, A.M., Mukhin, D.N., Orekhov, A.N., Koteliansky, V.E., and Smirnov, V.N. 1988. Modulation of human aortic smooth muscle cell phenotype: a study of muscle-specific variants of vinculin,

caldesmon, and actin expression. *Proc. Natl. Acad. Sci. USA* **85**:9542-9546.

Glukhova, M., Koteliansky, V., Fondacci, C., Marotte, F., and Rappaport, L. 1993. Laminin variants and integrin laminin receptors in developing and adult human smooth smooth muscle. *Dev. Biol.* **157**:437-447.

Goldberg, I., Rosen, E., Shapiro, H., Zoller, L., Myrick, K., Levenson, S., and Christenson, L. 1984. Isolation and culture of a tetraploid subpopulation of smooth muscle cells from normal rat aorta. *Nature (Lond.)* **226**:559-561.

Goldie, R.G., Spina, D., Henry, P.J., Lulich, K.M., and Paterson, J.W. 1986. In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, β-adrenoreceptor agonists and theophylline. *Br. J. Clin. Pharmacol.* **22**:669-679.

Gorski, D.H., LePage, D.F., Patel, C.V., Copeland, N.G., Jenkins, N.A., and Walsh, K. 1993. Molecular cloning of a diverged homeobox gene that is rapidly downregulated during the G_0/G_1 transition in vascular smooth muscle cells. *Mol. Cell. Biol.* **13**:3722-3733.

Graceffa, P. 1987. Evidence for interaction between smooth muscle tropomyosin and caldesmon. *FEBS Lett.* **218**:139-142.

Grainger, D.J., Hesketh, T.R., Metcalfe, J.C. and Weissberg, P.L. 1991. A large increase in non-muscle myosin occurs at first entry into M phase in rat vascular smooth muscle cells. *Biochem. J.* **277**:145-151.

Grainger, D.J., Witchell, C.M., Watson, J.V., Metcalfe, J.C., and Weissberg, P.L. 1993. Heparin decreases the rate of proliferation of rat vascular smooth muscle cells by releasing transforming growth factor β-like activity from serum. *Cardiovasc. Res.* **27**:2238-2247.

Green, W.F., Konnaris, K., and Woolcock, A.J. 1993. Effect of salbutamol, fenoterol, and sodium cromoglycate on the release of heparin from sensitized human lung fragments challenged with *Dermatophagoides pteronyssmus* allergen. *Am. J. Respi. Cell. Mol. Biol.* **8**:518-521.

Gröschel-Stewart, U., Schreiber, J., Mahlmeister, C., and K. Weber. 1976. Production of specific antibodies to contractile proteins and their use in immunofluorescence microscopy. I. Antibodies to smooth and striated chicken muscle myosins. *Histochemistry.* **46**:229-236.

Gunning, P., Gordon, M., Wade, R., Gahlmann, R., Lin, C-S., and Hardeman, E. 1990. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulatory compensation control mechanism.

Dev. Biol. **138**:443-453.

Halayko, A.J., and Stephens, N.L. 1994. Potential role for phenotypic modulation of bronchial smooth muscle cells in chronic asthma. *Can. J. Physiol. Pharmacol.* **72**:1448-1457.

Halayko, A.J., Salari, H., Ma, X., Stephens, N.L. 1996a. Markers of airway smooth muscle cell phenotype. *Am. J. Physiol.* **270**:L1040-L1051.

Halayko, A.J., Rector, E., and Stephens, N.L. 1996b. Airway smooth muscle is composed of phenotypically heterogeneous subpopulations of smooth muscle cells. *Am. J. Respir. Crit. Care Med.* **153**:A254 (Abstract).

Hall, I.P. and Kotlikoff, M. 1995. Use of cultured airway myocytes for study of airway smooth muscle. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* **268**:L1-L11.

Hall, I.P., Widdop, S.,Townsend, P., and Daykin, K. 1992. Control of cyclic AMP content in primary cultures of human tracheal smooth muscle cells. *Br. J. Pharmacol.* **107**:422-428.

Hamon, M., Bauters, C., Wernert, N., Courtin, P., Delcyre, C., Adamantidis, M., Lablanche, J.M., Bertrand, M.E., Dupuis, B., and Swynghedauw, B. 1993. Heparin does

not inhibit oncogene induction in rabbit aorta following balloon denudation. *Cardiovasc. Res.* **27**:1209-1213.

Hancock, K., and Tsang, V.C.W. 1983. India ink staining of proteins on nitrocellulose paper. *Anal. Biochem.* **133**:157-162.

Hayashi, K., Fujio, Y., Kato, I., and Sobue, K. 1991. Structural and functional relationships between *h*- and *l*-caldesmons. *J. Biol. Chem.* **266**:355-361.

Hedin, U., Bottger, B.A., Luthman, J., Johansson, S., and Thyberg, J. 1989. A substrate of the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser) is sufficient to promote transition of arterial smooth muscle cells from acontractile to a synthetic phenotype. *Dev. Biol.* **133**:489-501.

Hedin, U., Holm, J., and Hansson, G. 1991. Induction of tenascin in rat arterial injury. *Am. J. Pathol.* **139**:649-656.

Helper, D.J., Lash, J.A., and Hathaway, D.R. 1988. Distribution of isoelectric variants of the 17,000-dalton myosin light chain in mammalian smooth muscle. *J. Biol. Chem.* **263**:15748-15753.

Hirst, S.J., Barnes, P.J., and Twort, C.H. 1992. Quantifying proliferation of cultured

human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am. J. Respir. Cell. Mol. Biol.* 7:574-581.

Hirst, S.J., and Twort, C.H.C. 1992. The proliferative response of airway smooth muscle. *Clin.Exp.Allergy* **22**:907-915.

Hirst, S.J., Webb, B.L.J., Giembycz, M.A., Barnes, P.J., and Twort, C.H.C. 1995. Inhibition of fetal calf serum-stimulated proliferation of rabbit cultured tracheal smooth muscle cells by selsective inhibitors of protein kinase C and protein tyrosine kinase. *Am. J. Respir. Cell Mol. Biol.* **12**:149-161.

Holifield, B., Helgason, T., Jemelka, S., Taylor, A., Navran, S., Allen, J., and Seidel, C. 1996. Differentiated vascular myocytes: Are they involved in neointimal formation. *J. Clin. Invest.* **97**:814-825.

Holme, G., and Piechuta, H. 1981. The derivation of an inbred line of rats which develop asthma-like symptoms following challenge with aerosolized antigen. *Immunology* **42**:19-24.

Hossain, S. 1973. Quantitative measurement of bronchial muscle in men with asthma. *Am. Rev. Respir. Dis.* **107**:99-109.

Hultgardh-Nilsson, A., Nilsson, J., Jonzon, B., and Dalsgaard, C.-J. 1988. Coupling between inositol phosphate formation and DNA synthesis in smooth muscle cells stimulated with neurokinin A. *J. Cell. Physiol.* **137**:141-145.

Humphrey, M.B., Herrera Sosa, H., Gonzalez, G., Lee, R., and Bryan, J. 1992. Cloning of cDNAs encoding human caldesmons. *Gene* **112**:197-204.

Hungerford, J.E., Owens, G., Argraves, W.S., and Little, C.D. 1996. Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. *Dev. Biol.* **178**:375-392.

Ikebe, M., and Reardon, S. 1988. Binding of caldesmon to smooth muscle myosin. *J. Biol. Chem.* **263**:3055-3058.

Imai, H., Lee, K.J., Lee, S.K., Lee, K.T., Oneal, R.M., and Thomas, W.A. 1979. Ultrastructural features of aortic cells in mitosis in control and cholesterol-fed swine. *Lab. Invest.* **23**:401-415.

Ingraham, H.A., Chen, P.R., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L. and Resenfeld, M.G. 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* **55**:519-529.

Ishida, K., Parè, P.D., Thompson, R.J., and Schellenberg, R.R. 19990. Increased in vitro responses of trachealis smooth muscle from hyperresponsive guinea pigs. *J. Appl. Physiol.* **68**:1316-1320.

Ishimitsu, T., Uehara, Y., Ishii, M., and Sugimoto, T. 1988. Enhanced generation of vascular thromboxane A_2 in spontaneously hypertensive rats and its role in the rapid proliferation of vascular smooth muscle cells. *Am. J. Hypertension* **1**:38S-40S.

Jackson, C.L., and Schwartz, S.M. 1992. Pharmacology of smooth muscle cell replication. *Hypertension* **20**:713-736.

Jahn, L., Fouquet, B., Rohe, K., and Franke, W.W. 1987. Cytokeratins in certain endothelial and smoooth muscle cells of two taxanomically distant vertebrate species, Xenopsis laevis and man. *Differentiation* **36**:234-254.

Jahn, L., and Franke, W.W. 1989. High frequency of cytokeratin-producing smooth muscle cells in human atherosclerotic plaques. *Differentiation* **40**: 55-62.

Jain, M.K., Fujita, K.P., Hsieh, C.M., Endege, W.O., Sibinga, N.E.S., Yet, S.F., Kashiki, S., Lee, W.S., Perrella, M.A., Haber, E., and Lee, M.E. 1996. Molecular cloning and characterization of SmLIM, a developmentally regulated LIM protein preferentially expressed in aortic smooth muscle cells. *J. Biol. Chem.* **271**:10194-10199.

Jalkanen, M., Jalkanen, S. and Bernfield, M. 1991. Binding of extracellular effector molecules by cell surface proteoglycans. *In* Receptors For Extracellular Matrix. *Edited by* J.A. McDonald and R.P. Mecham. Academic Press, Inc., San Diego, CA. pp. 1-37.

James, A.L., Paré, P.D., and Hogg, J.C. 1989. The mechanisms of airway narrowing in asthma. *Am. Rev. Respir. Dis.* **139**:242-246.

Jiang, H., Rao, K., Halayko, A.J., Liu, X., and N.L. Stephens. 1992. Ragweed sensitization-induced increase of myosin light chain kinase content in canine airway smooth muscle. *Am. J. Respir. Cell. Mol. Biol.* 7:567-573.

Johnson, P.R.A., Armour, C.L., Carey, D., and Black, J.L. 1995. Heparin and PGE_2 inhibit DNA synthesis in human airway smooth muscle cells in culture. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* **269**:L514-L519.

Jones, P.A., Scott-Burden, T., and Gevers, W. 1979. Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **76**:353-357.

Jonzon, B., Nilsson, J., and Fedholm, B.B. 1985. Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J. Cell Physiol.* **124**:451-456.

Kameshita, I., and Fujisawa, H. 1989. A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. *Anal. Biochem.* **183**:139-143.

Kapanci, Y., Burgan, S., Pietra, G., Conne, B., and Gabbiani, G. 1990. Modulation of actin isoform expression in alveolar myofibroblasts (contractile interstitial cells) during pulmonary hypertension. *Am. J. Pathol.* **136**:881-889.

Kastan, M.B., Stone, K.D., and Civin, C.I. 1989. Nuclear oncoprotein expression as a function of lineage, differentiation stage, and proliferative status of normal human hematopoietic cells. *Blood* **74**:1517-1524.

Katoh, Y., Loukianov, E., Kopras, E., Zilberman, A., and Periasamy, M. 1994. Identification of functional promoter elements in the rabbit smooth muscle myosin heavy chain gene. *J. Biol. Chem.* **269**:30538-30545.

Katoh, Y. and Periasamy, M. 1996. Growth and differentiation of smooth muscle cells during vascular development. *Trends Cardiovasc. Med.* **6**:100-106.

Kawamoto, S. and R.S. Adelstein. 1987. Characterization of myosin heavy chains in cultured aorta smooth muscle cells. A comparative study. *J. Biol. Chem.* **262**:7282-7288.

Kelleher, M.D., Abe, M.K., Chao, T.-S.O., Jain, M.,Green, J.M., Solway, J., Rosner, M.R., and Hershenson, M.B. 1995. Role of MAP kinase activation in bovine tracheal smooth muscle mitogenesis. *Am. J. Physiol. (Lung Cell Mol Physiol)* **268**:L894-L901.

Kelley, C.A., Takahashi, M., Yu, J.H., and Adelstein, R.S. 1993. An insert of seven amino acids confers functional differences between smooth muscle myosins from the intestines and vasculature. *J. Biol. Chem.* **268**:12848-12854.

Kilfeather, S.A., Tagoe, S., Perez, A.C., Okona-Mensa, K., Matin, R., and Page, C.P. 1995. Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans. *Br. J. Pharmacol.* **114**:1442-1446.

Kocher , O., and Gabbiani, G. 1986. Expression of actin mRNA in rat aortic smooth muscle cells during development, experimental intimal thickening, and culture. *Differentiation* **32**:245-251.

Kocher, O., Skalli, O., Cerutti, D., Gabbiani, F. and Gabbiani, G. 1985. Cytoskeletal features of rat aortic cells during development: An electron microscopic, immunohistochemical, and biochemical study. *Circ. Res.* **56**:829-838.

Kocher, O., Gabbiani, F., Gabbiani, G., Reidy, M.A., Cokay, M.S., Peters, H., and Huttner, I. 1991. Phenotypic features of smooth muscle cells during the evolution of

experimental carotid artery intimal thickening. Biochemical and morphologic studies. *Lab. Invest.* **65**:459-470.

Kolbeck, R.C., and Spier, W.A., Jr. 1987. Regional contractile responses in pulmonary artery to α- and β-adrenoreceptor agonists. *Can. J. Physiol. Pharmacol.* **65**:1165-1170.

Komuro, I. and Izumo, S. 1993. *Csx*: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc. Natl. Acad. Sci. USA* **90**:8145-8149.

Kong, S-K., and Stephens, N.L. 1981. Mechanical properties of pulmonary arteries from sensitized dogs. *J. Appl. Physiol.* **55**:1669-1673.

Kong, S.-K., Halayko, A.J., and Stephens, N.L. 1990. Increased myosin phosphorylation in sensitized canine tracheal smooth muscle. *Am. J. Physiol. (Lung Cell Physiol.)* **259**:L53-L56.

Koteliansky, V.E., Ogryzko, E.P., Zhidkhova, N.I., Weller, P.A., Critchley, D.R., Vancompernolle, K., Vandekerckhove, J., Strasser, P., Way, M., Gimona, M., and Small, J.V. 1992. An additional exon in the human vinculin gene specifically encodes meta-vinculin-specific difference peptide. Cross species comparison reveals variable and conserved motifs in the metavinculin insert. *Eur. J. Biochem.* **204**:767-772.

Kuro-o, M., Nagai, R., Nakahara, K., Katoh, H., Tsai, R.-C., Tsuchimochi, H., Yazaki, Y., Ohkubo, A., and Takaku, F. 1991. cDNA cloning of a myosin heavy chain isoform in embryonic smooth muscle and its expression during vascular development and in arteriosclerosis. *J. Biol. Chem.* **266**:3768-3773.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**:680-685.

Laitinen, L.A., Heino, M., Laitinen, A., Kava, T., and Hashtela, T. 1985. Damage of the epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis.* **131**:599-606.

Lark, M.W., Yeo, T.K., Mar, H., Lara, S., Hellstrom, I., Hellstrom, K.E., and Wight, T.N. 1988. Arterial chondroitin sulphate proteoglycan: localization with monoclonal antibody. *J. Histochem. Cytochem.* **36**:1211-1221.

Lau, C.L., and Chacko, S. 1996. Identification of two types of smooth muscle cells from rabbit urinary bladder. *Tissue and Cell* **28**:339-355.

Lazaar, A.L., Albelda, S.M., Pilewski, J.M., Brennan, B., Pure, E., and Panettieri, R.A., Jr. 1994. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J. Exp. Med.* **180**:807-816.

Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.)* **283**:249-283.

Le Lièvre, C.S., and Le Douarin, N.M. 1975. Mesenchymal derivatives of the neural crest: analysis of chimeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **34**:125-154.

Leff, A.R. 1991. Inflammation and cell-to-cell interactions in airway hyperresponsiveness. *Am. J. Physiol (Lung. Cell Mol. Physiol.)* **260**:L189-L206.

Lemire, J.M., Covin, C.W., White, S., Giachelli, C.M., and Schwartz, S.M. 1994. Characterization of cloned aortic smooth muscle cells from young rats. *Am. J. Pathol.* **144**:1068-1081.

Lemire, J.M., Potter-Perigo, S., Hall, K.L., Wight, T.N., and Schwartz, S.M. 1996. Distinct rat aortic smooth muscle cells differ in versican/PG-M expression. *Arterioscler. Thromb. Vasc. Biol.* **16**:821-829.

Leslie, K., Mitchell, J., Woodcock-Mitchell, J., and Low, R. 1990. Alpha smooth muscle actin expression in developing and adult human lung. *Differentiation* **44**:143-149.

Letourneur, D., Caleb, B.L., and Castellot, J.J.,Jr. 1995. Heparin binding, internalization,

and metabolism in vascular smooth muscle cells. 1. Upregulation of heparin binding correlates with antiproliferative activity. *J. Cell. Physiol.* **165**:676-686.

Letourneur, D., Caleb, B.L., and Castellot, J.L., Jr. 1995b. Heparin binding, internalization, and metabolism in vascular smooth muscle cells: II. Degradation and secretion in sensitive and resistant cells. *J. Cell. Physiol.* **165**:687-695.

Lew, D.B., Nebigil, C., and Malik, K.U. 1992. Dual regulation by cAMP of ß-hexosaminidase-induced mitogenesis in bovine racheal myocytes. *Am. J. Respir. Cell Mol. Biol.* 7:614-619.

Lew, D.B., and Rattazzi, M.C. 1991. Mitogenic effect of lysosomal hydrolases on bovine tracheal myocytes in culture. *J. Clin. Invest.* **88**:1969-1975.

Lewis, E.B. 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* **276**:565-570.

Li, L., Miano, J.M., Cserjesi, P., and Olson, E.N. 1996a. SM22α, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ. Res.* **78**:188-195.

Li, L., Miano, J.M., Mercer, B., and Olson, E.N. 1996b. Expression of the *SM22α*

promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol.* **132**:849-859.

Li, Z.L., Lilienbaum, A., Butler-Browne, G., and Paulin, D. 1989. Human desmin - coding gene: complete nucleotide sequence, characterization and regulation of expression during myogenesis and development. *Gene* **78**:243-254.

Liddell, R., Syms, M., and McHugh, K. 1993. Heterogeneous isoactin gene expression in the adult rat gastrointestinal tract. *Gastroenterology* **105**:347-356.

Lindner, V., and Reidy, M.A. 1991. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **88**:3739-3743.

Lindner, V., Giachelli, C., Schwartz, S., and Reidy, M. 1995. A subpopulation of smooth muscle cells in injured rat arteries expresses platelet-derived growth factor-B chain mRNA. *Circ. Res.* **76**:951-957.

Lyons-Giordano, B., Brinjer, J.M., and Kefalides, N.A. 1989. Heparin increases mRNA levels of thrombospondin but not fibronectin in human vascular smooth muscle cells. *Biochem. Biophys. Res. Comm.* **162**:1100-1104.

Ma, X., Li, W., and Stephens, N.L. 1996. Detection of two clusters of mechanical properties of smooth muscle along the airway tree. *J. Appl. Physiol.* **80**:857-861.

Majack, R.A., and Bornstein, P. 1984. Heparin and related glycosaminoglycans modulate the secretory phenotype of vascular smooth muscle cells. *J. Cell Biol.* **99**:1688-1695.

Majack, R.A., and Clowes, A.W. 1984. Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. *J. Cell Physiol.* **118**:253-256.

Majack, R.A., Cook, S.C., and Bornstein, P. 1986. Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. *Proc. Natl. Acad. Sci. U.S.A.* **83**:9050-9054.

Majack, R.A., Grieshaber, N.A., Cook, C.L., Weiser, M.C.M., McFall, R.C., Grieshaber, S.S., Reidy, M.A., and Reilly, C.F. 1996. Smooth muscle cells isolated from the neointima after vascular injury exhibit altered responses to platelet-derived growth factor and other stimuli. *J. Cell. Physiol.* **167**:106-112.

Majesky, M.W., Benditt, E.P, and Schwartz, S.M. 1988. Expression and developmental control of platelet derived growth factor A-chain and B-chain/sis genes in rat aortic smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **85**:1524-1528.

Majesky, M.W., Giachelli, C.M., Reidy, M.A., and Schwartz, S.M. 1992. Rat carotid neointimal smooth muscle cells reexpress a developmentally regulated mRNA phenotype during repair of arterial injury. *Circ. Res.* **71**:759-768.

Majesky, M.W., Linder, V., Twardick, D.R., Schwartz. S.M., and Reidy, M.A. 1991. Production of transforming growth factor ß1 during repair of arterial injury. *J. Clin. Invest.* **88**:904-910.

Malmqvist, U. 1994. Effect of long term portal hypertension on structure, active force and content of contractile and structural proteins in smooth muscle of the rat portal vein. *Acta Physiol. Scand.* **150**:171-179.

Malmqvist, U., and Arner, A. 1991a. Correlation between isoform composition of the 17kDa myosin light chain and maximal shortening velocity in smooth muscle. *Pflugers Arch.* **418**:523-530.

Malmqvist, U., Arner, A., and Uvelius, B. 1991b. Lactate dehydrogenase activity and isoform distribution in normal and hypertrophic smooth muscle tissue from the rat. *Pflugers Arch.* **419**:230-234.

Manderson, J.A., Mosse, P.R.L., Safstrom, J.A., Young, S.B., and Campbell, G.R. 1989. Balloon catheter injury to rabbit carotid artery. I. Changes in smooth muscle cell

phenotype. *Arteriosclerosis* **9**:289-298.

Maples, J.A. 1985. A method for the covalent attachment of cells to glass slides for use in immunochemical assays. *Am. J. Clin. Pathol.* **83**:356-363.

Marston, S.B., and Smith, C.J.W. 1985. The thin filaments of smooth muscle. *J. Muscle Res. Cell Motil.* **6**:669-708.

McGowan, S.E. 1992. Extracellular matrix and the regulation of lung development and repair. *FASEB J.* **6**:2895-2904.

McHugh, K.M. 1995. Molecular analysis of smooth muscle development in the mouse. *Dev. Dynam.* **204**:278-290.

Meer, D.P. and Eddinger, T.J. 1996. Heterogeneity of smooth muscle myosin heavy chain expression at the single cell level. *Am. J. Physiol. (Cell Physiol.)* **270**:C1819-C1824.

Miano, J.M., Cserjesi, P., Ligon, K.L., Periasamy, M., and Olson, E.N. 1994. Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. *Circ. Res.* **75**:803-812.

Miano, J.M., Firulli, A.B., Olson, E.N., Hara, P., Giachelli, C.M., and Schwartz, S.M. 1996. Restricted expression of homeobox genes distinguishes fetal from adult human smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **93**:900-905.

Minoo, P., and King, R.J. 1994. Epithelial mesenchymal interactions in lung development. *Am. Rev. Physiol.* **56**:13-45.

Mironov, A.A., Rekhter, M.D., Kolpakov, V.A., Andreeva, E.R., Polishchuk, R.S., Bannykh, S.I., Filippov, S.V., Peretjatko, L.P., Kulida, L.V., and Orekhov, A.N. 1995. Heterogeneity of smooth muscle cells in embryonic human aorta. *Tissue and Cell* **27**:31-38.

Mitchell, R.W., and Halayko, A.J. 1996. Airway smooth muscle structure. *In* Asthma. *Edited by* P.J. Barnes, M.M. Grunstein, A.R.Leff, and A.J. Woolcock. Raven Press, NewYork. In press.

Mitchell, R.W., Kelly, E., and Leff, A.R. 1991. Reduced activity of acetylcholinesterase in canine tracheal smooth muscle homogenates after active immune-sensitization. *Am. J. Respir. Cell. Mol. Biol.* **5**:56-62.

Mitchell, R.W., Ruhlman, E., Magnussen, H., Leff, A.R., and Rabe, K.F. 1994. Passive sensitization of human bronchi augments smooth muscle shortening velocity and

capacity. *Am. J. Physiol. (Lung Cell. Physiol.)* **267**:L218-222.

Moessler, H., Mericskay, M., Li, Z., Nagl, S., Paulin, D., and Small, J.V. 1996. The *SM 22* promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. *Development* **122**:2415-2425.

Mohammed, M.A., and Sparrow, M.P. 1989. The distribution of heavy chain isoforms of myosin in airways smooth muscle from adult and neonate humans. *Biochem. J.* **260**:421-426.

Monical, P.L., Owens, G.K. and Murphy, R.A. 1993. Expression of myosin regulatory light-chain isoforms and regulation of phosphorylation in smooth muscle. *Am. J. Physiol (Cell Physiol.)* **264**:C1466-C1472.

Muthuchamy, M., Pajak, L., Howles, P., Doetschman, T., and Wieczorek, D.F. 1993. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Mol. Cell. Biol.* **13**:3311-3323.

Nagai, R., Kuro-o, M., Babij, P., and Periasamy, M. 1989. Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis. *J. Biol. Chem.* **264**:9734-9737.

Nanaev, A.K., Shirinsky, V.P., and Birukov, K.G. 1991. Immunofluorescent study of heterogeneity in smooth muscle cells of human fetal vessels using antibodies to myosin, desmin, and vimentin. *Cell Tissue Res.* **266**:535-540.

Naureckas, E.T., Hershenson, M.B., Abe, M.K., Kelleher, M.D., Fliorio, C., Heisler, S.I., Absher, M., Evans, J.N., Samsel, R.W., and Solway, J. 1995. Bronchioalveolar lavage fluid from immature rats with hyperoxia-induced airway remodelling is mitogenic for airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.* **12**:268-274.

Nemecek, G.M., Coughlin, S.R., Handley, D.A., and Moskowitz, M.A. 1986. Stimulation of aortic smooth muscle cell mitogenesis by serotonin. *Proc. Natl. Acad. Sci. USA* **83**:674-678.

Neylon, C.B., Avdonin, P.V., Dilley, R.J., Larsen, M.A., Tkachuk, V.A., and Bobik, A. 1994. Different electrical responses to vasoactive agonists in morphologically distinct smooth muscle cell types. *Circ. Res.* **75**:733-741.

Ngai, P.K., and Walsh, M.P. 1984. Inhibition of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity by caldesmon. *J. Biol. Chem.* **259**:13656-13659.

Nikkari, S.T., Jarvelainen, H.T., Wight, T.N., Ferguson, M., and Clowes, A.W. 1994. Smooth muscle cell expression of extracellular matrix genes after arterial injury. *Am. J.*

Pathol. **144**:1348-1355.

Nilsson, J., von Euler, A.M., and Dalsgaard, C.-J. 1985. Stimulation of connective tissue growth by substance P and substance K. *Nature* **315**:61-63.

Nishida, W., Kitami, Y., and Hiwada, K. 1993. cDNA cloning and mRA expression of calponin and SM22 in rat aortic smooth muscle cells. *Gene* **130**:297-302.

Noveral, J.P., and Grunstein, M.M. 1992. Role and mechanism of thromboxane-induced proliferation of cultured airway smooth muscle cells. *Am. J. Physiol. (Lung Cell Mol. Physiol.)* **263**:L555-L561.

Noveral, J.P., Rosenberg, S.M., Anbar, R.A., Pawlowski, N.A., and Grunstein, M.M. 1992b. Role of endothelin-1 in regulating proliferation of cultured rabbit airway smooth muscle cells. *Am. J. Physiol.* **263**:L317-L324.

Nyce, J.W., and Metzger, W.J. 1997. DNA antisense therapy for asthma in an animal model. *Nature* **385**:721-725.

O'Byrne, P.M. 1993. Airway hyperresponsiveness. *In* Allergy: Principles and Practice, 4th Ed. *Edited by* E. Middleton Jr., C. Reed, N.F. Adkinson, J.W. Yunginger, and W.W. Busse. Mosby, St. Louis. pp.1203-1213.

Ollerenshaw, S., Jarvis, D., Woolcock, A., Sullivan, C. and Scheibner, T. 1989. Absence of immunoreactive vasoactive intestinal polypeptide in tissue from the lungs of patients with asthma. *N. Engl. J. Med.* **320**:1244-1248.

Orekhov, A.N., Tertov, V.V., Kudryashov, S.A., Khashimov, K.H.A., and Smirnov, V.N. 1986. Primary culture of human aortic intimal cells as a model for testing antiatherosclerotic drugs: effect of cyclic AMP, prostaglandins, calcium antagonists, antioxidantants, and lipid-lowering agents. *Atherosclerosis* **60**:101-110.

Osborn, M., Caselitz, J., and Weber, K. 1981. Heterogeneity of intermediate filaments in vascular smooth muscle in vivo. *Differentiation*. **20**:196-202.

Ottlinger, M.E., Pukac, L.A., and Karnovsky, M.J. 1993. Heparin inhibits mitogen-activated protein kinase activation in intact rat vascular smooth muscle cells. *J. Biol. Chem.* **268**:19173-19176.

Owens, G.K. 1989. Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am. J. Physiol. (Heart Circ. Physiol.)* **257**:H1755-H1765.

Owens, G.K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75**:487-517.

Owens, G.K., Geisterfer, A.A.T., Yang, Y. W.-H., and Komoriya, A. 1988. Transforming growth factor-ß-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. *J. Cell. Biol.* **107**:771-780.

Owens, G.K., and Thompson, M.M. 1986. Developmental changes in isoactin expression in rat aortic smooth muscle cells in vivo. Relationship between growth and cytodifferentiation. *J. Biol. Chem.* **261**:13373-13380.

Page, C.P. 1993. An explanation of the asthma paradox. *Am. Rev. Respir. Dis.* **147**:S29-S32.

Palmberg, L., Claesson, H-E., and Thyberg, J. 1989. Effects of leukotrienes on phenotypic properties and growth of arterial smooth muscle cells in primary culture. *J. Cell Sci.* **93**:403-408.

Panettieri, R.,A., Jr, Murray, R.K., Bilgen, G., Eszterhas, A.J., and Martin, J.G. 1995. Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cells in vivo. *Chest* **107** (Suppl 3):94S-95S.

Panettieri, R.A., Murray, R.K., DePalo, L.R., Yadvish, P.A., and Kotlikoff, M.I. 1989. A human airway smooth muscle cell line that retains physiological responsiveness: *Am J Physiol (Cell Physiol.)* **256**:C329-C335.

Panettieri, R.A., Yadish, P.A., Kelly, A.M., Rubenstein, N.A., and Kotlikoff, M.I. 1990. Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am. J. Physiol. (Cell Physiol.).* **259**:L365-L371.

Patel, C.V., Gorski, G.H., Lepage, D.F., Lincecum, J., and Walsh, K. 1992. Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. *J. Biol. Chem.* **267**:26085-26090.

Pease, D.C., and Paule, W.J. 1960. Electron microscopy of elastic arteries. *J. Ultrastuct. Res.* **3**:469-483.

Phillips, G.N. Jr., and Chacko, S. 1996. Mechanical properties of tropomyosin and implications for muscle regulation. *Biopolymers* **38**:89-95.

Picker, L.J., Nakache, M., and Butcher, E.C. 1989. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J. Cell Biol.* **109**:927-937.

Pietilä, K., Moilanen, T., and Hikkari, T. 1980. Prostaglandins enhance the synthesis of glycosaminoglycans and inhibit growth of rabbit aortic smooth muscle cells in culture. *Artery* 7:509-518.

Pukac, L.A., Ottlinger, M.E., and Karnovsky, M.J. 1992. Heparin suppresses specific second messenger pathways for protooncogene expression in rat vascular smooth muscle cells. *J. Biol. Chem.* **267**:3707-3711.

Pyne, S., and Pyne, N.J. 1993. Bradykinin stimulates phospholipase D in primary cultures of guinea pig tracheal smooth muscle. *Biochem. Pharmacol.* **45**:595-603.

Raines, E.W., Dower, S.K., and Ross, R. 1989. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. *Science* **243**:393-396.

Reihsaus, E., Innis, M., MacIntyre, N., and Liggett, S.B. 1993. Mutations in the gene encoding for the β_2-adrenergic receptor in normal and asthmatic subjects. *Am. J. Respir. Cell Mol. Biol.* **8**:334-339.

Reilly, C.F., Fritze, L.M.S., and Rosenberg, R.D. 1986. Heparin inhibition of smooth muscle cell proliferation: A cellular site of action. *J. Cell. Physiol.* **129**:11-19.

Reilly, C.F., Fritze, L.M.S., and Rosenberg, R.D. 1987. Antiproliferative effects of heparin on vascular smooth muscle cells are reversed by epidermal growth factor. *J. Cell Physiol.* **131**:149-157.

Rennard, S. I. 1996. Extracellular matrix. *Am. J. Resp. Crit. Care. Med.* **153** (Suppl. 6):

S14-S15.

Roche, W.R., Brewster, C.E., Djukanovic, R., Wilson, J., and Howarth, P.H. 1990. Myofibroblasts are responsible for basement membrane thickening in bronchial asthma. *Am. Rev. Respir. Dis.* **141**:A501 (abstract).

Roche, W.R., Beasley, R., Williams, J.H., and Holgate, S.T. 1989. Subepithelial fibrosis in bronchi of asthmatics. *Lancet* **1**:520-524.

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.)*. **362**:801-809.

Ross, R., and Klebanoff, S.J. 1971. The smooth muscle cell. I. In vivo synthesis of connective tissue proteins. *J. Cell Biol.* **50**:159-171.

Rovner, A.S., Murphy, R.A. and Owens, G.K. 1986. Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells. *J. Biol. Chem.* **261**:14740-14745.

Rubbia, L. and Gabbiani, G. 1989. The cytoskeletol of arterial smooth muscle cells during development, atheromatosis, and tisue culture. *J. Cardiovasc. Pharmacol.* (Suppl.) **6**:S9-S11.

Rubin, K., Hansson, G.K., Rönnstrand, L., Claeson-Welsh, L., Fellström, B., Tingström, A., Larson E., Klareskog, L., Heldin, C.H., and Terracio, L. 1988. Induction of B-type receptors for platelet-derives growth factor in vascular inflammation: Possible implications for development of vascular proliferative lesions. *Lancet* **1**:1352-1356.

Ruderman, J.V. 1993. MAP kinase and the activation of quiescent cells. *Curr. Opinion Cell Biol.* **5**:207-213.

Sandford, A., Weir, T., and Paré, P. 1996. The genetics of asthma. *Am. J. Rev. Crit. Care Med.* **153**:1749-1765.

Salari, H., Chen-Yeung, M., Howard, S., and Schellenberg, R.R. 1992. Increased tracheal contraction from hyperresponsive guinea pigs correlates with enhanced polyphosphoinositide hydrolysis and intracellular calcium release. *J. Allergy Clin. Immunol.* **90**(6 Pt. 1):918-926.

Saltis, J., Thomas, A.C., Agrotis, A., Campbell, J.H., Campbell, G.R., and Bobik, A. 1995. Expression of growth factor receptors on arterial smooth muscle cells. Dependency on cell phenotype and serum factors. *Atherosclerosis* **118**:77-87.

Samuel, M., Kim, Y., Horiuchi, K.Y., Levin, R.M., and Chacko, S. 1992. Smooth muscle myosin isoform distribution and myosin ATPase in hypertrophied urinary bladder.

Biochem. Int. **26**:645-652.

Sappino, A.P., Schürch, W., and Gabbiani, G. 1990. Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as markers of phenotypic modulations.*Lab Invest.* **63**:144-161.

Sartore,S., Scatena, M., Chivegato, A., Faggin, E., Giuriato, L., and Pauletto, P. 1994. Myosin isoform expression in smooth muscle cells during physiological and pathological vascular remodelling. *J Vasc. Res.* **31**:61-81.

Sawtell, N.M., and Lessard, J.L. 1989. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the α-vascular but not the γ-enteric isoform in differentiating striated myocytes. *J. Cell. Biol.* **109**:2929-2937.

Schellenberg, R.R., and Foster, A. 1984. In vitro response of human asthmatic airway and pulmonary vascular smooth muscle. *Int. Arch. Allergy Appl. Immunol.* **75**:237-241.

Schonherr, E., Jarvelainen, H.T., Kinsella, M.G., Sandell, L.J., and Wight, T.N. 1993. Platelet-derived growth factor and transforming growth factor-β1 differentially affect the synthesis of buglycan and decorin by monkey arterial smooth muscle cells. *Arterioscler. Thromb.* **13**:1026-1036.

Schonherr, E., Jarvelainen, H.T., Sandell, L.J., and Wight , T.N. 1991. Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chrondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* **266**:17640-17647.

Schwartz, L.B., and Austen, K.F. 1980. Enzymes of the mast cells. *J. Invest. Dermatol.* **74**:349-353.

Schwartz, S.M., Campbell, G.R., and Campbell, J.H. 1986. Replication of smooth muscle cells in vascular disease. *Circ. Res.* **58**:427-444.

Schwartz, S.M., Foy, L., Bowden-Pope, D., and Ross, R. 1990. Derivation and properties of platelet-derived growth factor-independent rat smooth muscle cells. *Am. J. Pathol.* **136**:1417-1428.

Schwartz, S.M., and L. Liaw. 1993. Growth control and morphogenesis in the development and pathology of arteries. *J. Cardiovasc. Pharm.* **21**:S31-S49.

Scott, M.P., Tamkun, J.W. and Hartzell, G.W. 1989. The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989**:25-48.

Sears, M.R. 1996. Epidemiological trends in asthma. *Can. Respir. J.* **3**:261-268.

Seto, M., Yano, K., Sasaki, Y., and Azuma, H. 1993. Intimal hyperplasia enhances myosin phosphorylation in rabbit carotid artery. *Exp. Mol. Path.* **58**:1-13.

Shanahan, C.M., Weissberg, P.L., and Metcalfe, J.C. 1993. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Circ. Res.* **73**:193-204.

Shimizu, R.T., Blank, R.S., Jervis, R., Lawrenz-Smith, S.C., and Owens, G.K. 1995.The smooth muscle α-actin gene promoter is differentially regulated in smooth muscle *versus* non-smooth muscle cells. *J. Biol. Chem.* **270**:7631-7643.

Sigurdsson, S.B., Chitano, P., and Stephens, N.L. 1996. The Schultz-Dale response of sensitized canine bronchial smooth muscle. *Resp. Physiol.* **102**:79-87.

Simons, M., Leclerc, G., Safian, R.D., Isner, J.M., Weir, L., and Baim, D.S. 1993. Relation between activated smooth-muscle cells in coronary-artery lesions and restenosis after atherectomy. *N. Engl. J. Med.* **328**:608-613.

Simons, M. and Rosenberg, R.D. 1992. Antisense nonmuscle myosin heavy chain and c-myb oligonucleotides suppress smooth muscle cell proliferation in vitro. *Circ. Res.* **70**:835-843.

Singh, J.P., Wiernicki, T.R., and Gupta, S.K. 1993. Role of serine/threonine kinase casein

kinase-II in vascular smooth muscle cell proliferation and inhibition by heparin. *Drugs Dev. Res.* **29**:129-136.

Small, J.V. 1995. Structure-function relationships in smooth muscle:the missing links.*BioEssays* **17**:785-792.

Small, J.V., Fürst, D.O., and Thornell, L.-E. 1992. The cytoskeletal lattice of muscle cells. *Eur. J. Biochem.* **208**:559-572.

Smith, P.G., Janiga, K.E., and Bruce, M.C. 1994. Strain increases airway smooth muscle cell proliferation. *Am. J. Respir. Cell Mol. Biol.* **10**:85-90.

Snape, W.J., and Kao, H.W. 1988. Role of inflammatory mediators in colonic smooth muscle function in ulcerative colitis. *Dig. Dis. Sci.* **33**(Suppl.):65S-70S.

Snetkov, V.A., Hirst, S.J., Twort, C.H.C., and Ward, J.P.T. 1996. Ion channels in freshly isolated and cultured human bronchial smooth muscle cells. *Am. J. Respir. Crit. Care Med.* **153**:A166 (Abstract).

Sobue, K., Muramoto, Y., Fujita, M., and Kakiuchi, S. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA* **78**:5652-6555.

Sobue, K., and Sellers, J.R. 1991. Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin sysytems. *J. Biol. Chem.* **266**:12115-12118.

Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morrisey, E.E., Ip, H.S., and Parmacek, M.S. 1996. Structure and expression of a smooth muscle cell-specific gene, SM22α. *J. Biol. Chem.* **270**:13460-13469.

Somlyo, A.P., Devine, C.E., Somlyo, A.V., and Rice, R.V. 1973 Filament organization in vertebrate smooth muscle. *Philos. Trans. R. Soc. Lond. Ser. B.* **265**:223-229.

Somlyo, A.P., and Somlyo, A.V. 1994. Signal transduction and regulation in smoothmuscle. *Nature (Lond.)* **372**:231-236.

Stephens, N.L., Jiang, H., and Halayko, A. 1993. Role of airway smooth muscle in asthma: Possible relation to the neuroendocrine system. *Anat. Rec.* **236**:152-163.

Stephens, N.L., Halayko, A.J., and Swynghedauw, B. 1991a. Myosin heavy chain isoform distribution in normal and hypertrophied rat aortic smooth muscle. *Can. J. Physiol. Pharmacol.* **69**:8-14.

Stephens, N.L., Jiang, H., Xu, J., and Kepron,W. 1991b. Airway smooth muscle mechanics and biochemistry in experimental asthma. *Am. Rev. Respir. Dis.* **143**:1182-

1188.

Strasser, P., Gimona, M., Moessler, H., Herzog, M.,and Small, J.V. 1993. Mammalian calponin: Identification and expression of genetic variants. *FEBS Lett.* **330**:13-18.

Sturgill, T.W., and Wu, J. 1991. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim. Biophys. Acta* **1092**:350-357.

Sun, H., and Tonks, N.K. 1994. The coordinated action of protein tyrosine phosphatases and kinases in cell signalling. *Trends Biochm. Sci.* **19**:480-485.

Swynghedauw, B. 1986. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscle. *Physiol. Rev.* **66**:710-770.

Takahashi, K., Miwa, A., Takagi, M., Kojima, A., Eshita, Y., Nara, Y., Yamori, Y., Hirabayashi, M., Kato, O., Awata, N., Kobayshi, T., and Shibata, N. 1995. Overexpression of calponin in transgenic rats inhibits intimal arteial smooth muscle cell hyperplasia in the carotid artery model of restenosis. *Circulation* **92**:I-295 (Abstract).

Takahashi, K., Takagi, M., Ohgami, K., Nakai, M., Kojima, A., Nadal-Ginard, B., and Shibata, N. 1993. Inhibition of smooth muscle cell migration and proliferation caused by

transfection of the human calponin gene is associated with enhanced cell matrix adhesion and reduced PDGF responsiveness. *Circulation* **88**:I-74 (Abstract).

Takahashi, K., Tazunoki, T., Okada, T., Ohgami, K., Miwa, T., Miki, A., and Shibata, N. 1996. The 5'-flanking region of the human smooth muscle cell calponin gene contains a *cis*-acting domain for interaction with a methylated DNA-binding transcription repressor. *J. Biochem.* **120**:18-21.

Tom-Moy, M., Madison, J.M., Jones, C.A., de Lanerolle, P., and Brown, J.K. 1987. Morphologic characterization of cultured smooth muscle cells isolated from the tracheas of adult dogs. *Anat. Rec.* **218**:313-328.

Tomlinson, P., Wilson, J. and Stewart, A. 1994. Inhibition by salbutamol of the proliferation of human airway smooth muscle in culture. *Br. J. Pharmacol.* **111**:641-647.

Topouzis, S., and Majesky, M.W. 1996. Smooth muscle lineage diversity in the chick embryo: Two types of aortic smooth muscle cells differ in growth and receptor-mediated transcriptional responses to transforming growth factor-β. *Dev. Biol.* **178**:430-445.

Towbin, H., and Gordon, J. 1984. Immunoblotting and dot immunoblotting-current status and outlook. *J. Immunol. Methods* **72**:313-340.

Trabelsi-Terzidis, H., Fattoum, A. Represa, A., Dessi, F., and Ben-Ari, Y. 1995. Expression of an acidic isoform of calponin in rat brain: Western blots of one- or two-dimensional gels and immuno-localization in cultured cells. *Biochem. J.* **306**:211-215.

Tso, J.Y., Sun, X-H., Kao, T-H., Reece, K.S., and Wu, R. 1985. Isolation and characterization of rat and human glyceraldehyde 3-phosphate dehydrogenase cDNA's: genomic complexity and molecular evolution of the gene. *Nucleic Acid Res.* **13**:2485-2502.

Turla, M.B., Thompson, M.M., Corjay, M.H., and Owens, G.K. 1991. Mechanisms of angiotensin II- and arginine vasopressin-induced increases in protein synthesis and content in cultured rat aortic smooth muscle cells. *Circ. Res.* **68**:288-299.

Twort, C.H., and Van Breeman, C. 1989. Human airway smooth muscle in cell culture: control of the intracellular calcium store. *Pulm. Pharmacol.* **2**:45-53.

Uehara, Y., Fujiwara, T., Nakashiro, S., and Shan, Z.D. 1990. Morphology of smooth muscle and its diversity as studied with scanning electron microscopy. *In* Ultrastructure of Smooth Muscle. *Edited by* P.M. Motta. Kluwer Academic Publishers, Boston. pp. 119-136.

Ueki, N., Sobue, K., Kanda, K., Hada, T. and Higashino, K. 1987. Expression of high and

low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **84**:9049-9053.

Upadhya, A., Samuel, M., Cox, R.H., Bagshaw, R.J., and Chacko, S. 1993. Characteristics of arterial myosin in experimental renal hypertension in the dog. *Hypertension* **21**:624-631.

Uvelius, B., Arner, A., and Malmqvist, U. 1989. Contractile and cytoskeletal proteins in detrusor muscle from obstructed rat and human bladder. *Neurourol. Urodyn.* **8**:396-398.

van der Loop, F.T.L., Schaart, G., Timmer, E.D.J., Ramaekers, F.C.S., and van Eys, G.J.J.M. 1996. Smoothelin, a novel cytoskeletal protein specific for smooth muscle cells. *J. Cell Biol.* **134**:401-411.

Vanhoutte, P.M. 1978. Heterogeneity in vascular smooth muscle. *In* Microcirculation. Edited by G. Kaley and B.W. Altura. University Park Press, pp.181-309.

Villanove, X., Marthan, R., Tunon de Lara, J.M., Johnson, P.R.A., Savineau, J.P., McKay, K.O., Alouan, L.A., and Black, J.L. 1993. Sensitization decreases relaxation in human isolated airways. *Am. Rev. Respir. Dis.* **148**:107-112.

Walker, L.N., Bowden Pope, D.F., Ross, R., and Reidy, M.A. 1986. Production of

platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. *Proc. Natl. Acad. Sci. USA* **83**:7311-7315.

Walsh, M.P. 1994. Calmodulin and the regulation of smooth muscle contraction. *Mol. Cell. Biol.* **135**:21-41.

Walsh, M.P., Carmichael, J. D., and G.J. Kargacin. 1993. Characterization and confocal imaging of calponin in gastrointestinal smooth muscle. *Am. J. Physiol. (Cell Physiol.)* **265**:C1371-C1378.

Watson, M.H., Venance, S.L., Pang, S.C, and Mak, A.S. 1993. Smooth muscle cell proliferation. Expression and kinase activities of $p34^{cdc2}$ and mitogen-activated protein kinase homologues. *Circ. Res.* **73**:109-117.

Weissberg, P.L., Grainger, D.J., Shanahan, C.M., and Metcalfe, J.C. 1993. Approaches to the development of selective inhibitors of vascular smooth muscle cell proliferation. *Cardiovasc. Res.* **27**:1191-1198.

Whicker, S.D., Armour, C.L., and Black, J.L. 1988. Responsiveness of bronchial smooth muscle from asthmatic patients to relaxant and contractile agonists. *Pum. Pharmacol.* **1**:25-31.

White, S.L. and Low, R.B. 1996. Identification of promoter elements involved in cell-specific regulation of rat smooth muscle myosin heavy chain gene transcription. *J. Biol. Chem.* **271**:15008-15017.

White, S., Martin, A.F., and Periasamy, M. 1993. Identification of a novel smooth muscle myosin heavy chain cDNA: isoform diversity in the S1 head region. Am. J. Physiol. **264**:C1252-C1258.

Widdop, S.K., Daykin, K., and Hall, I.P. 1993. Expression of muscarinic M_2 receptors in cultured airway smooth muscle cells. *Am. J. Respir. Cell. Mol. Biol.* **9**:541-546.

Wieczorek, D.F., Smith, C.W., and Nadal-Ginard, B. 1988. The rat alpha-tropomyosin gene generates a minimum of six different mRNAs coding for striated, smooth, and nonmuscle isoforms by alternative splicing. *Mol. Cell. Biol.* **8**:679-694.

Wiggs, B.R., Bosken, C., Paré, P.D., James ,A., and Hogg, J.C. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Resp. Dis.* **145**:1251-1258, 1992.

Wight, T.N. 1989. Cell biology or arterial proteoglycans. *Arteriosclerosis* **9**:1-20.

Winder, S.J., and Walsh, M.P. 1990. Smooth muscle calponin. Inhibition of actomyoin

MgATPase and regulation by phosphorylation. *J. Biol. Chem.* **265**:10148-10155.

Wilcox, J.N., Smith, K.M., Williams, L.T., Schwartz, S.M., and Gordon, D. 1988. Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. *J. Clin. Invest.* **82**:1134-1143.

Wissler, R.W. 1968. The arterial medial cell, smooth muscle or multifunctional mesenchyme? *J. Athero. Res.* **8**:201-213.

Wohrley, J.D., Frid, M.G., Moiseeva, E.P., Orton, E.C., Belknap, J.K., and Stenmark, K.R. 1995. Hypoxia selectively induces proliferation in a specific subpopulation of smooth muscle cells in the bovine neonatal pulmonary arterial media. *J. Clin. Invest.* **96**:273-281.

Wolf, Y.G., Rasmussen, L.M., and Ruoslahti, E. 1994. Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. *J. Clin. Invest.* **93**:1172-1178.

Woodcock-Mitchell, J., White, S., Stirewalt, W., Periasamy, M., Mitchell, J. and Low, R.B. 1993. Myosin isoform expression in developing and remodeling rat lung. *Am. J. Respir. Cell Mol. Biol.* **8**:617-625.

Woolcock, A.J., Salome, C.M., and Yan, K. 1984. The shape of the dose-response curve to histamine in asthmatic and normal subjects. *Am. Rev. Respir. Dis.* **130**:71-75.

Wright, T.C., Pukac, L.A., Castellot, J.J., Jr., Karnovsky, M.J., Levine, R.A., Kim-Park, H.-Y., and Campisis, J. 1989. Heparin suppresses the induction of c-fos and c-myc mRNA in murine fibroblasts bt selective inhibition of a protein kinase C-dependent pathway. *Proc. Natl. Acad. Sci. USA* **86**:3199-3203.

Xie, Y.N., Gerthoffer, W.T., Reddy, S.N., Cominelli, F., Eysselein, V.E., and Snape, W.J., Jr. 1992. An abnormal rate of crossbridge cycling in colonic smooth muscle associated with experimental colitis. *Am. J. Physiol. (Gastrointest. Liver Physiol.)* **262**:G921-G926. 251-1258.

Yang, C.M., Chou, S.-P., Sung, T.-S., and Chien, H.-J. 1991. Regulation of functional muscarininc receptor expression in tracheal smooth muscle cells. *Am. J. Physiol. (Cell Physiol.)* **261**:C1123-C1129.

Yao, L.Y., Moody, C., Schonherr, E., Wight, T.N., and Sandell, L.J. 1994. Identification of the proteoglycan versican in aorta and smooth muscle cells by DNA sequence analysis, in situ hybridization and immunohistochemistry. *Matrix Biol.* **14**:213-225.

Zanellato, A.M.C., Borrione, A.C., Tonello, M., Scannapieco, G., Pauletto, P., and

Sartore, S. 1990. Myosin isoform expression in smooth muscle cell heterogeneity in normal and atherosclerotic rabbit aorta. *Arteriosclerosis* **10**:996-1009.