Phenotype-Dependent Integrin Expression in Canine Airway Smooth Muscle Cells

By Shan Li

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ABSTRACT

Background &Aim: Airway smooth muscle (ASM) cells exhibit phenotype plasticity and are capable of modulating between a contractile state and a synthetic/proliferative state. As extracellular matrix proteins may influence airway smooth muscle (ASM) phenotype mediated via integrin receptors, we hypothesized that integrin expression on canine ASM cells is phenotype dependent.

Methods: On proliferating and serum-deprived contractile primary cultured airway myocytes, expression of integrin β1, α3, α5, α6, α7, β4 and their ligands, laminin, and fibronectin were analyzed by indirect immunofluorescence, Western blotting and reverse-transcriptase polymerase chain reaction. Immunohistochemistry was used to detect the integrin expression in canine and human bronchial tissues. Expression of integrin associated CD151 molecule was also investigated by Western blotting, immunocytochemistry and immunohistochemistry.

Results: RT-PCR revealed the presence of gene transcripts for β1, α3, α5, α6, α7 and their ligands laminin and fibronectin except β4 in canine airway smooth muscle cells; mRNA for β4 integrin was absent. Furthermore, two isoforms of α6 and α7 integrin were detected: An α6A subunit was present on both serum-fed and serum-deprived myocytes whereas α6B subunit was exclusively expressed by serum-fed myocytes. The α7B subunit was expressed on both serum-fed and serum-deprived canine myocytes whereas α7A subunit was absent. Double fluorescent immunocytochemistry for smMHC and laminin or fibronectin revealed exclusive co-localization of laminin with elongate,

smMHC-positive myocytes. Conversely, fibronectin associated with all myocytes. Staining for α6 and α7 integrins, acting as laminin-specific receptors, appeared exclusively on elongated, smMHC and laminin-positive myocytes. Conversely, α5 integrin, which is a fibronectin-specific receptor subunit, was widely distributed. Also, CD151 protein was expressed exclusively by contractile phenotype canine airway smooth muscle cells and tissues.

Conclusion: Expression of laminin-specific integrin receptors α6β1 and α7β1 was phenotype dependent, being present in high abundance exclusively in contractile state canine and human airway myocytes. CD 151, associated protein that associates with laminin-binding integrins was also expressed exclusively by contractile state myocytes. These data suggest that ligation of myocytes with extracellular laminin via α6β1 and/or α7β1 may be a critical determinant of the maturation of contractile ASM. Moreover, a potential role for CD151 in integrating signal transduction from α6β1 and α7β1 is implicated. These results provide rationale for future studies to dissect the role of α6β1, α7β1 and CD151 in ASM differentiation.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
SMC	Smooth muscle cell
CASMC	Canine airway smooth muscle cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
FBS	Fetal bovine serum
FITC	Fluoro-isothiocyanate
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
PAGE	Polyacrylamide gel electrophoresis
mM	Millimolar
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonylfluoride
SDS	Sodium dodecyl sulfate
smMHC	Smooth muscle myosin heavy chain
TBS-T	Tris buffered saline (150m NaCl) containing 0.1% Tween-20
TGF-β	Transforming growth factor-β
RT-PCR	Reverse transcriptase polymerase chain reaction
LN	Laminin

FN	Fibronectin
Col I	Collagen I
HCS	Homologous Cell substrate
MMPs	Metalloproteinases
TIMPs	Tissue-specific inhibitors of MMPs
CAMs	Cell adhesion molecules
PKC	Protein kinase C
MAP	Mitogen activated protein

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CHAPTER 1. LITERATURE REVIEW

1.1 Pathophysiology of Asthma

Asthma is a chronic disease characterized by reversable airway obstruction, inflammation and structural changes in the bronchial walls referred to as a remodeling. Typically, airways remodeling includes epithelial damage, thickening of the lamina reticularis, increased vascularity of the sub-epithelial spaces, fibrosis of the subepithelial spaces in parallel with a significant increase in myofibroblast number, thickening of the airway smooth muscle layer that encircles the bronchi, hyperplasia of mucous glands, and increased numbers of mast cells (Holgate et al, 2000). These changes have been considered to be a secondary phenomena, developing late in the disease process as a consequence of persistent inflammation (Holgate et al, 2001). So-called epithelial mesenchymal trophic unit (EMTU) in the airway wall has been implicated. The EMTU, consisting of the epithelial layer and mesenchymal cells in the airway wall, reaches a physiological equilibrium in the airways after birth, but can become activated as result of inflammation-induced epithelial damage in asthma; this appears to drive the remodeling process (Holgate et al, 2001). Epithelial damage and cytokines of a TH2 phenotype can act in concert to cause a functional disturbance of the EMTU, which leads to myofibroblast activation and induction of inflammatory and remodelling response characteristic of chronic asthma (Holgate et al, 2001). Many cellular components of the airway wall are involved in airway remodelling. The epithelial, mesenchymal and neural cells and local extracellular matrix interact to initiate many processes, including growth, tissue repair, and regulation of the inflammatory process (Evans et al, 1999). Fibroblasts that make up the mesenchymal component of the epithelial-mesenchymal unit are

involved in remodelling. In patients with asthma, the bronchial epithelium is structurally and functionally abnormal, with an increase in the thickening and density of collagen in subepithelial basement membrane (Holgate et al, 2003).

Thickening of the reticular basement membrane is a characteristic feature of the asthmatic bronchus, occurring early on in the disease process. Electron microscopy has demonstrated that airway wall "thickening" is the result of a fibrotic response predominantly in the lamina reticularis (McCarter et al, 1996). This response is characterized by the enhanced accumulation of fibronectin, tenascin, laminin 2 and Type I, III, and V collagens (Bradding et al, 1997). The fibrotic response may play a role in the generation of partially reversible and/or fixed airway obstruction as a significant correlation between methacholine sensitivity and the intensity of airway fibrosis has been reported (Rybalkin et al, 2002). Importantly, fibrosis is thought to be subserved by resident stromal cells, in particular those of mesenchymal origin such as fibroblasts, myofibroblasts and smooth muscle cells. Significant fibrosis due to deposition of collagen and elastin around smooth muscle cells has been described.

1.1.1 Airway Smooth Muscle Thickening

Asthmatics exhibit airway wall thickening, as has been assessed by high-resolution computed tomography scanning, and disease severity is correlated with the degree of airway wall thickening (Awadh et al, 1998). Airway wall thickness is due to an increase in muscle mass, extracellular matrix, and vessel area leading to markedly and permanently reduced airway caliber and distensibility (Johns et al, 2000). Morphometric

studies of airway smooth muscle in asthma have demonstrated that there is an increase in airway smooth muscle mass within the airway wall (Ebina et al, 1993). This is the key etiologic factor in airway narrowing that accompanies asthma (Lambert et al, 1993). Although there are some discrepancies, increased muscle mass appears to result from both cellular hypertrophy and hyperplasia, though hyperplasia is the more consistent observation (Ebina et al, 1993). Ebina et al (1993) studied airway smooth muscle thickness and smooth muscle cell number of patients with fatal asthma with state-of-the-art stereological methods. Two asthmatic sub-types were found; one in which the smooth muscle thickness was increased only in the central bronchi (Type I) as the result of cellular hyperplasia, and a second in which the quantity of the smooth muscle was increased throughout the airway tree (Type II), with myocyte hyperplasia occurring in central airways and hypertrophy predominated in small airways.

Increased subepithelial myofibroblasts have been noted in the airways of patients with chronic severe asthma (Brewster et al, 1990) as well as those undergoing allergic challenge (Gizyki et al, 1997). Recent studies suggest that these cells may drive subepithelial fibrosis in patients with chronic severe asthma (Morishima et al, 2001). Though there are no definite data that myofibroblasts contribute to increased airway smooth muscle mass in asthma, this has also been suggested as a mechanism for ASM hypertrophy (Holgate et al, 2003).

Airway smooth muscle cells exhibit phenotype plasticity and are capable of modulating between a contractile state and a synthetic/proliferative state in which the cells synthesize

abundant extracellular matrix (ECM) and proinflammatory mediators (Halayko et al, 1994). The signals for converting airway smooth muscle cells into a proliferative and secretory state in asthma are unknown, but may include mediators associated with virus or allergen induced airway inflammation (Busse et al, 1990). Increased abundance of epidermal growth factor (EGF), a mitogen for human airway smooth muscle, has been found in asthmatic airways (Vignola et al, 1997). In bronchoalveolar lavage fluid, basic fibroblast growth factor concentrations are also higher in subjects with atopic asthma than in control subjects (Redington et al, 2001). Moreover, bronchoalveolar lavage fluid from asthmatic airways has been shown to increase the extracellular signal regulated kinase (ERK) activation, cyclin D1 protein abundance, [3H]-thymidine incorporation and proliferation of cultured human airway smooth muscle cells (Naureckas et al, 1999). EGF appears to be a "good candidate" for promoting airway smooth muscle (ASM) proliferation in vivo; other potential candidate factors include EGF, TGF-β and platelet-derived growth factor. Panettieri et al (1998) showed that EGF activates ErbB-2 and stimulates phospatidylinositol 3-kinase in human airway smooth muscle (ASM) cells, a mechanism that induces transcription of cyclin D1, thereby inducing cells through G1 checkpoints of the cell cycle.

Cell-culture studies disclosed a wide range of soluble factors that can promote proliferation of human ASM cells, suggesting that through autocrine loops, these cells may also regulate their own proliferative rate (Johnson et al, 2001). Several recent reports suggest that airway smooth cells (ASM) may also generate pro-inflammatory mediators

and express cell adhesion molecules, thus implicating airway smooth muscle cells as contributors to the inflammatory mechanisms of asthma (Johnson et al, 2001).

1.2 Smooth Muscle Cells

1.2.1 Characteristics of Differentiated Smooth Muscle Cells (SMC)

Mature, differentiated airway smooth muscle cells (ASMCs) found in the airway wall exist in a quiescent differentiated state with a low proliferative index. Smooth muscle has remarkable functional plasticity necessary for growth and repair during injury when additional functions such as proliferation and secretion of ECM and biological mediators are apparent. Thus, myocytes are not terminally differentiated as they retain the ability to undergo reversible changes in their phenotype, a process known as phenotypic modulation (switch from contractile state to synthetic state) and maturation (switch from synthetic state to contractile state) (Campbell et al, 1987).

Contractile smooth muscle cells (SMCs) are characterized by the presence of a high-volume fraction of myofilaments, and they express numerous smooth muscle specific genes encoding contractile proteins and proteins that regulate contraction (Campbell et al, 1987; Halayko et al 1996; 1997; 1998). Synthetic SMCs are characterized by increased mitogenic activity and abundance of intracellular organelles associated with synthesis, such as rough endoplasmic reticulum and Golgi apparatus (Campbell et al, 1987). There is also a simultaneous decrease in the intensity of immuno-staining for smooth muscle-specific contractile proteins, although the total amount of actin and myosin remains relatively unaltered due to the accumulation of non-muscle isoforms (Owens et al, 1986).

Halayko et al (1996) have examined the relative distribution of a number of contractile (e.g. smooth muscle myosin heavy chain, smooth muscle α-actin), cytoskeletal (e.g. non-muscle myosin heavy chain, vimentin, desmin) and regulatory proteins (e.g. tropomyosin, calponin, caldesmon, myosin light chain kinase, protein kinase C) under non-proliferating (e.g. contractile phenotype) and proliferating (i.e. synthetic phenotype) conditions. The intermediate filament protein, vimentin was highly expressed in synthetic proliferating tracheal smooth muscle cells. In non-dividing, contractile tracheal smooth muscle cells, smooth muscle myosin heavy chain and α-actin were highly expressed. The function of the contractile phenotype is to constrict and maintain tension, whereas the function of more synthetic cells is dedicated to the synthesis and deposition of extracellular matrix components, as well as production of paracrine and autocrine growth-promoting factors (Campbell et al, 1993; Halayko and Solway, 2001).

1.2.2 Phenotypic Modulation of SMCs in Culture

Phenotype plasticity is a feature of cells committed to the smooth muscle lineage and is manifest as reversible modulation and maturation of individual myocytes (Halayko et al, 1998). The phenotype modulation is a characteristic response of primary cultured mature SMCs (Chamley-Campbell et al, 1979; Halayko et al., 1996); reversion to the contractile state also occurs at confluence and with prolonged serum deprivation (Halayko et al, 1999). Phenotypic maturation of cultured SMCs is marked by increased myofilament and contractile apparatus-associated protein content, reacquisition of typical pharmacological responsiveness, and decreased abundance of synthetic organelles (Halayko et al, 1999). In prolonged serum-free culture only a distinctive subset of airway SMCs (~20%)

develop a functionally contractile phenotype with elongated morphology, fully reconstituted contractile apparatus, abundant contractile protein content, and cell surface re-coupling of muscarinic M3 receptors for acetylcholine (Halayko et al, 1999).

Phenotypic plasticity of SMCs requires the differential expression of a repertoire of phenotype-specific genes and subsequent accumulation of the proteins that they encode. They are regulated both at the level of transcription and protein translation of SMC phenotype-specific markers. Some of the best characterized markers for SMCs of the contractile phenotype include smooth muscle α-actin, smooth muscle γ-actin, smMHC, calponin, h-caldesmon, SM22, smoothelin, metavinculin, and α1-integrin (Halayko et al, 2001). The expression of caldesmon, calponin, SM22, β-tropomyosin and α1 integrin is regulated by transcription. Alternative splicing mechanisms determine the pattern of expression of specific isoforms of caldesmon, α-tropomyosin, vinculin, metavinculin, and smooth muscle myosin heavy chain (smMHC). Translation and accumulation of proteins unique to contractile phenotype appears to be controlled, in part, by a phosphatidylinositide-3-kinase (PI3 kinase) signaling cascade including Akt1, mTOR and p70 S6 kinase (Halayko et al, 2004).

Phenotypic plasticity in combination with a spatially and temporally diverse developmental and differentiation repertoire, appear to produce divergent SMC populations both within and between smooth muscle-associated organs (Halayko et al, 1997). Heterogeneity in pharmacological responsiveness and contractile properties of different sized airways has been described (Ma et al, 1996). In addition, topologic

differences in SMC hyperplasia and hypertrophy, associated with increased bronchial smooth muscle mass in asthma have been reported (Ebina et al, 1993), suggesting that bronchial myocytes from different airways respond distinctly to mitogenic and inflammatory mediators. Halayko et al (1997) have reported the existence of heterogeneous subpopulations of airway SMCs differing in phenotypic marker protein content, attachment efficiency to cell culture plates, and proliferative capacity. They further demonstrated that airway smooth muscle is composed of distinct SMC populations differing in mitogen and anti-proliferative mediator responsiveness (Halayko et al, 1998). Using flow cytometry, Halayko et al (1997) examined inter-and intra-airway heterogeneity of canine smooth muscle cells. Their data showed that tracheal and bronchial smooth muscles are composed of differentially distributed sub-populations of myocytes. The sub-populations differed in contractile protein and DNA content suggesting that they may possess different capabilities for contraction and/or proliferation. In addition, the distribution pattern of phenotypically divergent contractile myocytes in large and small airways appeared to correlate with observed heterogeneity in contractile and pharmacological properties of the airways.

1.2.3 Regulation of SMC Phenotype and Function

The tendency of SMCs to modulate to a synthetic phenotype in primary culture has made the effort to understand and determine the mechanisms involved in regulating SMC differentiation challenging. There has been no "master" regulatory gene identified in smooth muscle similar to ones found in skeletal muscle (Owens et al, 1995). While the complex regulation of SMCs is not well understood, it is thought that maintenance of the

"differentiated" state relies on regulation from various extracellular cues (Blau et al, 1994).

Halayko et al (1996) showed that the changes in contractile protein phenotype occur when canine tracheal smooth muscle cells are placed in culture. The abundance of a number of smooth muscle proteins (e.g. smooth muscle-α-actin and smMHC) falls rapidly when the cells become proliferative, while the content of several non-smooth muscle proteins increases, suggesting modulation of phenotype is induced in response to serum-rich media. Wong et al (1998) confirmed these studies by showing that the expression of smooth muscle actins (α and γ) and smMHC is regulated by growth conditions in cultured rat tracheal smooth muscle cells. The expression of these proteins was less in proliferating than in non-proliferating cells. A recent study from Gosens et al (2002) observed the occurrence of phenotype switching in organ-cultured intact bovine tracheal smooth muscle, showing that serum and growth factors are capable of shifting bovine tracheal smooth muscle phenotype toward a less contractile phenotype, which is linearly related to their mitogenic response. Serum withdrawal leads to reconstitution of the contractile phenotype in primary cultured airway SMC (Halayko et al., 1999). Ma et al (1998) have also ucceeded in inducing a hypercontractile canine airway smooth muscle phenotype in serum-free media.

Multiple signaling pathways regulate smooth muscle cell differentiation including Rho signaling. Rho proteins are regulators of the actin cytoskeleton, gene transcription, cell cycle progression and adhesion (Sotiropoulos et al, 1999). Gosens et al (2004)

investigated the role of Rho-kinase in phenotype switching and proliferation of bovine tracheal smooth muscle. The results showed that although Rho-kinase acts as a major regulator involved in basal maintenance of contractility in bovine trachea smooth muscle, RhoA dependent actin cytoskeleton dynamics also regulate smooth muscle-specific gene expression and thus modulate changes in the cell phenotype (Halayko et al, 2001). The actin cytoskeleton is shown to regulate SRF (Liu et al, 2003; Panel et al, 2003), which in turn regulates the expression of smooth muscle cell–specific genes.

Gosens et al (2002) investigated the effects of insulin on bovine tracheal smooth muscle phenotype in vitro, using both intact tissue and isolated cells, in which they measured contractility and proliferative responsiveness, respectively as parameters for smooth muscle phenotype. Insulin was acutely mitogenic for smooth muscle cells in the presence of other growth factors. In contrast, insulin pretreatment induces a phenotype shift towards a hypercontractile and less proliferative phenotype (Gosens et al, 2003). TGF-β has been shown to up-regulate the expression of smooth muscle α-actin (Mitchell et al, 1993). Desmouliere et al (1992) has shown this is due at least partly to a change in rate of transcription. Interleukin-1 (IL-1) β also is a known regulator of smooth muscle phenotype. Zhang et al (1997) have shown that IL-1 β down regulates smooth muscle α-actin expression. IL-1 β also may decrease proliferation and provide an example of dissociation of changes in SMC phenotype from cell replication (Zhang et al, 1997). Retinoic acid (RA) has been shown to be an inductive signal for producing and maintaining SMCs of the contractile phenotype (Blank et al, 1995). RA binds to intracellular RA receptors that 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 et al, 1995).

The ECM also influences the ability of SMCs to migrate, proliferate and differentiate (Owens, 1995). ECM components of basement membrane, chiefly laminin and collagen type IV induce a delay in the spontaneous phenotypic modulation and proliferative index of contractile SMCs from a variety of tissues when seeded in primary culture (Hirst et al, 2000; Thyberg et al, 1994). Conversely, other matrix components such as fibronectin, thrombospondin and collagen type I promote the transition of contractile smooth muscle cells to the synthetic phenotype (Thyberg et al, 1994). Hayashi et al (1998) reported that prolonged maintenance of the contractile phenotype of chicken gizzard myocytes seeded on laminin matrix in serum-free conditions only occurred if culture medium was also supplemented with insulin-like growth factor (IGF)-1. Under these conditions IGF-1 activated PI3 kinase and was required for maintenance of the differentiated phenotype. As noted earlier, a recent study revealed a requirement for signaling via the PI(3) kinase-Akt1-mTor-p70S6 kinase pathway for accumulation of contractile proteins in ASM cells (Halayko et al, 2004)

1.2.4 Role of ECM in ASM Cell Proliferation

The intricate network of macromolecules forming the ECM not only plays an important role in maintaining structure and function of the airway but also has the potential to regulate a variety of cellular functions including proliferation (Underwood et al, 1998).

Hirst et al (2000) showed that different ECM proteins determined different growth patterns in human ASM cells, with fibronectin and collagen promoting proliferation and laminin inhibiting proliferation. Adhesion to fibronectin is anti-apoptotic for human ASM cells (Freyer et al, 2001), further emphasizing the importance of cell-matrix interactions in maintaining cell viability and growth. Adhesion-induced ERK activation, which may require ligand-integrin binding, may be involved in the regulation of airway smooth muscle proliferation. It has been shown that microinjection of endothelial cells with a kinase-inactive FAK reduces DNA synthesis (Gilmore et al, 1996), suggesting that adhesion-induced cellular signalling may influence cell growth. Another mechanism regulating smooth muscle proliferation is represented by matrix metalloproteinase (MMP)-2, which plays an important autocrine role required for human airway smooth muscle proliferation (Johnson et al, 1999). It is known that TIMPs are involved in modulating ASM cell proliferation by counteracting the proteolytic activity of the MMPs. Johnson et al (1999) observed the production of TIMP-1 but not of TIMP-2 or -3 from human airway smooth muscle cells in culture. MMP-2 is increased, whereas TIMP-1 is decreased in bronchial fluids obtained from asthmatic subjects.

The effects of mitogens are mediated through at least two distinct receptor systems: tyrosine kinase-linked receptors and G-protein-coupled receptors (GPCRs). Down-stream of these two receptor types, the Shc-Grb2-Sos complex or (PI) 3 kinase is activated. This leads to activation of p21 Ras, which induces Raf, which is translocated to the plasma membrane, where it phosphorylates mitogen-activated protein kinase (MEK). A group of key regulators of growth, the mitogen-activated protein (MAP) kinases, is

then stimulated. One of the MAP kinases, extracellular signal-regulated protein kinase (ERK), plays a key role in human ASM cell growth (Lee et al, 2001). Mitogens activating both tyrosine kinase and GPCRs cause upregulation of active ERK and this is associated with an increase in thymidine uptake, an indicator of DNA synthesis during cell proliferation. Inhibition of MEK with specific antagonists such as UO126 or with antisense DNA decreases ERK activation and also inhibits thymidine incorporation (Lee et al, 2001). Activated ERK activates transcription factors such as c-Jun, c-Fos, and c-Myc in the nucleus and this may occur via activation of p90 ribosomal S6 kinase. Others have reported the existence of ERK-independent pathways for proliferation that are mediated via stimulation of PI 3-kinase (Krymskaya et al, 2000). In addition, there is synergy between ERK and PI3-kinase pathways (Krymskaya et al, 2000). Protein kinase C (PKC)-ξ, one of the atypical isoforms of this enzyme, is specially increased when human ASM cells are stimulated with growth factor mitogens (Carlin et al, 1999). Moreover, an antisense oligonucleotide directed to PKC-ξ, which can be activated by PI 3-kinase and Ras and may directly activate Raf-1, significantly inhibits proliferation of HASM cells induced by platelet-derived growth factor (PDGF) (Krymskaya et al, 2000).

1.2.5 Role of ECM in ASM Phenotype

A dynamic relationship exists between cells and the extracellular matrix (ECM) because cells in culture either proliferate or differentiate depending on the type and density of ECM proteins on which they grow (Carlin et al, 1999). In the intact wall of the mature airway, ASM cells remain in a quiescent and fully differentiated state which is characterized in part by well-developed contractile elements and a stable ECM

environment (Hirst et al, 2000). Thus, ASM phenotype appears to depend, in part, on cell adhesion-extracellular matrix interactions. The interaction of ASM with extracellular matrix creates mechanical forces that appear critical in regulating smooth muscle-specific gene expression during ASM development and differentiation (Yang et al, 1998). Biopsy and post-mortem studies report increases in deposition of fibronectin, collagen I, III, and V, laminin-2, tenascin, versican, and hyaluronan in the airways of individuals with asthma (Roche et al, 1989). Not only do airway myofibroblasts produce extracellular matrix proteins and glycoproteins, but Johnson et al (2002) have demonstrated that airway SMCs in culture can be stimulated to increase production of fibronectin, perlecan, laminin-γ1, and chondroitin sulfate above baseline by the addition of asthmatic serum.

Little is known about the mechanisms mediating the effects of altered matrix on the SMCs. In vivo and in vitro studies have shown that several structural components of the extracellular matrix modulate smooth muscle phenotype. The interstitial ECM proteins such as fibronectin (FN) and collagen I (Col I) promote proliferation of human airway smooth cells and induce loss of contractile protein expression (Hedin et al, 1989) whereas basement membrane elements such as laminin (LN) inhibit proliferation and support a more contractile phenotype (Yamamoto et al, 1993). Shuger's group has shown that laminin α1- and α2-chains are required for normal morphogenesis and elongation of airway myocytes in developing lung (Yang et al, 1998). Conversely, other matrix components such as fibronectin, thrombospondin and collagen type I promote the transition of contractile smooth muscle cells to the synthetic phenotype (Thyberg et al, 1994). Moreover, the synthetic cells can produce extracellular matrix molecules and also

autocrine growth-promoting factors (Baskin et al, 1993), and these could in turn influence contractile or synthetic phenotype. Clearly, smooth muscle cells from the airways may constitute a source of growth factors, cytokines and extracellular matrix products capable of inducing a greater proportion of synthetic phenotype smooth muscle cells.

Histological studies have shown that fibronectin and collagen deposition as well as "myofibroblast " proliferation may contribute to subepithelial fibrosis in the bronchi of asthmatics (Relan et al, 1999). Roche et al (1990) have suggested that myofibroblasts are responsible for collagen deposition and basement thickening in bronchial asthma. These "myofibroblasts" are speculated to be a phenotypic variant derived from airway smooth muscle cells, perhaps in the guise of the synthetic phenotype. Moreover, the production of collagen type I and fibronectin by these myofibroblasts may be under the control of activated bronchial epithelial cells (Beasley et al, 1989). Epithelial cell shedding, a characteristic finding in the airways of even mild asthmatics (Beasley et al, 1989) could result in marked changes in the matrix composition and in turn promote changes in myofibroblast/smooth muscle phenotype.

Tao et al (2003) developed a novel cell culture system in which primary rat airway smooth muscle cells were plated on an ethanol-fixed, confluent monolayer of homologous smooth muscle cells (HCS). Cells grown on HCS exhibited morphological and functional characteristics consistent with a differentiated phenotype. Smooth muscle-specific α-actin immunostained diffusely in cells on HCS, whereas it appeared as stress fibres in cells on glass. Unlike cells on glass, cells on HCS contracted in response to

methacholine, which is typical of contractile smooth muscle cells. Agonists stimulated intracellular Ca2+ oscillations in cells on HCS, whereas they elicited biphasic peak and plateau transients in cells on glass. The distinct ultrastructure of cells grown on HCS might have been regulated by a distinct spatial distribution of contact sites and the complex geometric arrangement of the HCS ECM network.

Beqaj et al (2002) have suggested a novel mechanism by which laminin-2 regulates smooth muscle differentiation. They showed that RhoA, a small GTPase signalling protein, is abundant in undifferentiated embryonic mesenchymal cells and high RhoA activity was required to maintain the round undifferentiated mesenchymal cell phenotype. This was associated with localization of the myogenic transcription factor serum response factor (SRF) to the cytoplasm of the cells where it is transcriptionally inactive. RhoA levels decreased rapidly when cells were grown on laminin-2 but not on other main components of the extracellular matrix, resulting in cell elongation and translocation of SRF to the nucleus, where it activates transcription of many smooth muscle specific genes (Halayko & Solway 2001; Camuretti-Mercado et al, 2000; Liu et al, 2003). The possible mechanism controlling SMC phenotype is that ECM components exert their effect by interaction with integrin receptors and this not only produces a physical linkage to the cells but also generates signals that affect cell behaviour. The signalling systems activated by their respective receptors haven't completely been defined. However, ASM cells at least in vitro are equipped with distinct receptors for these ECM molecules. Evidence has shown that SMCs adhere to FN primarily through the integrin $\alpha 5\beta 1$ while they adhere to LN through the integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ (Hirst et al, 2000; Albelda

and Buck, 1991). Thus, adhesion of SMCs to FN may lead to the activation of different signaling pathways than adhesion of cells to LN.

1.2.6 ECM and Integrins in Asthma

One of the prominent structural changes associated with asthma involves enhanced matrix deposition and remodelling within subepithelial region of the airway wall (Roberts et al, 1995). In asthma there are quantitative and qualitative abnormalities in the ECM. In patients with severe, persistent asthma, the architecture of the airway wall undergoes prominent structural changes including the increased deposition of fibronectin and collagen type III and V into the subepithelial layer and loss of airway epithelium (Hirst et al, 2000). Studies of asthmatic patients have repeatedly demonstrated a 2-3 fold increase in the thickness of the basement membrane (Roberts et al, 1995). The levels of ECM such as fibronectin, laminin γ chain, percelcan and chondroitin sulfate are also increased when airway smooth muscle cells derived from a non-asthmatic patient are exposed to serum from an atopic asthmatic patient (Johnson et al, 1999). The general profile of matrix proteins from asthmatic volunteers differs from that in non-asthmatic subjects in that they express increased perlecan and collagen I, and decreased laminin α1 chain, collagen IV and chondroitin sulfate (Johnson et al, 2002).

The mechanism by which enhanced ECM deposition may contribute to the pathogenesis of asthma is not known. It has been proposed that increased deposition of fibronectin and collagen into the subepithelial matrix alters geometric and biomechanical properties of the airways (Roche et al, 1989). Mathematical models suggest that the accumulation of

subepithelial ECM will increase the thickness of the airway wall and thereby enhance airway narrowing in response to smooth muscle contraction (Pare et al, 1997). In addition, collagen fibrils within the subepithelial matrix appear thicker and more densely packed in asthmatic patients than in control subjects (Roche et al, 1989). Other studies (Bousquet et al, 1992) have identified irregularly arranged collagen fibrils in the ECM of asthmatic patients. It has been proposed that alterations in collagen deposition and organization increase the tensile stiffness of the airway and in turn decrease airway distensibility (Hirst et al, 2000). Also, in patients with asthma, the enhanced deposition of fibronectin into the subepithelial space may alter the deposition and /or arrangement of collagen fibrils and, in turn, may modify the mechanical properties of the airway wall (Schuger et al, 1997).

Johnson et al (2000) & 2002) have shown the composition of the ECM is tightly controlled and involves a dynamic process of matrix deposition and degradation. In asthma, this balance is disturbed, resulting not only in an abnormal amount of matrix deposition, but also in an altered composition of matrix components. ASMs secrete matrix-degrading enzymes, metalloproteinases (MMPs) as well as endogenous tissue-specific inhibitors of MMPs (TIMPs) (Johnson et al, 2000). There is an imbalance between induction, expression, activation, and inhibition of MMPs in asthma. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid (BALF) from asthmatics has been reported (Kelly et al, 2000). MMP-9 and its tissue inhibitors of MMPs (TIMP-1) are expressed in an unbalanced ratio in bronchoalveolar fluid of asthma patients (Mautino et al, 1997). MMP-2, the major MMP secreted by human ASM cells is

constitutively released, but remains inactive because of high levels of tissue inhibitor of metalloproteinases (TIMP)-2 on the ASM cell membrane (Foda et al, 1999). Evidence now demonstrates a significant inverse correlation between levels and activation of BALF MMP-8 and FEV1 value in asthma patients (Prikk et al, 2002). These findings support the notion that MMPs contribute to extracellular matrix and basement membrane degradation in asthma. Moreover, TIMP-1 levels are raised in asthma suggesting an imbalance between the production of MMP-9 and TIMP-1 may also occur in asthmatics and contribute to a pro-fibrotic microenvironment (Mautino et al, 1997).

TGF-β, a multifunctional protein, is one of the most potent regulators of inflammation and connective tissue synthesis. TGF-β exists in three isoforms in mammals: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 appears to be the most common isoform associated with disorders characterized by inflammation and fibrosis due to the observation that at the sites of injury, TGF-β1 is released in large quantities by platelets and inflammatory cells such as macrophages (Khalil et al, 1999). TGF-β1 is a chemoattractant for inflammatory cells and fibroblasts as well as a mitogen to immature fibroblasts (Garret et al, 1997). Mckay et al (1998) has found that the cytokine TGFβ is implicated in pro-fibrotic events and in airway remodeling in asthma. Minshall et al (1997) demonstrated that TGF-β1 mRNA and immunoreactivity were increased in the submucosal eosinophils of asthmatics. In addition, Redington et al (2001) demonstrated an increase in the quantity of TGFβ1 in the bronchoalveolar lavage fluid from asthmatic patients and that the levels of TGFβ were further increased in asthmatics after exposure to allergen. Together, these

observations suggest that, in asthmatic airways, there is an increase and release of TGF-β1 that may be important in the pathogenesis of airway fibrosis and remodeling.

On the basis of animal models of injury, it has been observed that there is recruitment and activation of inflammatory cells before subepithelial fibrotic changes are seen (Garret et al, 1997). Activated inflammatory cells and structural cells are induced to release a number of proinflammatory and fibrotic cytokines including TGF-β (Garret et al, 1997). Recently, Coutts et al (2001) demonstrated that the release of biologically active TGF-β induces the synthesis of collagen I by ASM cells. In addition, Burgess et al (1996) recently reported that TGFβ can upregulate CTGF (connective tissue growth factor) mRNA and protein, which promotes gene expression of collagen I and fibronectin. Significantly, levels of CTGF mRNA and protein are increased in airway smooth muscle cells cultured from patients with asthma compared to patients without asthma (Burgess et al, 2003). These data suggest that local changes in TGFβ from airway smooth muscle or inflammatory cells may regulate not only growth of the muscle (Cohen et al, 2000) but also the immediate smooth muscle extracellular environment.

Attention has been directed toward the role of cell adhesion molecules (CAMs) in mediating the asthmatic response (Pickering et al, 2000). Previous studies showed increased expression of β1 and β2 integrins, very late antigen (VLA-4), and lymphocyte function-associated antigen 1 (LFA-1) as well as of CD44, a receptor for hyaluronic acid (HA), on leukocytes recovered from the airways of patients with asthma (Peroni et al, 1993). The expression and activation of a cascade of CAMs that include selectins,

integrins, and CD31, as well as the local production of chemoattractants, lead to leukocyte adhesion and transmigration into lymph nodes and site of inflammation involving non-lymphoid tissues. Furthermore, Wegner et al (1990) demonstrated that antibodies against intercellular adhesion molecule (ICAM-1), a counter receptor for LFA-1, decreased eosinophil infiltration, and attenuated bronchial hyperresponsiveness in a primate model of asthma.

Some evidence has shown that although extravasation of lymphocytes from the circulation is a relatively well defined early step in establishing a local inflammatory response, the subsequent interactions of the infiltrating lymphocytes with other cell types in the bronchial submucosa or extracellular matrix may be important for sustaining the inflammatory response. For example, Lazaar et al (1994) has shown that ASM, both in vivo and in vitro, constitutively expresses high levels of CD44, and can be stimulated to express ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) in response to inflammatory mediators such as TNF-α. Activated T cells adhere to cultured ASM, and stimulation of the ASM with TNF enhanced this adhesion. Adhesion was partially blocked by a monoclonal antibody specific for LFA-1 and VLA-4 on T cells and ICAM-1 and VCAM-1 on ASM cell. The adhesion of activated T lymphocyte induced DNA synthesis in growth-arrested ASM cells. Thus, the interaction between T cells and ASM may provide insight into the mechanisms that induce bronchial inflammation and possibly ASM cell hyperplasia seen in asthma.

1.2.7 Integrin Expression in Airway Smooth Muscle

Airway smooth muscle cells reportedly express several members of the integrin family including α1β1, α2β1, α3β1, α6β1, α8β1, α9β1, αvβ3, αvβ5, α9β1 (See Table 1)(Charalambopoulos et al, 2000). Flow cytometry analysis from Freyer et al (2001) confirmed that α5, αv, and β1 integrin subunits are universally expressed in primary cultured human airway smooth muscle cells and about half of the cells express α6. Less than a third of cells have detectable surface α1, α3 and α4 expression. There are some similarities and some differences in the observations of Nguyen et al (2002) in that these authors found high levels of α2, α5, β1 and β2 integrins, low levels of α1 and α6 and intermediate levels of α4 and α5 in human airway smooth muscle cells. The specific roles these integrins play in contraction and/or proliferation of airway smooth muscle cells have not yet been examined (Panettieri et al, 1998). It is likely that integrins play a regulatory role in airway smooth muscle proliferation. Airway smooth muscle cells can be induced to express both ICAM-1 and VCAM-1, with pro-inflammatory mediators, and these receptors mediate interactions between smooth muscle and leukocytes during inflammation (Hynes, 1992).

1.3 Integrin and Extracellular Matrix

1.3.1 Integrins As Adhesion Receptors

Integrins are the major family of cell surface receptors that mediate attachment to extracellular matrix molecules and specific classes of integrins also mediate important cell-cell adhesive interactions. Cell adhesion assays, usually beginning with assays of adhesion to culture plate coated with specific matrix components have identified many of the putative ligands to which specific integrin bind. Integrin-mediated adhesion is rapidly

and precisely regulated. Cells can regulate integrin-mediated adhesion by changing receptor affinity for ligand.. Affinity-independent mechanisms also exist, including changes in cell shape; leading to increased resistance to shear stresses and increased cell area available to interact with substrates (Lotz et al, 1989), cooperative interactions promoted by integrin clustering (Hato et al, 1998), and changes in the diffusion coefficient of integrins in the plane of the membrane (Kucik et al, 1996). Integrin linkage with the cytoskeleton can affect ligand affinity, diffusion coefficients, clustering, and cell shape (Bennett et al, 1999; Kucik et al, 1996; Yanch et al, 1997). In combination, multiple elements specify the "strength of adhesion" which can be quantified by direct measurement of the force required to detach cells from substrate (Lotz et al, 1989).

1.3.2 Integrin Structure

The integrin receptor family of vertebrate cells is composed of at least 18 distinct alpha subunits and 8 or more beta subunits that interact noncovalently in a restricted manner to form more than 20 family members (Hynes, 1992). Each receptor is a non-covalent heterodimer consisting of one α and one β polypeptide subunit. Each subunit contains three domains: a glycosylated extracellular domain, a hydrophobic transmembrane domain, and a cytoplasmic domain (Argraves et al, 1987) (see Figure 1). At the extracellular NH2-terminus, all α-subunits have seven repeated homologous domains (I-II), each of which consists of ~50 amino acid residues. Bivalent cation binding sites located in the central part of each repeat exhibit the highest homology. For collagen-specific integrins, α-subunits contain an insertion domain (I-domain) of 200 amino acid residues between

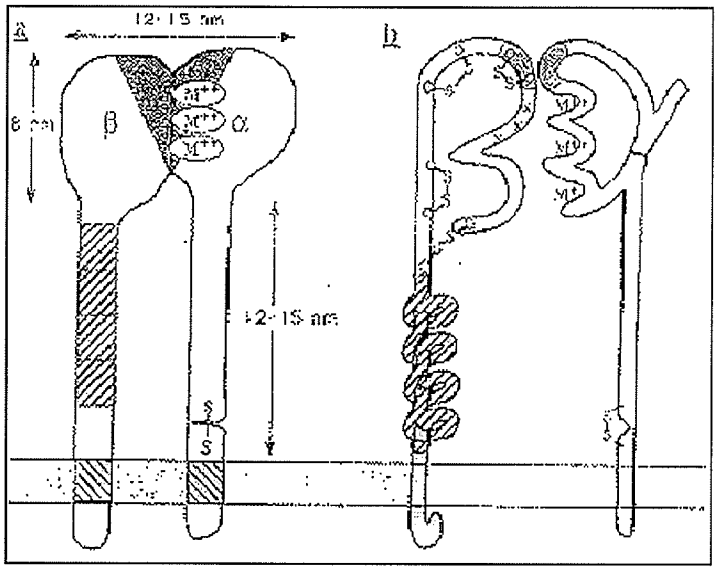
Figure 1. Structure of integrin α and β subunits.

A (top panel): Schematic showing the tertiary structure of an integrin dimer and putative locations of the cysteine-rich repeats in the β subunit (crosshatched) and metal-binding sites in the α subunit (M++). The shaded areas represents the ligand-binding regions that are comprised by both subunits based on crossing-linking and binding data. On the β-subunit cysteine repeats are internally folded and the head region of the β subunit contains internal disulfide bridges (from Hynes, 1992).

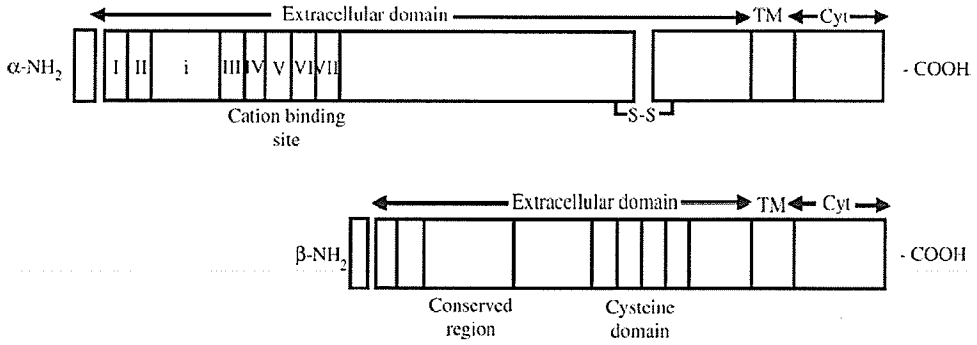
B (bottom panel): Schematic representation of the domain arrangement of the polypeptide chains for α (top) and β integrins (bottom).

Legend: –NH2 = amino terminal (extracellular domain), TM = transmembrane domain; Cyt = cytoplasmic domain; –COOH = carboxy terminal (intracellular domain); i = insert-domain; S–S = disulfide linkage (from Berman, et al, 2003).

A.


a
12-15 nm
b
β
α
12-15 nm
S
S

B.


Extracellular domain
TM
Cyt
α-NH2
I
II
i
III
IV
V
VI
VII
- COOH
Cation binding site
S-S
Extracellular domain
TM
Cyt
β-NH2
- COOH
Conserved region
Cysteine domain

the second and the third I-II domains. The I-domain is involved in the interaction of these integrins with corresponding ligands (Mould et al, 2000). Their binding involves a so-called MIDAS-site (metal ion-dependent adhesion site), also located in the I-domain. Many α-subunits lacking an I-domain undergo posttranslational cleavage of the polypeptide chain followed by subsequent linkage of the two fragments by a disulfide bond. In this case, the mature α-chain consists of larger amino-terminal and smaller carboxy-terminal fragments of molecular masses of 125 and 25 kD.

The transmembrane domain of each α-subunit adopts α-helical configuration; it traverses the lipid bilayer and links the extracellular and intracellular domains. Among various α-subunits this region is characterized by high homology. The cytoplasmic domain of α-subunits include relatively short amino acid stretches of 20-50 residues that significantly differ in primary structure. The existence of several splicing variants increases the heterogeneity of cytoplasmic domains of α-subunits. Cytoplasmic domains play a primary role in ligand and signal properties of integrins, and variability of these sites is a basis for diverse functions of the whole integrin family and unique functions of certain receptors (Loster et al, 2001).

Primary structure of β–subunits is less variable than that of α-subunits. Each β–subunit contains a bivalent cation binding site that is located at the distance of 100 residues from the amino-terminus. This site is required for ligand-receptor interaction. The transmembrane domain of β–subunit adopts β–helical configuration and traverses the membrane only once. In contrast to cytoplasmic domains of α-subunits, the cytoplasmic

domains of β–subunits share high homology. All variants of cytoplasmic domains of β-subunits contain a conserved HDRR sequence flanking the transmembrane domain. The HDRR sequence forms a complex with a conserved GFFKR sequence in the cytoplasmic domains of α-subunits and thus participates in heterodimer assembly (Van der Flier et al, 2001).

1.3.3 The Integrin-ECM Relationship

Integrins connect cells to the extracellular matrix (ECM). The ECM consists of several classes of biomolecules, including structural proteins such as collagens and elastin; adhesion proteins such as fibronectins, laminins, and entactin; proteoglycans; and glycosaminoglycans. This complex matrix doesn't simply surround cells, and hold them together; it also directly or indirectly mediates a number of critical biological processes. The regulation of cell proliferation, differentiation, survival, and immediate gene expression is influenced by integrin-ECM interaction. The disruption of epithelial and endothelial cell interactions with the ECM induces programmed cell death, whereas fibroblast integrin adhesion can affect cell cycle activities by influencing cyclin-A and D expression (Juliano et al, 1993).

Integrins link the ECM to the cytoskeleton across the plasma membrane. In cells unattached to a substrate integrins are diffusely distributed in the plasma membrane and they do not exhibit signal activity. Interaction with matrix proteins causes morphological changes of the plasma membrane and intracellular cytoskeletal structures. The rearrangements result in formation of focal adhesions, which contain clustered integrins

bound to matrix proteins at the extracellular surface, and attached indirectly to actin microfilaments via actin-binding proteins on the cytoplasmic side. Integrins bind to ECM ligands in a manner that is dependent upon both affinity and avidity and is influenced by the composition of the integrin heterodimer, and ligand conformation. Different ligands, or different forms of a particular ligand, can transmit distinct signals to a cell through the same integrin (Gui et al, 1990), as most integrins do not exhibit unique ligand specificity, although they can demonstrate specificity in a cell-dependent fashion (Juliano 1993) (See Figure 2 and Table 1). Correspondingly, the inhibitory effect on cell adhesion by individual anti-integrin antibodies is rarely reported as 100% (Gui et al, 1990). This is likely due to the presence of multiple integrins on the cell that recognize the same ligands, thus bestowing overlapping specificities. The reason for redundancy in ligand specificity is unclear; however, cell cooperative interactions and transmission of different information from the environment remain viable possibilities. Integrin receptors that exhibit common ligand specificity appear to transduce an array of intracellular signals that control physiological reactions (Humphries et al, 2000).

Integrin-ligand interactions are determined by several factors. One major determinant of integrin binding to ligand is the presence of integrin recognition sequences in ligand (Pieschbacher et al, 1984). Integrins bind many ligands in a divalent-cation-dependent manner (see Figure 2). Despite the structural diversity of integrin ligands, they have in common an exposed aspartic acid or glutamic acid residue critical for their binding (Leahy et al, 1996). The ligand aspartic acid or glutamic acid is present within defined recognition sequences found in one or more copies within a ligand

Figure 2: The integrin family of adhesion receptors and their main ligands. There are 18 α and 8 β mammalian subunits tht assemble to form 24 different heterodimers. Although integrins are the main receptors for ECM proteins, they also bind cellular counter receptors (e.g. ICAMs, VCAM), soluble molecules (fibrinogen, complement components) and pathogens (e.g. viruses and bacteria). Subfamilies (based on sequence alignments, functional properties and expression pattern) include: the RGD-binding integrins (blue), the laminin-binding integrins (red), the leukocyte integrins (black), the I-domain integrins (green), and α4 group.

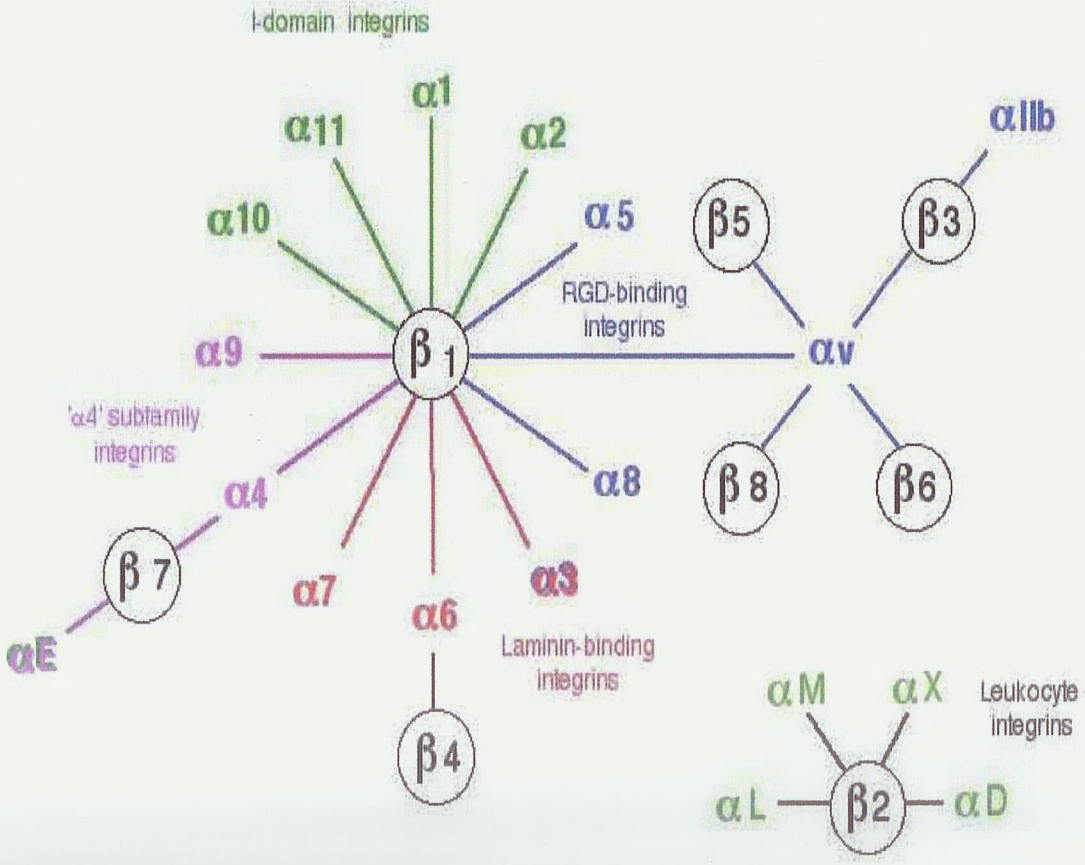


Table 1. Integrins expressed by smooth muscle cells and their specificity for extracellular matrix protein ligands (from Charalambopoulos et al, 2000).

Integrin	**Ligand(s)**
α1β1	collagen
α2β1	collagen, laminin, tenascin
α3β1	collagen, fibronectin, laminin
α6β1	laminin
α8β1	fibronectin, vitronectin
α9β1	tenascin
αvβ3	fibronectin, vitronectin, thrombospondin, tenascin, denatured collagen, osteopontin, fibrinogen
αvβ5	vitronectin

(Ruoslahti et al, 1996). Aspartic-acid-based sequences (e.g. RGD, LDV, KGD, RTD and KQAGD) bind to the majority of integrins (Ruoslahti et al, 1996).

The second factor that affects integrin-ligand interactions is divalent cations. The glutamic acid or aspartic acid in ligands forms a ternary complex with receptor-bound divalent cations in αA–domain-containing or lacking integrins (Lee et al, 1995). While Mg2+ and Mn2+ support physiological ligand binding at a broad range of concentrations, Ca2+ does so in the micromolar range in both αA-containing and –lacking integrins (Hu et al, 1996). It has been suggested that millimolar concentrations of Ca2+ bind at an allosteric Ca2+-specific binding site in the β subunit and increase the rate of ligand dissociation (Hu et al, 1996).

The third factor that affects ECM-integrin binding is the activation state of an integrin. Integrins must be activated to undergo adhesion and binding to the ECM and the switching of integrins from low to high affinity is triggered normally by inside-out signals acting to release intracellular constraints exerted on the integrin cytoplasmic tails (Hughes et al, 1996). Tertiary and quaternary rearrangements occur extracellularly as a result. Integrin activation can also be induced artificially by deleting the transmembrane/cytoplasmic tails, by the use of certain mAbs, the divalent cation Mn2+, by breaking extracellular constraints, or by limited proteolysis of integrin ectodomain (Arnaout et al, 2002).

In the best-studied cases, changes in affinity reflect changes in integrin structure rather than changes in the surrounding plasma membrane environment. These structural changes can also be identified with conformation specific antibodies (Hughes et al, 1996). Direct evidence for conformational changes in integrin ectodomains includes increasing energy transfer between fluorophores on αIib and β3 integrin subunits with treatment of platelets with agonists that activate αIIbβ3. Probing of chemical and conformational differences between resting and activated integrins, mapping of natural mutants that switch integrins into high affinity and use of a few apparently activation-sensitive mAbs have yielded potentially interesting findings regarding sites in integrins that participate in the conformational change leading to activation.

Ligand binding of integrins is thought to be controlled by a mechanism requiring either a) receptor clustering alone; b) ligand occupancy plus receptor clustering; or c) clustering, ligand occupancy, and tyrosine kinase activation (Akiyama et al, 1996). Activation of integrins occurs by local stimuli such as soluble mediators or by solid interfaces (ECM or other cells). Thus, cell activation may involve adhesion by clusters of various stimulated integrins culminating in outside-in signals triggered by local events in the cellular environment (Niessen et al, 1994). In the course of this process, adhesion plaques form at the cytoplasmic face of action of integrin of the cell membrane that serve as focal points for recruitment of proteins to provide cascade interfaces for actin, G-proteins, tyrosine kinases, and transcription factors (Niessen et al, 1994). Equally important, integrins must then be inactivated to avoid cell adhesion and ECM binding at inopportune times and locations (Niessen et al, 1994).

1.3.4 Integrins as Signaling Receptors

The short cytoplasmic domain of the α and β integrin subunit have no intrinsic enzymatic activity and appear to function by interactions with cytoplasmic molecules that nucleate the formation of focal adhesions where integrins link to intracellular cytoskeletal complexes and bundles of actin filaments (Arnaout et al, 2002). The recent resolution of the crystal structure of integrins demonstrates a potential basis for signal initiation, with a massive change in conformation of the cytoplasmic domain occurring when the integrin is occupied by ligand.

Integrin clustering induces focal adhesion assembly with cytoskeletal proteins and induces the focal concentration of specific protein kinases. Substrates that are phosphorylated as a result of integrin activation include proteins involved in focal adhesion formation (e.g. tensin and paxillin), and protein kinases located in focal adhesions (FAK, Src, Ab1, etc.) (Howe et al, 2002). Focal adhesion kinase (FAK) plays a key role in transduction of integrin-mediated signals. Mouse embryos of fibronectin or FAK knockout transgenic mice die at the gastrula stage due to common developmental defects (Ilic et al, 1997). FAK is a 125-KDa protein that binds to the integrin β-subunit cytoplasmic domain and to multiple other components of focal adhesions and is highly concentrated at these sites. Integrin ligation causes autophosphorylation of FAK at tyrosine-397 (Assoian et al, 2001). The phosphorylated Tyr 397 binds to the SH2-domain (Src homology domain) of the proto-oncoprotein Src, which is also a non-receptor tyrosine kinase (Schlaepfer et al, 1998). Binding to FAK activates Src, which catalyzes

phosphorylation of other tyrosine residues in the FAK molecule, resulting in complete activation of FAK (Schlaepfer et al, 1998). At this stage, the adaptor protein, crk-associated substrate (CAS) binds to the FAK-Src complex (Schlaepfer et al, 1998), thereby enabling engagement of multiple other signalling proteins to the site. FAK also has binding sites for the SH2 domain of the ras-associated protein, Grb2, PI (3)-kinase, and phospholipase C and for a number of other adaptor proteins such as paxillin that can further expand the potential downstream targets of integrin signalling (Schlaepfer et al, 1998).

Other protein kinases have been shown to be involved in integrin signaling pathways. The serine/threonine protein kinase C (PKC) family are activated when fibronectin binds to integrins (Hood et al, 2002). This results in recruitment of PKC to the plasma membrane region, where PKC participates in focal adhesion formation, FAK phosphorylation, and cell spreading (Woods et al, 1992). Activation of the PAK family of serine/threonine protein kinases is also linked with substrate–dependent and RTK-dependent activation of ERK (Howe et al, 2002). The effect of PAK on integrin-and RTK-dependent signal transduction is realized via direct phosphorylation of Raf and MEK kinases (Howe et al, 2002). Raf kinase and MEK are common components for these signaling pathways. PI (3) kinase also represents a family of protein kinases activated during the interaction of integrins with matrix proteins (Hood et al, 2002). Signaling cascades initiated by PI (3) kinase are involved in regulation of cell proliferation, locomotion, vesicular transport, apoptosis, and oncogenesis (Hood et al, 2002). A feedback mechanism exists between PI (3) kinase and integrins, and in some

cases receptor activation requires catalytic activity of this enzyme (Hood et al, 2002). In addition, ILK (integrin-linked kinase), which binds directly with integrins, catalyzes phosphorylation of Ser790 in the cytoplasmic domain of the integrin β1-subunit; this is required for receptor localization to focal adhesions (Wu and Dedhar, 2001). This kinase also initiates formation of a branched network of protein-protein interactions mediating integrin links with many structural and signaling proteins (Wu and Dedhar, 2001).

1.3.5 Regulation of Integrin Expression

1.3.5.1 Mediators that Regulate Integrin Levels

TGFβ increases β1 integrin expression on several cell types at both the protein and mRNA level (Ignotz et al, 1987). In some cases, however, increases of β1 integrin dimers occur not due to increased synthesis of β1 but due to increases of its α subunit partner (Heino et al, 1989) or to faster maturation of β1 subunit. In these cases, it has been suggested that the β subunits are normally expressed in excess and that stimulation by TGFβ increases assembly with the limiting α partner. Indeed, Jaspers et al (1994) showed that over expression α4 integrin by transfection with α4 cDNA increased the rate of maturation of the β1integrin precursor as well as the quantity of β1 integrins on the cell surface. Also, β5 integrin can be regulated by TGFβ. Previous reports showed that a TGFβ response element exists between nucleotides –63 and –44 of the β5-integrin promoter and it is required for Smad 4 mediated expression of the gene in a murine osteoblast cell line (Kintscher et al, 2002). On TGFβ stimulation, Smad 4 binds to Smad 2 and 3, and translocates to the nucleus where it binds to Sp1 transcription factors resulting in increased binding of Sp1/Sp3/Smad 2, 3, 4 complexes to a Sp1/Sp3 binding

site within the TGFβ response region of the β5 integrin promoter (Lai et al, 2000). Many of the effects of TGFβ are cell-type specific. Integrin α3 is increased on guinea pig airway epithelial cells after stimulation with TGFβ (Sheppard et al, 1992) but decreased on keratinocytes (Zambuno et al, 1995).

In vascular SMCs, expression of the β1 integrin subunit is not modulated significantly by PDGF-BB but expression of both α1 and α5 integrin subunits which can associate with β1 is enhanced. However, PDGF-BB is able to increase β3 mRNA level, which is responsible for enhanced αvβ3 integrin expression, since the αv integrin mRNA level is constitutively expressed. These results suggest that regulation of the β1 and β3 family of integrins by PDGF-BB occurs through distinct mechanisms, with the β1 family of receptors regulated by change in α subunit levels, while β3 itself is directly responsive to PDGF-BB (Janat et al, 1992)

Retinoic acid has also been shown to modify integrin expression. Medhora et al (2000) showed that the β1 integrin subunit mRNA and protein was induced after treatment with all-trans retinoic acid (ATRA) in two different rat vascular smooth muscle cell lines. A significant upregulation of the β1integrin subunit was observed in vivo. Treatment of these cells with ATRA also showed increased adhesion to fibronectin.

Witzenbichler et al (1999) reported that over expression of the homeobox transcription factor Gax (growth-arrest specific homeobox) led to loss of αvβ3 and αvβ5 via the specific suppression of β3 and β5 subunits in cultured VSMCs and in SMCs after acute

vascular injury in vivo. These observations suggest a pleiotropic action of Gax over expression on growth and survival may be mediated, at least in part, through its effects or integrin expression by vascular cells. Recently, Kintscher et al (2002) reported the regulation of integrin expression by ligands of the peroxisome proliferator-activated receptor (PPAR) class, a member of the nuclear receptor superfamily. The two different PPARα ligands (WY-14643 and ETYA) inhibit TGF-β-induced β5 integrin expression in rat VSMCs. They further demonstrated that a PPARα/Smad4 association, which is likely mediated by additional nuclear proteins, may prevent formation of Sp1/Sp3/Smad2, -3 or -4 complexes on a TGF-β response element (-63/-44) on the β5 integrin promoter to block transcriptional activity. The data suggested a more specific effect of ligand-activated PPARα on the TGF-β-regulated transcription factor Smad4 as the mechanism for repressing TGF-β stimulated β5 integrin transcription. Other nuclear hormone receptors including the glucocorticoid receptor and vitamin D receptor have also been shown to regulate integrin expression in a variety of cells (Cao et al, 1993).

1.3.5.2 Regulation of Ligand Affinity

Regulation of integrin receptors involves changes of the cell surface expression as well as alterations in the affinity of the receptor for ligands. This change results from alterations of receptor conformations and by events involving integrin-associated intracellular components such as cytoskeletal proteins and signalling proteins effectors (Seki et al, 1996). An intracellular change (inside-out signaling) can activate integrin molecules on the cell surface. In this case, conformational changes of the receptor and receptor clustering accompany activation (Phillips et al, 1988). Seki et al (1996) report that β1-

integrin-dependent adhesion is increased by short-term exposure to PDGF. Using a specific antibody mAB15/7, which binds to an activation-dependent epitope on the β1-intgerin, they demonstrated that PDGF-mediated increase adhesion was induced by changes in the affinity/avidity of the β1-integrins. Similarly, Kappert et al (2000) reported that expression of β1-integrin was unchanged after treatment with angiotensin II and PDGF-BB but β1-integrin-mediated cellular responses were augmented. Therefore, it appears that expression alone of a particular integrin does not control functional responses to ligand exposure.

1.3.5.3 Alternative Splicing

Another mechanism by which cells can regulate the profile integrin expression and function is by alternative splicing. Alternative splicing has been reported for several integrins including α3, α6, α7, β1, β4, β3, and αIIb (Tamura et al, 1991). Niessen et al (1994) found no change in ligand specificity with different isoforms of α6. However, the modulation of adhesive strength, influence on cell morphology, and migration of macrophages is different in cells transfected with α6A or α6B isoforms, indicating functional responses are isoform-dependent (Shaw et al, 1993). Others (Jiang et al, 1995) reported that α6B is strongly expressed on undifferentiated F9 embryonic carcinoma cells at cell-cell boundaries, and that upon differentiation; the expression of α6B is decreased, whereas α6A expression is greatly increased. Balzac et al (1993) investigated the function of the β1B isoform and found no isoform-dependent differences in ligand binding though β1A localized to focal adhesion, whereas β1B did not. Moreover, they found that transfected β1B did not induce tyrosine phosphorylation in CHO cells, as was

the case for endogenous or transfected human β1A. Transfection of the β1B subunit resulted in reduced adhesion and migration suggesting a dominant-negative effect on β1A (Balzac et al, 1994). Fornaro at al (1995) showed that cells expressing the β1C subunit showed less ability to proliferate in response to serum stimulation. Meredith et al (1995) showed that when the β1C subunit was transfected into mouse fibroblasts, it inhibited cell cycle progression in late G1 near the G1/S transition.

Three cytoplasmic variants of α7 integrin (α7A, -B, and -C) and two extracellular forms (X1 and X2) have been described. Schober et al (2000) demonstrated that expression of the two extracellular splice variants of α7X1 and α7X2 in HEK293 cells conferred different motilities on laminin isoforms: Whereas α7X2B promotes cell migration on both laminin-1 and laminin-2, α7X1B supported motility only on laminin-2. These results suggest a α7 splice variant-specific roles in cell adhesion and migration on laminin.

1.3.5.4 Control of Gene Expression

In general, integrin promoters contain multiple Sp1, AP1, and/or AP2 binding sites, but not TATA and CCAAT boxes, as cis-elements. Sp1 serves to anchor TFIID, which is critical for initiation of mRNA synthesis in the absence of TATA boxes. Another putative transcription factor-binding site that is common to multiple integrin promoters is specific for the Ets family of transcription factors (Rosen et al, 1994). Within the α4 promoter, a single Ets site is sufficient to bind GABPα/GABPβ which are critical for promoter activity. An adjacent upstream site acts as an enhancer of α4 transcription. This indicates

that Ets site is functional in transcriptional regulation and may be important in controlling tissue-specific expression. The α6 integrin gene maps to human chromosome 2q (Hogervorst, et al, 1991). The gene consists of 27 exons, one of which (exon 5B) is an additional exon present only in certain tissue-specific transcripts. The α6 promoter directs transcription initiation from a primary site 202 nucleotides from the translation initiation codon (Hogervorst et al, 1991). Unlike most other integrin gene promoters, the α6 promoter has a TATA box (GATAAA), which is located 22 nucleotides upstream from the primary transcription initiation site (Lin et al, 1997). A 190-bp region upstream from the TATA box is rich (78%) in C and G nucleotides and contains several Sp1 and AP2 binding sequences (Hogervorst, et al, 1991). However, full promoter activity requires only 78bp of this C/G-rich sequence (Lin et al, 1997). Genomic analysis comparing α6-expressing cells with those do not express α6 suggests that DNA methylation is not involved in the silencing of the α6 gene (Lin et al, 1997). DNase I footprint analysis has confirmed the binding of Sp1 and AP2 to their cognate sequences (Lin et al, 1997). A nuclear extract of α6-expressing HBL-100 cells also produced significant binding to these sites, suggesting that the two transcription factors are probably involved in the positive regulation of the α6 promoter (Lin et al, 1997).

The α7 integrin gene is composed of at least 27 exons spanning a region of about 22.5 kb (Wang et al, 1995). The 5'-flanking sequences near the transcriptional start site lacks both a TATA and a CCAAT box (Vignier et al, 1999). Transcription from promoters without TATA and CCAAT boxes can be initiated at a consensus sequence defined as the initiator sequence (Inr), which is present in α7 promoter. The transcription start site for

the α7 gene appears to start not in the Inr-like sequence but in close proximity to it, 2 bps upstream of the Inr-like sequence (Vignier et al, 1999). The Inr, in combination with nearby Sp1/GC boxes, directs correct transcription initiation. Also, numerous binding sites for ubiquitous, developmental, and cell type-specific transcription factors including five putative Sp1 binding sites, eight consensus E-boxes (CANNTG) provide binding sites for basic helix-loop-helix (bHLH), two AP-1 binding sites, one AP-2 binding site, and one Ets binding site (Vignier et al, 1999). There are five A/T-rich sites in the α7 promoter that may also function as binding sites for several transcription factors such as MEFS, Mef2a-d and serum response factor (Vignier et al, 1999). The MCAT motif located at position -585 is the binding site for transcriptional enhancer factor-1 (TEF-1) or a TEF-like protein and is believed to be important in directing cardiac-specific expression.

The regulation of chicken α1 integrin expression in smooth muscle cells has been studied and α1 integrin is down-regulated in phenotype modulated cultured SMCs during serum-stimulated dedifferentiation (Obata et al, 1997). The 5'-upstream sequences of the α1 integrin gene share structural similarities with other α integrin genes; both TATA and CCAAT boxes are absent, and consensus binding sites for AP1 and AP2 are present. The comparative analysis of a series of deleted α1 integrin promoter-CAT constructs revealed that the basal promoter activity of the gene was attributed to the region from +77 to +173 and that the 5'-upstream sequence (-516 to +281) produced sufficient transcriptional activity in differentiated gizzard SMCs compared to that in dedifferentiated SMCs. Site-directed mutagenesis revealed that a CArG box-like motif was important for the

transcriptional activation in differentiated SMCs, whereas binding sites for AP1 or AP2 were not, suggesting that the CArG box-like motif serves as a positive element only in a differentiated phenotype of SMCs.

1.4 CD151 (PETA-3)

The tetraspanins are a family of widely expressed four-transmembrane-domain proteins, with at least 28 distinct family members in mammals (Hemler et al, 1996). These proteins organize into specific protein and lipid-containing membrane microdomains; the assembly of microdomains depends on *primary* (direct, high stoichiometry) interactions of specific tetraspanins with other proteins. Also, *secondary* interactions arise due to the tendency of tetraspanins to oligomerise and to associate with other tetraspanins. Tetraspanins form complexes with a number of membrane proteins including integrins, to form a network termed the "tetraspanin web". Numerous reports link tetraspanins with regulation of a variety of cellular processes including cell activation, migration, cell-cell interactions and membrane fusion. To date, the precise mechanisms whereby tetraspanins function have not been determined. Despite their association with integrins, tetraspanins do not modulate integrin-dependent cell adhesion but rather appear to modulate cell migration, fusion, and signaling (Berditchevski, 2001). A general working hypothesis is that tetraspanins act as molecular facilitators or transmembrane linkers, using intracellular and/or transmembrane domains to recruit key signaling enzymes, whereas extracellular domains engage other transmembrane proteins in specific lateral associations.

CD 151 (PETA-3/SFA-1) is a cell surface protein that belongs to the tetraspanin 4 superfamily (TM4SF). CD 151 stands out because of its stable association with several β1 and other integrins in a variety of cell types (Sterk et al, 2000), with the primary interaction believed to be mediated by the integrin α-chain (Yauch et al, 2000; Yauch and Hemler, 2000). Moreover, CD 151 appears to specifically complex with laminin-binding integrins including α3β1, α6β1, α6β4 and α7β1, and is co-distributed with these integrins in many tissues at sites of cell-matrix interactions (Sterk et al, 2002). There is no evidence that the association with tetraspanins can directly affect integrin affinity for ECM ligands, integrin clustering or linkage of integrins to the actin cytoskeleton, all of which are key factors in establishing strong adhesive contacts with ECM substrates. In most cells, CD151 is absent from focal contacts-complex adhesion structures primarily responsible for strong attachment to the ECM. It has been shown that the CD151 molecules are involved in adhesion-dependent signaling mediated by integrins and the ligation of CD151 stimulates integrin-dependent signaling pathways. Thus TM4SF proteins appear to be important components of the integrin signal-transducing mechanism. Although molecular details are still sketchy, the CD151 molecules are reported to alter integrin-dependent signaling through FAK, in parallel with rearrangement of the cortical actin cytoskeleton (Hemler, 2001). Since the CD151 molecule uses extracellular domains to associate with partner proteins and cytoplasmic or transmembrane domains to associate with intracellular signaling enzymes such as conventional PKC isoforms, it also functions as an integrin-associated "transmembrane linker". To date phenotype-dependent expression of tetraspanins such as CD151, which

associates with integrins that bind laminin and are expressed in abundance by contractile phenotype smooth muscle cells, has not been investigated.

1.5 Statement of Problem and Hypothesis

Smooth muscle has remarkable phenotype and functional plasticity necessary for growth and repair during injury when additional functions such as proliferation and secretion of ECM and biological mediators are apparent. Smooth muscle cells from the airways constitute a source of growth factors, cytokines and extracellular matrix products associated with airway remodeling in asthma. In addition, the smooth muscle cell itself can synthesize locally active cytokines and ECM components, which alter the matrix composition that determines ASM phenotype.

Studies suggest opposing roles for laminin and fibronectin in regulation of differentiated properties of airway smooth muscle cells. The abundance and isoforms expression profile for laminin is altered in airways from asthmatic patients. Laminin and other basement components promote the expression of a contractile differentiated smooth muscle phenotype, whereas fibronectin and collagen I stimulate the cells to adopt a proliferative phenotype, and to promote myocyte migration and further synthesis of ECM and pro-inflammatory molecules. While the precise molecular mechanisms for phenotypic modulation in airway smooth muscle remains unknown, these may be mediated, in part, via a family of cell-surface glycoprotein receptors. Thus, the ECM and specific repertoire of integrins expressed by airway SMC can define and determine phenotype and function of individual myocytes.

As our laboratory has previously generated significant data and understanding in using cell culture models as a means to dissect mechanisms that regulate phenotype and function of airway smooth muscle cells, we proposed studies using these systems to characterize and compare phenotype-dependent expression of laminin receptors, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$ and the fibronectin receptor, $\alpha 5\beta 1$, in proliferating and contractile canine airway smooth muscle cells (CASMC) in primary culture and in tissue specimens. As almost nothing is known about how matrix specific integrins mediate phenotype switching or mainitenance in airway smooth muscle cells, we also proposed to investigate expression of the tetraspanin CD151, which associates with laminin-binding integrins and mediates intracellular signalling.

Hypothesis: Laminin binding integrin expression by airway smooth muscle in primary culture and in tissue is phenotype-dependent.

Objectives: Our goal was to characterize and compare differential expression of subunits of the laminin receptors, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, and the fibronectin receptor, $\alpha 5\beta 1$ in serum-fed, proliferating and serum-deprived contractile canine airway myocytes. To achieve this goal we completed experiments to meet Four Specific Aims:

1) To analyze the integrin protein and mRNA abundance in serum-fed and serum-deprived canine airway myocytes by Western Blotting and RT-PCR techniques respectively.

2) To determine the relative cellular distribution of integrins and their ligands (laminin and fibronectin) by confocal microscopy.

3) To characterize the integrin expression profile of airway myocytes in canine and human bronchial tissues, using immunohistochemistry.

4) To examine CD151 expression in canine airway myocytes, canine bronchi and human bronchi by Western blot, immunocytochemistry and immunohistochemistry.

CHAPTER 2. MATERIALS AND METHODS

2.1 Reagents

Tissue culture materials (Dulbecco's modified Eagle's media (DMEM), pencillin-streptomycin-glutamine, antibiotic-antimycotic, gentamicin reagent solution, fetal bovine serum (FBS) and 0.053M EDTA, 0.05% Trypsin were obtained from Gibco/BRL (Grand Island, N.Y., USA). Elastase and protease were obtained from Sigma Chemical (St. Louis, MO.). Type 1 Collagenase was purchased from Calbiochem (San Diego, USA). Aprotinin and pepstatin were purchased from Sigma Chemical (St. Louis, MO). Purchased from Jackson ImmunoResearch (West Grove, PA) were bovine serum albumin (IgG free) and normal donkey serum. Other purchases included ECL detection system (Amersham Biosciences, UK) and all reagents for protein electrophoresis (Bio-Rad, Hercules, CA). Prolong antifade kit, TOTO-3 iodide and propidium iodide were obtained from Molecular Probes (Eugene, Oregon).

2.2 Canine Tracheal Smooth Muscle Cell (SMC) Primary Culture

We used the method described by Halayko et al. (1996, 1999) for tracheas excised from anesthetized mongrel dogs and placed into ice-cold calcium free, aerated Kreb's solution (126mM NaCl, 25mM $NaHCO_3$, 1.38mM NaH_2PO_4, 4.7mM KCl, 2.46mM $MgSO_4$, 1.91mM $CaCl_2$, 5.56mM dextrose). Trachealis muscle was dissected, cleaned of serosa and epithelia at room temperature, and washed four times under aseptic conditions in cold Hank's Balanced Salt Solution (HBSS/HEPES, 100 units/ml Penicillin G sodium, 100μg/ml streptomycin sulfate and 0.25μg/ml amphotericin B). The muscle was minced thoroughly with fine scissors and cells were isolated from tissue in 12ml of sterile filtered

digestion buffer (HBSS/HEPES containing 600U/ml collagenase, 5U/ml elastase and 2U/ml Nagarse protease). The suspension was incubated at 37°C for 45 minutes with a vigorously shaking water bath after, which tissue pieces were disrupted with gentle trituration using a 1mm pore 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% FBS and penicillin-streptomycin-glutamine (PSG) (100 units/ml penicillin G sodium, 100μg/ml streptomycin sulfate, 0.292 mg/ml L-glutamine, 0.1mM sodium citrate in 0.14% NaCl) and then the cells were pelleted by centrifugation at 600 × g for 5 minutes at room temperature. The resulting supernatant was discarded and pelleted cells were re-suspended in 5ml of fresh DMEM/10% FBS plus PSG and stored on ice.

A fresh aliquot (12mls) of digestion buffer was added to the tube containing the debris that remained after the first digestion. After mixing, the debris was incubated for a further 40 minutes to isolate the remaining SMCs. 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/PSG, centrifuged and re-suspended in fresh DMEM in the same manner that the first digestion 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/PSG. Cell number was estimated by counting using a haemocytometer. The cells were plated onto 100 mm plastic culture dishes at a density of 8000-10000 cells/cm^2. Cells were grown at 37°C in DMEM/ 10%FBS/PSG in a

humidified 95% air/5% CO_2 incubator and culture medium was replaced every 72hrs. Cultures reached confluence normally within 6-7 days.

For preparation of growth arrested smooth muscle cells, culture media was aspirated and plates were washed with PBS three times then serum-free Ham's F-12 medium supplemented with insulin-transferring-selenium A (ITS) and penicillin-streptomycin-glutamine (PSG) (100 units/ml penicillin G sodium, 100μg/ml streptomycin sulfate, 0.292 mg/ml L-glutamine, 0.1mM sodium citrate in 0.14% NaCl) was added. Cultures were arrested for 7-15 days to induce differentiation of smooth muscle cells. Culture media was changed every 72 hours. Elongated contractile cells normally emerged by the third day of serum deprivation, and increased in number for a further 4-7 days. Thereafter elongate cell number was stable. For all subsequent experiments, cells from passage 1 to passage 2 were used.

2.3 Protein Analysis

2.3.1 Preparation of Protein Lysates

Medium was aspirated from tissue culture dishes. Cells were rinsed three times with ice-cold PBS (Phosphate-buffered Saline)(2.68mM KCl, 1.47mM KH_2PO_4, 136mM NaCl, 8.1mM Na_2HPO_4) and then cells were lysed by addition of 200μl ice cold RIPA buffer (40mM Tris-HCl, pH 8.0, 150mM NaCl, 1%NP-40, 1% deoxycholic acid) containing freshly added protease inhibitors (2mM PMSF (phenylmethlsulfonylfluoride), 5μg/ml aprotinin and 5μg/ml pepstatin A) and phosphatase inhibitors (1mM Na_3VO_4 and 1mM NaF). Cells were scraped from the plates with a cell scraper (on ice) and the lysates were

transferred to a 1.5 ml micro-centrifuge tube. Cell lysates were sonicated with 2 pulses for 10 seconds at setting 2# using a probe sonicator model 100 (Fisher Scientific, Ottawa, ON), then centrifuged at 14000×g for 15 minutes in a microcentrifuge at 4°C. The supernatant was transferred to a new 1.5ml microcentrifuge tube and stored at –20°C until used in subsequent experiments.

2.3.2 Protein Assay

Total protein concentration was determined using the Bio-Rad DC Protein Assay Kit. Four dilutions of the samples (1:5, 1:10, 1:15, 1:20) were prepared using double distilled water. Bovine Serum Albumin (BSA) was used as a standard in each assay (ranging from 0.195-1.35mg/ml). Absorbance was measured at 750nm with a plate reader (Bio-Tek instruments, Winooski, VT) using KC junior software.

2.3.3 SDS-PAGE

Proteins in samples were resolved by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The Mini-Protean 3 cell (Bio-Rad, Hercules, CA) was employed. Upper gels and lower gels were prepared from a 30% acrylamide/bis-acrylamide monomer stock solution. Separating (lower) gel contained: 1) acrylamide/bis-acrylamide (concentration depending on requirement), 2) 1.5M Tris-HCl, pH 8.8 and, 3) 10% SDS (w/v). Stacking gel (upper gel) included: 1) 4% (w/v) acrylamide/bis-acrylamide, 2) 0.5M Tris-HCl, pH 6.8 and, 3) 10% (w/v) SDS. Samples were diluted 1:4 in SDS/sample buffer (0.5M Tris-HCl pH 6.8, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 25% (v/v) glycerol, 5%(v/v) β-mercaptoethanol) and boiled for 5 minutes to

denature the proteins. For some proteins non-denaturing sample buffer was used in which β-mercaptoethanol was omitted. Equal amounts of total proteins were loaded onto mini-gels using a Hamilton syringe and thereafter separated by electrophoresis at constant voltage (200V) for 45 minutes at room temperature using electrode buffer with 25mM Tris, 0.192M glycine, 0.1%(w/v) SDS.

2.3.4 Western Blotting

Protein homogenates were separated by SDS-PAGE on 8x10cm mini-gels as indicated in Section 2.3.3 above. Proteins were electroblotted to presoaked nitrocellulose membranes at constant voltage (100V) for 90 minutes in transfer buffer (20% methanol, 25mM Tris, 130mM glycine) in a Mini-Protean 2 Trans-Blot apparatus (Bio-Rad, Hercules, CA) with ice cooling. Immediately after transfer, blots were washed for 5 minutes in TBS (20mM Tris-base, 150mM NaCl, pH 8.0) for 5 minutes. For immunodetection, blots were blocked in TBS/0.1% Tween-20/5% skim milk powder for 2hrs at room temperature then incubated with primary antibody diluted in TBST/1% skim milk overnight at 4°C on a shaker. Primary antibodies and dilution used are listed in Table 2. Blots were washed 3 × 10 minutes with TBS/0.1% Tween-20. Horseradish peroxidase (HRP) conjugated secondary antibodies were added and incubated for 2 hours at room temperature. Secondary antibodies and dilution used are listed in Table 3. After washing 3 × 10 minutes with TBS/0.1% Tween-20, blots were incubated in enhanced chemiluminescence (ECL) substrates for 1 minute. Chemilumigrams were exposed to HyperFilm-ECL and developed in an automatic film processor.

2.3.5 Densitometry

Developed film was scanned with an EPSON TWAIN 5 scanner using Photoshop software. Image analysis and quantification were performed using background subtraction with Total Lab V2.01 software (Nonlinear Dynamics Ltd, UK). Relative protein abundance for each film was determined and used for subsequent statistical analyses.

2.4 Immunocytochemistry

Cells were studied at two points in culture: (1) 50%-70% confluent in DMEM/10% FBS, and (2) 7-10 days after confluent cultures had been maintained in serum-free media. Cells were plated in 6-well cell culture dishes containing pre-cleaned 25 mm round glass. Attached cells were rinsed three times with CB buffer (10mM MES, 150mM NaCl, 5mM EGTA, 5mM MgCl2, 5mM glucose, pH 6.1) and fixed in 3% paraformaldehyde for 15 minutes at 4°C and subsequently permeablized with 3% paraformaldehyde/0.3% Triton X-100 for 5 minutes at 4°C. Coverslips were then rinsed two times with CB buffer and stored in sterile Cyto-TBS (20mM Tris base, 154mM NaCl, 2mM EGTA, 2mM MgCl2, pH 7.2) for up to 4 weeks in the dark.

Prior to staining, coverslips were incubated in blocking solution (Cyto-TBS containing 1% BSA and 2% normal donkey serum) for 2 hours at room temperature in a humidified chamber. The normal serum was matched to the species in which the secondary antibody was raised (e.g. if the secondary antibody was from goat, normal goat serum was used). Each coverslip was inverted onto 50 μl of primary antibody diluted in Cyto-

TBS/0.1%Tween-20 on a Parafilm-covered culture dish and incubated overnight at 4°C. The primary antibodies used are listed in Table 2. Negative controls were incubated in Cyto-TBS/0.1%Tween-20 without primary antibody. After primary antibody incubation, coverslips were then washed four times, for 15 minutes each with Cyto-TBS/0.1% Tween-20. Thereafter, fluorochrome-conjugated secondary antibodies were applied.

All secondary antibodies were diluted 1:100 in Cyto-TBS/0.1%Tween-20. The secondary antibodies used are listed in Table 4.1. Each coverslip was inverted onto 50μl of secondary antibody solution on a fresh Parafilm surface. Cells were then incubated in the dark for two hours at room temperature. Coverslips were then washed four times for 15 minutes each with Cyto-TBS/0.1% Tween-20 and two times for 10 minutes each with distilled water.

Nuclear staining using 0.5 M Toto-3 in distilled water was done immediately after the final water wash. Thereafter, cells were rinsed with double-distilled water and mounted, cell side down onto pre-cleaned microscope slides using Prolong Antifade mounting medium (Molecular Probes, Eugene, ON). The prepared slides were sealed with nail polish and finally stored in the dark at 4°C until they were viewed and photographed using an Olympus Fluoview 2.0 confocal laser-scanning microscope system (Olympus Optical Co. Ltd, Tokyo, Japan) equipped with associated software.

2.5 Histology

2.5.1 Tissue Dissection

Bronchi obtained from anaesthetized mongrel dogs were carefully dissected out in Krebs-Henseleit solution under dissecting microscope with fine forceps and scissors. Human central bronchi (0.5-1cm diameter) from the patients receiving surgery were transported to the laboratory on ice for use in subsequent experiments.

2.5.2 Cryosectioning

Canine and human lung specimens were acquired according to animal and human ethics protocols approved by the University of Manitoba Central Animal Care Services and Human Research Ethics Board, respectively. Bronchial segments were embedded in OCT (optimum cutting temperature) compound using biopsy cryomolds, and then frozen in a dry ice/ethanol bath. Blocks were stored at -70°C until serial sections were cut at a thickness of 10μm in a ThermoShandon cryotome. Cryostat chamber temperature was set at -25°C. The sections were mounted onto Fisherbrand Superfrost/Plus microscope slides and (-20°C) until stained. Slides were removed from –20°C, warmed to room temperature, then placed into slide chambers containing cold 3% paraformaldehyde for 15 minutes. Slides were then rinsed 3 × 10 minutes with cold Cyto-TBS to eliminate residual paraformaldehyde, then sections were permeablized with cold 0.3% Triton X-100 in Cyto-TBS for 20 minutes and rinsed 2 × 10 minutes with ice-cold Cyto-TBS.

For each antigen, two serial sections were processed. Immunohistochemical staining was performed using indirect immunofluorescence. Nonspecific antibody binding was blocked by placing 100 μl of 3% donkey serum in Cyto-TBST (CytoTBS/0.1% Tween) over each section then covering with Parafilm for 1 hour at room temperature. After

rinsing, diluted primary antibodies (see Table 2) in Cyto-TBST were applied to the sections in a humidified chamber overnight at 4°C. The slides were then washed with Cyto-TBST 3 × 20 minutes. All secondary antibodies were diluted 1:100 in Cyto-TBST. The secondary antibodies used are listed in Table 4.2. The slides were incubated with secondary antibodies in Cyto-TBST for 1 hour at room temperature in the dark. After washing 3 × 20 minutes in Cyto-TBST, nuclei were stained with 4.1μg/ml propidium iodide for 20 minutes in the dark. The slides were then rinsed in water and allowed to airway-dry before the addition of 25μl of Prolonged Anti-fade mounting medium (Molecular Probes, Eugene, ON). The prepared slides were sealed with nail polish and finally stored in the dark at 4°C until they were viewed and photographed using an Olympus Fluoview 2.0 confocal laser-scanning microscope with associated software. For each batch of sections processed for immunohistochemistry, two parallel sections of each tissue were included as a negative control in which primary antibody was omitted.

2.6 Analysis of mRNA

2.6.1 Primer Design for PCR

Integrin primer pairs were designed based on previously published reports for α3, α6, and α7 (Star and Quaranta, 1992; Collo et al, 1993; Tennenbaum et al 1995). However, this approach was inefficient to produce specific canine integrin α5 and β1 primers. Thus, we designed new primers by manually aligning all the sequences from human, rat, mouse genes downloaded from GenBank (http:www.ncbi.nlm.nih.gov/) and identified areas of highest inter-species homology. We used the Clustal program (http://clustalw.genome.ad.jp/sit-bin/nph- clustalw) to perform this alignment analysis.

Specific areas of homology were then used to design primers using either Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or BLAST (http://www.ncbi.nlm.nih.gov/BLAST) programs. All primer sequences (synthesized by Invitrogen Custom primers) are listed in Table 5.

2.6.2 RNA Preparation

Total RNA was extracted from cells cultured on 100-mm dishes using TRIzol reagent. Cells were rinsed 1-2x with PBS and 1.0ml TRIzol was added for 5 minutes. Plates were scraped and the cell lysate was transferred to a 1.5 ml microcentrifuge tube. The sample was then centrifuged at 12000 × g for 10 minutes at 4°C to remove insoluble material. The supernatant containing RNA was transferred to fresh microfuge tubes. Chloroform (200µl) was added and the tubes then inverted gently to mix, followed by centrifugation at 15000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube. Care was taken to avoid sampling the interface that contained DNA and proteins. RNA was precipitated with 500µl isopropanol and incubated at room temperature for 10 minutes. Precipitated RNA (white pellet) was collected by centrifugation at 12000 × g for 10 minutes at 4°C. The pellet was then washed with 1ml 75% ethanol and re-centrifuged at 7500 × g for 5 minutes at 4°C. The RNA was air-dried by inverting the tubes for 30 minutes, and then reconstituted in 30-50 µl of autoclaved distilled water previously treated with diethyl pyrocarbonate (DEPC). To evaluate RNA quantity and quality, 2 µl of sample was diluted to 50µl with DEPC water and the absorbance was measured at 260nm in a UV-spectrophotometer (Berthold Technologies, USA) to estimate concentration (see formula below). An absorbance of 1 unit at 260nm corresponds to

~40μg of RNA/ml. We used the ratio of absorbance at 260nm and 280nm to estimate of RNA purity; only RNA that had an A260/A280 ratio of 1.6-1.8 was used for study. To calculate RNA concentration, the following formulae were used: i) Concentration of RNA sample (μg/ml) = 40μg/ml × A260 × Dilution factor (2:48); ii) Total amount RNA (μg) = Concentration of RNA (μg/ml) × Volume of sample (ml). To further assess the integrity of the purified RNA we also performed electrophoresis. Equal amounts of total RNA for each sample were run on 1.0% agarose gels in 1xTAE buffer (40mM Tris-HCl, 20mM acetic acid, 1mM EDTA) and the integrity of 18S and 28S rRNA bands was assessed by eye on a UV transilluminator.

2.6.3 RT-PCR

A sample mixture was made containing 2 μg total RNA, 1μl Oligo dT Primer (500μg/ml) and subjected to 5 minutes heating at 65°C, chilled on ice and briefly centrifuged. A reaction mixture consisting of 1μl M-MLV Reverse Transcriptase, 1μl dNTPs (10mM each of dATP, dCTP, dGTP, dTTP), 2μl 0.1 M DTT, 1μl RNaseOUT (40 units/μl), and 4μl of 5× first-strand buffer was prepared for each sample. The reaction mixture was added into the sample mixture and first strand synthesis was then carried out at 42°C for 70 minutes, followed by heating at 70°C for 15 minutes to inactivate the reserve transcriptase and denature RNA/cDNA complexes and primer dimers. For subsequent PCR reactions, a master mix was made containing 2.5μl 10× PCR buffer, 0.5μl 0.2 mM dNTPs, 0.25μl 2.5 units of Platinum TaqDNA Polymerase, 1μl 10nM forward primer and 1μl 10nM reverse primer. One μl of cDNA from RT reactions was added to the above master mix to a final volume of 25μl. PCR was performed in a DNA thermal cycler

(Teche Incorporated, Princetin, NJ, USA). Conditions for PCR are outlined in Table 5 for each primer used. After the final cycle, the samples were kept at 72°C for 10 minutes. As a negative control for the PCR reaction, the above reaction mixture was prepared without the cDNA template and treated under the same condition. The housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as internal controls as they are constitutively expressed in most cells.

2.6.4 Analysis of PCR Amplified Products

The PCR products were analyzed by agarose gel electrophoresis, which provided a quick method to estimate the size of a DNA band from PCR amplification. A horizontal gel electrophoresis apparatus was used. Agarose (1.5gm/100ml) was dissolved in TAE buffer and poured in casts to form gels. All gels also contained ~10ng/ml ethidium bromide. Prior to each run, gels were equilibrated 30 minutes in TAE as running buffer at room temperature; 3μl of each PCR product was mixed with 1ul of 6xDNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose in water). On all gels, a molecular marker ladder to estimate the size of the PCR product was also included. Electrophoresis was performed at 100V at room temperature until the bromophenol blue has reached a distance approximately halfway between the sample wells and the cathode edge of the gel. DNA bands were visualized using a UV trans-illuminator. Photographs were taken using a FisherBiotech Photo-Documentation camera with an FB-PDH-1012 hood and an orange or green filter employing Polaroid 667 black and white instant film. For subsequent presentation of gels, photographs were scanned using an EPSON scanner to generate digital images.

2.7 Statistical Analysis

All measurements made from chemilumigrams were completed using 2-4 replicates from each of three cell lines. Data were expressed as mean ± SEM. The GraphPad Prism analysis program (GraphPad Software, Inc.) was used to perform a parametric one-way analysis of variance (ANOVA) on Western blot data obtained by densitometry. Statistical significance for all analysis was accepted at $P<0.05$.

Table 2. Primary antibodies used for immunocytochemistry, immunoblotting and immunohistochemistry.

Antigen	Type	Species	Clone	Dilution			Source
				ICC	WB	IHC	
Integrinα6	Mab	Rt	GoH3	1:300	N/A	1:300	Coulter/Immunotech
Integrinα6A	Pab	Rb	392	1:100	N/A	1:100	Dr. I. Curtis
Integrinα6B	Pab	Rb	1142	1:100	1:500	1:100	Dr. I. Curtis
Integrinα6A	Pab	Rb	6844	1:100	N/A	1:100	Dr. L. Reichardt
Integrinα6B	Pab	Rb	382	1:100	1:500	1:100	Dr. L. Reichardt
Integrinα7A	Pab	Rb	SV2	1:200	N/A	1:100	Dr. G. Tarone
Integrinα7B	Pab	Rb	ZE8	1:200	N/A	1:100	Dr. G. Tarone
Integrinα3	Pab	Rb		1:250	1:400	1:250	Chemicon
Integrinα5	Pab	Rb		1:500	1:500	1:500	Chemicon
Integrinα5β1	Mab	Ms	JBS5	1:300	N/A	1:300	Dr. J. Wilkins
Integrinβ1	Mab	Ms	JB1A	1:75	1:500	1:75	Dr. J. Wilkins
Integrinβ4	Mab	Rt		1:400	N/A	1:400	Chemicon
Integrinα7	Mab	Rt	Cy8	1:400	N/A	1:400	Dr. R. Kramer
Laminin	Pab	Rb		1:200	1:500	1:200	Chemicon
Fibronectin	Pab	Rb		1:500	1:500	1:500	Chemicon
SM myosin heavy chain	Mab	Ms	hSM-V	1:500	1:500	1:500	Sigma
Gizzard myosin (HC)	Pab	Rb		1:2500	1:2500	1:2500	Dr. M. Walsh
Calponin	Mab	Ms	hcp	N/A	1;2000	N/A	Sigma
SM22	Pab	Rb		N/A	1:750	N/A	Dr. J. Solway
SM-α-actin	Mab	Ms		N/A	1:500	N/A	Sigma
CD151	Mab	Ms	11B1	5ug/ml	2.5ug/ml	5ug/ml	Dr. LK. Ashman
CD151	Mab	Ms	14A2.H1	5ug/ml	N/A	10ug/ml	Dr. LK. Ashman
CD151	Mab	Ms	14B5.H6	5ug/ml	N/A	10ug/ml	Dr. LK. Ashman
CD151	Mab	Ms	5C11	5ug/ml	N/A	10ug/ml	Dr. M. Helmer

Abbreviations: Rt=rat, Ms=mouse, Rb=rabbit, ICC=Immunocytochemistry, WB=Western Blotting, IHC=Immunohistochemistry

Table 3. Secondary antibodies used in Western Blotting

Secondary antibody	Dilution	Source
HRP-conjugate goat anti-mouse IgG	1:3000	Sigma
HRP-conjugate goat anti-rabbit IgG	1:5000	Sigma
HRP-conjugate goat anti-rat IgG	1:5000	Sigma

Table 4.1 Secondary antibodies used for immunocytochemistry

Secondary antibody	Dilution	Source
FITC-conjugated donkey anti-mouse IgG	1:100	Jackson ImmunoResearch
Cy3-conjugated donkey anti-rabbit IgG	1:100	Jackson ImmunoResearch
Cy3-conjugated donkey anti-rat IgG	1:100	Jackson ImmunoResearch

Table 4.2 Secondary antibodies used in immunohistochemistry

Secondary antibody	Dilution	Source
FITC-conjugated donkey anti-mouse IgG	1:100	Jackson ImmunoResearch
FITC-conjugated donkey anti-rabbit IgG	1:100	Jackson ImmunoResearch
FITC-conjugated donkey anti-rat IgG	1:100	Jackson ImmunoResearch

Table 5. Specific primer sequences and conditions used for polymerase chain reactions (PCR) analyses.

Gene	Primer sequence (5'-3') - Forward (Top) - Reverse (Bottom)	$MgCl_2$ mM	PCR product bp	Cycling profile
α3(Ham) **J05281**	AAGCCAAATCTGAGACTGTG GTAGTATCGGT CCGAATCT	1.5	660/510 (α3A/α3B)	94°C; 1min 55°C; 2min 72°C; 3min 40 cycles total
α5(M) **BC059829**	CCCAGACTTCTTTGGCTCTG GTTGAACATGGAGGGGAAGA	1.5	161	94°C; 40sec 55°C; 40sec 72°C; 45sec 40 cycles total
α6(M) **BC058095**	GTGAGGTGTGTGAACATCAG CATGGTATCGGGGAATGCT	1.5	540/410 (α6A/α6B)	94°C; 1min 55°C; 2min 72°C; 3min 40 cycles total
β1(H) **XM_134403**	GTCCCACTGGTCCAGACATC CCCATTTGGCATTCATTTTC	1.5	159	94°C; 40sec 55°C; 40sec 72°C; 45sec 40 cycles total
α7(M) **NM_008398**	GTTGTGGAAGGAGTCCC GTCTTCCCGAGGGATCTT	1.5	283/170 (α7A/α7B)	94°C; 1min 55°C; 2min 72°C; 3min 40 cycles total
β4 (M) **L04678**	CGCCGTCTGGTAAACATC AGTAGCTTCACCTGCAACTC	1.5	259	94°C; 1min 55°C; 1min 72°C; 1min 35 cycles total
β-actin(M) **BC013835**	TCACCCACACTGTGCCCATCTACGA CAGCGGAACCGCTCATTGCCAATGG	1.5	294	94°C; 40sec 60°C; 40sec 72°C; 45sec 25 cycles total
GAPDH(M) **NM002026**	AGCAATGCCTCCTGCACCACCAAC CCGGAGGGGGCCATCCACAGTCT	1.5	136	94°C; 1min 59°C; 1min 72°C; 1min 30 cycles total

Abbreviation: M = mouse, H = Human , Ham= Hamster

CHAPTER 3. RESULTS

3.1 Phenotype of Primary Cultured Canine Airway Myocytes

SMCs were isolated from the canine trachealis for generating primary cultures as described in Materials and Methods. At confluence, canine tracheal myocyte cultures exhibited a hill-and-valley appearance that is characteristic of SMCs growing in serum. Individual cells assumed a flattened, short, spindle shape. In post-confluent primary SMC culture with long-term serum deprivation up to 14 days two morphologically distinct subgroups of cells appeared. About one-sixth of the cells developed an elongated morphology and most aligned side-by-side and end-to-end to form bundles coursing through the culture dishes. The remaining myocytes instead were rather circular and flattened, randomly oriented into a continuous monolayer between bundles of elongated cells. These observations are entirely consistent with previous description of similar cultures in our laboratory (Halayko et al, 1999; Halayko et al, 2004). The organization of cells noted above can be seen in a number of the figures in this thesis (eg. Figure 5).

Previous reports have demonstrated that induction of contractile phenotype in cultured tracheal SMC is accompanied by a considerable increase in expression of contractile apparatus-associated protein (Halayko et al, 1996, 1999). As shown in Figure 3, we compared the presence of smooth muscle-specific protein markers in serum-fed, sub-confluent and confluent CASMC cultures with 7-day serum-deprived CASMC cultures. By Western blot, smMHC appears to be the most dependable definitive marker of the SMC lineage as immunoblot analysis revealed virtually no smMHC protein in serum-fed, sub-confluent CASMC cultures that express a proliferative phenotype. A rather low level

of smMHC was detected in serum-fed, confluent CASMC cultures; however, a significant accumulation of smMHC was evident in 7-day serum-deprived CASMC cultures. This pattern of expression is consistent with prior reports from our lab using this culture system (Halayko et al, 1999; Halayko et al, 2004).

Smooth muscle-α-actin is one of the earliest markers of smooth muscle differentiation, being one of the most abundant proteins in mature SMCs. Protein for sm-α-actin was abundant in myocytes at all culture, and increased significantly with serum deprivation (Figure 3). SM22, a 22kDa actin filament binding protein abundant in mature, contractile myocytes also increased significantly in 7-day serum-deprived cell cultures. The pattern of abundance of calponin, a smooth muscle specific actin regulatory protein that is abundant in contractile myocytes mimicked that seen for SM22 (Figure 3). Taken together, these data confirm that phenotype switching from a "synthetic" state to "contractile" state occurs readily and reproducibly with prolonged serum deprivation of primary cultured airway SMC.

3.2 Laminin Expression

To examine the expression of laminin in proliferating and contractile CASMC cultures, immunoblotting was employed. Total protein lysates, which included intracellular and extracellular laminin, were fractionated by 5% PAGE in reducing conditions. Blots were stained with a polyclonal antibody against laminin-α1 and -α2 chains. Typical results are shown in Figure 4. As expected, a band of ~200KDa was detected in lysates from 70%, 100% confluent and 7 day serum-deprived CASMC cultures. To compare laminin protein

level between serum-fed and serum-deprived cell cultures, blots from 3 different cultures were subjected to densitometry. Data were assessed by One-way ANOVA analysis. The densitometry (OD) values obtained per blot were normalized to the serum-fed, 70% confluent CASMC sample. No statistical difference in total laminin abundance was evident between serum-fed and serum-deprived CASMC cultures.

To compare the co-distribution of laminin and smMHC in individual canine airway smooth muscle myocytes, we used confocal immunofluorescent cytochemistry with dual-labeling using antibodies for laminin and smMHC (Figure 5). In proliferating myocyte cultures, laminin staining displayed a punctate intracellular distribution that appeared in almost all cells, and little secreted extracellular laminin was evident. As expected for serum-fed cells very low labeling for smMHC was evident. In contrast, contractile myocytes in serum 7-day deprived cultures expressed abundant extracellular and intracellular laminin. Only smMHC-positive elongated cells exhibited clear extracellular laminin staining. Laminin labeling in non-elongate, smMHC-deficient (non-contractile) myocytes was weak and appeared to be mostly intracellular. These observations confirm that coincident, abundant staining for laminin and smMHC is a unique character of contractile phenotype myocytes in primary culture. We also examined the expression of laminin by smMHC-rich contractile airway smooth muscle cells in intact tissue specimens. As shown in Figure 6A, contractile canine bronchial smooth muscle showed intensive staining for laminin. Contractile human bronchial smooth muscle also stained strongly with mAb specific for laminin (Figure 35). Of note, as expected tissue labeling

also revealed that laminin was enriched in the subepithelial basement membranes of the airways (Table 6).

3.3 Fibronectin Expression

The expression of fibronectin in proliferating or contractile CASMC cultures was evaluated by Western blotting. Samples were fractionated by 5% PAGE gel in reducing conditions. Blots were stained with a polyclonal antibody against fibronectin. Typical results are shown in Figure 7. A band of ~ 220KDa was detected in 70%, 100% confluent and 7 day serum-deprived CSAMC cultures. Analyses were completed for 3 individual cultures. To compare differences in fibronectin protein levels between serum-fed and serum-deprived cell cultures, we used scanning densitometry. Thereafter, data were assessed by One-way ANOVA analysis. The densitometry (OD) values obtained per blot were normalized to the serum-fed, 70% confluent CASMC sample, and were set to an arbitrary unit of one. The results for 3-4 experiments shown in Figure 7B show that though there was a trend for an increase in total fibronectin abundance coincident with increasing cell density, no statistically significant difference was measured between cell cultures at all conditions.

To compare the co-distribution of fibronectin and smMHC, canine airway smooth muscle myocytes were dual-labeled with antibodies against fibronectin and staining was assessed by confocal immunocytochemistry (Figure 5). In proliferating myocyte cultures, fibronectin staining displayed a fibrillar pattern for all cells. Fibronectin staining was also homogeneously distributed in 7-day serum-deprived myocyte cultures containing

elongated smMHC-positive cells. These observations indicate that though contractile myocytes express fibronectin, it is not a unique feature of these cells, as non-contractile myocytes also express the proteoglycan. The tissue distribution of fibronectin was also determined by immunohistochemical staining using monoclonal antibody specific for fibronectin. As shown in Figure 6D, contractile phenotype canine bronchi smooth muscle showed strong staining for fibronectin. Contractile human bronchi smooth muscle (Figure 35) also stained strongly for fibronectin. We also detected strong staining for fibronectin in submucosal regions devoid of smooth muscle. These observations confirm that fibronectin is expressed by airway smooth muscle cells but is not a unique marker of the contractile phenotype (Table 6).

3.4 Analysis of RNA Quality

Verification of the integrity of total RNA from each cell and tissue samples was assessed by visualization of the 28S and 18S rRNA bands in agarose gels stained with ethidium bromide (Figure 8). Total RNA (1μg) was isolated from serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures and loaded on a 1.5% agarose gel, subjected to electrophoresis with ethidium bromide. RNA was then visualized under UV transilluminator as described in Materials and Methods. The ratio and appearance of 28S:18S rRNA has been used as a reliable indicator of rRNA quality, with an apparent ratio of 2.0 being indicative of high quality, intact total RNA. As shown in Figure 8, the 18S and 28S ribosomal RNA bands are sharp and clearly visible and no significant smearing is evident. Cursory assessment also suggests that 28S:18S rRNA exists at a ratio of ~2:1. As a further step to assess RNA integrity, all samples were subjected to RT-

PCR for β-actin and GAPDH (Figure 8). Moreover this assessment allowed for assessing quality of sample RNA concentration, as both GAPDH and β-actin are constitutively expressed.

3.5 Integrin β1 Expression

Expression of β1 integrin relative to SMC phenotype was assessed in cultured canine airway myocytes by measuring β1 integrin RNA and protein levels using RT-PCR and Western blotting (Figures 9 and 10). RT-PCR was performed on cDNA prepared from 2μg total RNA that was isolated from serum-fed, sub-confluent, confluent serum-fed, and 7-day serum-deprived CASMC cultures. We designed primer sets specific for canine integrin β1 (Table 5). Transcript for β1 integrin was detected all CASMC cultures (Figure 9), with the reaction product corresponding to an expected product of 159 base pairs. We also performed RT-PCR using RNA isolated from intact canine and human bronchial smooth muscle tissue. Consistent with cell cultures, abundant PCR product corresponding to β1 integrin was obtained in tissues.

By Western blot, two broad bands of about 130KDa and 110KDa that correspond to the mature and immature integrin β1 forms respectively were seen consistently in CASMC cultures (Figure 10). The mature form protein is altered by post-translational modifications, including glycosylation and acetylation. To detect any differences in β1 integrin expression in serum-fed and serum-deprived cell cultures, densitometry data from immunoblots for 3 independent cell cultures were analyzed using one-way ANOVA analysis. The densitometry (OD) values obtained per blot were normalized to the serum-

fed, 70% confluent CASMC sample, which was thus set to arbitrary unit of one. Graphical representation of the results for 3-4 experiments employing anti-β1 integrin antibody are shown in Figure 10. Based on the analysis, there was no statistical difference between serum-fed and serum-deprived CASMC cultures.

To compare the co-distribution of integrin β1 with smMHC, and thus assess coincidence of β1 integrin expression with contractile phenotype myocytes, CASMCs were dual-labeled with antibodies against integrin β1 and smMHC and staining was assessed by confocal microscopy (Figure 11). In proliferating myocyte cultures, integrin β1 showed a punctate, evenly distributed pattern, widely distributed over the surface of all myocytes. As previously noted, virtually all canine tracheal myocytes in serum-fed, subconfluent cultures exhibited negligible immunoreactivity for smMHC. In 7-day serum-deprived canine myocyte cultures, integrin β1 was expressed in abundance and was widely distributed in all cells, including elongated SMC-positive cells that demonstrated strikingly positive immunoreactivity for smMHC. A staining signal for β1 integrin was also evident in flat non-contractile phenotype myocytes but labeling intensity was clearly less than that for elongated, contractile cells.

To pinpoint interactions between integrin β1and its ligands, laminin and fibronectin, CASMC cultures were also double immunostained for integrin β1 and laminin or fibronectin and examined by confocal microscopy (Figure 11). In contractile myocyte cultures, integrin β1 and laminin co-colocalize in laminin-positive elongated cells.

We also assessed the distribution of β1 integrin in intact canine and human bronchial smooth muscle tissue by immunohistochemical staining (Figures 12 and 34). As shown in Figure 12, diffuse staining of β1 integrin was apparent in canine bronchi smooth muscle. A similar pattern of staining was also evident in human bronchi smooth muscle (Figure 34). Collectively, these data reveal β1 integrin is expressed in a phenotype-independent fashion, though processing of the polypeptide to its mature form seems more readily apparent in contractile myocytes (Table 6).

3.6 Integrin α3 Expression

To determine whether serum-fed and serum-deprived CASMC culture expressed integrin α3, we performed RT-PCR using specific primer sets (Table 5) to assess the presence of α3 mRNA isoforms. The primers used distinguished A and B isoforms, which appeared as 660 and 516 bp products respectively (Figure 13). Serum-fed and serum-deprived CASMC cultures predominantly expressed the α3B transcript, though low abundance of the α3A transcript was detected in some serum-deprived samples (see Lane 5, Figure 13). Canine bronchial smooth muscle tissue expressed both the α3B and α3A isoforms, with the α3B band being more intense. Of note, using human bronchial tissue, ASM appeared to express only the α3A isoform. It is not clear why a species-specific difference in α3 isoform expression might exist.

To confirm the finding that α3 integrin was expressed in serum-fed and serum-deprived CASMC cultures, Western blot analysis was employed to examine the abundance of α3 protein. Samples were fractionated by SDS-PAGE, transferred onto nitrocellulose and

probed for anti-integrin α3 with a polyclonal antibody (Table 2). Figure 14, shows that two bands of ~ 140KDa and ~115KDa were detected in all cultures; these likely correspond to nascent and post-translation modified isoforms of the α3B and α3A integrins. We used one-way ANOVA to compare integrin α3 protein levels in different culture condition; however, no statistical difference was observed between canine myocytes at any stage of primary culture.

Double immunostaining analysis was carried out to assess the relative distribution of integrin α3 and smMHC in cultured CASMCs. Integrin α3, smMHC and merged immunofluorescence images are shown in Figure 15. Integrin α3 showed a fibrillar pattern that suggested an organizational association with stress fibres. A punctate staining pattern throughout all other parts of the cell was also apparent. Integrin α3 was clearly evident in contractile myocyte cultures and in particular was co-expressed in elongated smMHC-positive myocytes suggesting that α3 integrin may be a more-or-less unique discriminator of the contractile phenotype. The distribution of α3 integrin was further determined by immunohistochemical staining of intact canine and human bronchial smooth muscle tissues using a monoclonal antibody specific for α3 integrin (Figures 16 & 33). Staining of canine bronchial smooth muscle was readily visible, and notably, the smooth muscle of small vessel in submucosal area were also stained with for α3 integrin. Human bronchial smooth muscle showed intense immunoreactivity for integrin α3 that was similar to that seen for canine tissues. These data indicate α3 integrin is expressed in abundance by contractile ASM both in vitro and vivo. Moreover, RT-PCR analyses

suggest that species-and/or phenotype-specific expression of α3 isoforms may exist (Figure 13) (Table 6).

3.7 Integrin α5 Expression

Estimation of the presence of transcript levels for α5 integrin was performed using RT-PCR. Specific primers for canine α5 integrin were designed as described in Materials and Method. As illustrated in Figure 17, a product consistent in size predicted for the integrin α5 subunit (166bp) was readily evident in canine airway myocytes in serum-fed and serum-deprived conditions. Integrin α5 was also detected easily in intact canine bronchial smooth muscle, however the primers used detected only low levels of integrin α5 mRNA in human bronchial smooth muscle. The identity of a PCR product of ~700bp in size that was seen for most reactions is not known.

Western blot analysis was employed to examine integrin α5 protein expression (Figure 18). Samples were fractionated by SDS-PAGE, transferred onto nitrocellulose and probed with an anti-integrin α5 polyclonal antibody. A band corresponding to ~145KDa was detected in lysates from 70% and 100% confluent, and 7 day serum-deprived SAMC cultures. One-way ANOVA was used to compare integrin α5 levels measured by scanning densitometry. Western blot data was normalized and is representative of 3-4 independent experiments. No statistical difference was observed between serum-fed and 7 day serum-deprived canine myocytes.

Double immunostaining was carried out to compare the relative distribution of integrin α5 and smMHC in individual myocytes in primary culture. Integrin α5, smMHC and merged immunofluorescence images are shown in Figure 11 for proliferating and contractile CASMC cultures. Integrin α5 immunostaining was generally distributed in a punctate manner throughout the cell in subconfluent, serum-fed cultures. Integrin α5 was also widely distributed in serum-deprived cultures that contained smMHC-positive myocytes. However, integrin α5 was not expressed in a phenotype-dependent-fashion, as the protein was equally evident in both contractile and non-contractile phenotype myocytes. We also performed double immunostaining to compare the distribution of α5 with its ligands, laminin or fibronectin (Figure 11). Dual-labeling with antibodies against integrin α5β1 and laminin or fibronectin was used. In contractile myocyte cultures, integrin α5β1 was evident in laminin-positive elongated cells and co-localize with fibronectin in both elongated (contractile) and non-contractile myocytes. As integrin α5 protein was clearly expressed by canine tracheal myocytes, we assessed the protein's expression in intact canine and human bronchial smooth muscle. Immunohistochemistry revealed that both canine and human bronchial smooth muscle displayed intensive integrin α5 staining (Figures 19 and 33)(Table 6).

3.8 Integrin α6 Expression

The distribution of laminin-specific α6 integrin isoforms in serum-fed and serum-deprived canine airway smooth muscle cells was assessed by RT-PCR. The reaction was also performed using cDNA from intact canine and human bronchial smooth muscle tissue (Figure 20). A 540-bp fragment corresponding to the α6A isoform and a 410-bp

fragment corresponding to the α6B isoform were detected in serum-fed CASMC cultures, though the α6B product appeared to be in significantly lower abundance. In contrast, in serum-deprived CASMC in which elongate contractile myocytes accumulate, α6A was expressed exclusively. Canine bronchial tissue expressed both A and B isoforms, and an additional band visible at 520bp; this product is likely a hybrid resulting from re-annealing single strands of the 540-and 410-bp products.

To compare the co-distribution of α6A and smMHC, double immunostaining analysis was performed. Figure 21 shows the results of dual-labeled immunostaining with antibodies against integrin α6A and smMHC in proliferating and contractile CASMC cultures. In all myocytes from serum-fed cultures, integrin α6A displayed a punctate distribution pattern. However, clear and dramatically increased expression of α6A exclusively on smMHC positive, elongate contractile phenotype CASMC was evident in serum-deprived primary cultures. To further characterize the distribution of α6B subunit, immunostaining analysis was performed in proliferating and contractile CASMCs (Figure 22). The intensity of staining for α6B was similar to that of negative controls in all cultures indicating that little or no α6B immunostaining was detected. This was consistent with the results obtained by RT-PCR (Figure 20). Unfortunately, despite clear evidence for α6A mRNA RT-PCR and α6A protein by immunocytochemistry, we were unable to detect α6A protein by Western blotting in reducing or non-reducing conditions. Thus it appears the antibodies we employed (monoclonal GoH3, and polyclonal antibodies) were not suitable for immunoblot analyses.

To characterize α6 subunit distribution in ASM in vivo, we analyzed α6 integrin expression in canine and human bronchial tissues using immunohistochemistry with polyclonal antibodies for either α6A or α6B. We detected robust labeling for α6A in the smooth muscle layer of both canine and human bronchi (Figure 23 and 33). However no positive staining for α6B in smooth muscle cells in either tissue was observed (Figures 24 & 33). These results confirm that contractile phenotype ASM cells express almost exclusively α6A integrin, and that the proteoglycan may be a reliable marker of ASM contractile phenotype (Table 6).

3.9 Integrin α7 Expression

The laminin-specific α7 integrin has been described as a skeletal and cardiac muscle-specific integrin (Zoiber et al, 1993). However, other studies using RT-PCR or in situ hybridization have suggested α7 is also expressed in other non-striated muscle tissues and cell lines (Yao et al 1997). As shown in Figure 25, RT-PCR was performed on cDNA made from RNA extracted from serum-fed CASMC and serum-deprived CASMC cultures, and from canine and human bronchial smooth muscle tissue. A single band corresponding in size to the 170bp product predicted for α7B subunit mRNA was easily detected in all cultured cell and tissue samples assessed. Conversely, no evidence for integrin α7A subunit mRNA was detected using primers designed to identify the gene product.

To confirm RT-PCR analyses the localization of α7A and α7B integrin proteins in cultured SMC was assessed by confocal immunocytochemistry (Figures 26 & 27).

Consistent with the results of PCR analysis, little or no immunostaining of α7A was detected in either serum-fed or serum-deprived CASMC cultures. To compare the co-distribution of α7B and smMHC, double immunostaining analysis was performed for proliferating and contractile CASMC cultures (Figure 27). Dual labeling with antibodies against integrin α7B and smMHC revealed that integrin α7B was organized in a punctate distribution pattern on almost all cells. Of note, in serum-deprived cultures α7B was prominently and exclusively expressed on smMHC-positive elongated myocytes. Unfortunately, neither the monoclonal (Cy8) nor the polyclonal antibodies for α7 were suitable for Western blot analyses. Thus, despite clear evidence of α7B expression by RT-PCR and immunocytochemistry, we were unable to obtain quantitative data concerning α7A integrin abundance in different culture conditions or in tissues. The expression and distribution of the two α7 integrin subunit isoforms was further characterized in canine and human bronchial tissue cryosections using indirect immunofluorescence using polyclonal antibodies (Table 2) directed against the cytoplasmic domains of α7A and α7B (Figures 28, 29 & 34). In a manner similar to staining in serum-deprived cultures, α7B antibodies generated intense staining in contractile smooth muscle cells in the muscle layer encircling the bronchus for both canine and human tissues (Figures 29 & 34). Moreover, labeling for the α7A isoform was not detected, thus confirming that contractile phenotype ASM in vivo and in vitro express the α7B laminin-binding subunit exclusively (Table 6).

3.10 CD151 Expression

CD151 expression was characterized by Western blot. In Figure 30, a band corresponding to ~34kDa was detected on 70%, 100% confluent and 7-day serum-deprived CSAMC cultures. One-way ANOVA was used to compare CD151 levels; normalized content from 3-4 independent experiments revealed that total CD151 was highest in 100% serum-fed cultures, a value that was significantly different from that for 7-day serum deprived cultures. ($p<0.05$). The relative cellular distribution of CD151 and smMHC was also investigated by indirect immunofluorescence and confocal microscopy in proliferating and contractile CASMCs (Figure 31). Labeling with antibodies against CD151 and smMHC revealed that in serum-free cultures, CD151 was prominently and exclusively expressed on smMHC-positive (contractile phenotype), elongated myocytes. To gain insight into the localization of CD151 protein in tissue, intact canine bronchi and human bronchi were also processed for CD151 immunohistochemistry (Figures 32 & 36). Significant CD151 immunoreactivity was observed in the smooth muscle layer of both canine and human bronchi. Collectively these observations suggest that CD151, a tetraspanin scaffolding protein that associates specifically with laminin-binding integrin clusters, is a unique marker for contractile phenotype ASM cells (Table 6).

Table 6. Summary of results.

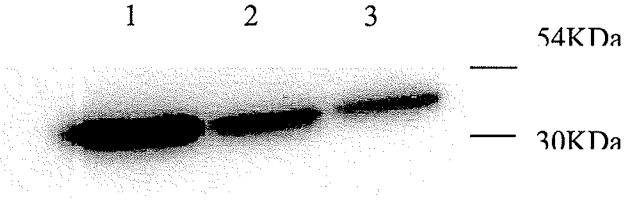
Integrin		Protein Expression			mRNA
		ICC	IHC	IB	RT-PCR
α3 integrin (A & B isoforms)	SF CASMCs	+/-		+	+B -A
	SD CASMCs	EM+ NEM+/-		+	+B +/-A
	Canine Bronchi		+		+B +/-A
	Human Bronchi		+		+B -A
α5 integrin	SF CASMCs	+		+	+
	SD CASMCs	EM+ NEM+		+	+
	Canine Bronchi		+		+
	Human Bronchi		+		+/-
α6A integrin	SF CASMCs	+/-			+
	SD CASMCs	EM+ NEM+/-			+
	Canine Bronchi		+		+/-
	Human Bronchi		+		+/-
α6B integrin	SF CASMCs	-			+/-
	SD CASMCs	EM+/- NEM-			-
	Canine Bronchi		–		+
	Human Bronchi		–		+
α7A integrin	SF CASMCs	–			–
	SD CASMCs	EM– NEM-			–
	Canine Bronchi		–		–
	Human Bronchi		–		–
α7B integrin	SF CASMCs	+/-			+
	SD CASMCs	EM+ NEM-			+
	Canine Bronchi		+		+
	Human Bronchi		+		+
β1 integrin	SF CASMCs	+		+	+
	SD CASMCs	EM+ NEM+/-		+	+
	Canine Bronchi		+		+
	Human Bronchi		+		+
CD151	SF CASMCs	+		+	
	SD CASMCs	EM + NEM+/-		+	
	Canine Bronchi		+		
	Human Bronchi		+		

Abbreviations: **SF CASMCs** = Serum-Fed CASMCs; **SD CASMCs** = Serum-Deprived CASMCs; **EM** = Elongated, contractile myocytes; **NEM** = Non-Elongated, Non-contractile myocytes; **ICC** = Immunocytochemistry; **IHC**=Immunohistochemistry; **IB**=Immunoblotting; + = positive expression/labeling; - = no expression/labeling detected; ▨ = no data.

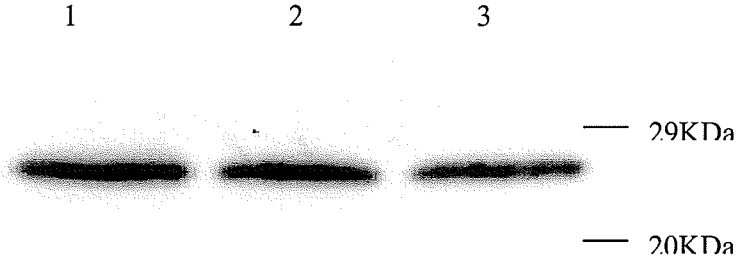
Figure 3. Comparison of abundance of smooth muscle-specific protein markers in serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures. Western blots showing **(A)** smMHC (200-204 kDa), **(B)** sm-α-actin (43 kDa), **(C)** SM22 (22 kDa), and **(D)** calponin (34 kDa). Total protein loaded to each lane was 20μg. 5 % SDS-PAGE gel was used to separate smMHC, 10% SDS-PAGE gel was used for all other markers. *(Lane 1)* 7-day serum-deprived CASMC; *(Lane 2)* 100% confluent, serum-fed CASMC culture; *(Lane 3)* 70% confluent serum-fed CASMC culture. Positions of molecular mass standards (kDa) are indicated.

A:

B:



C:



D:

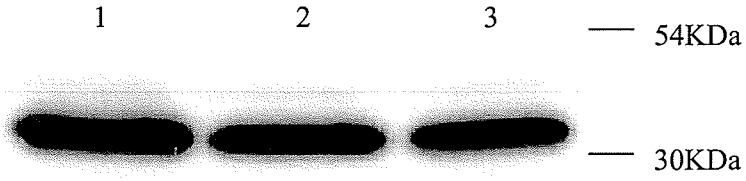
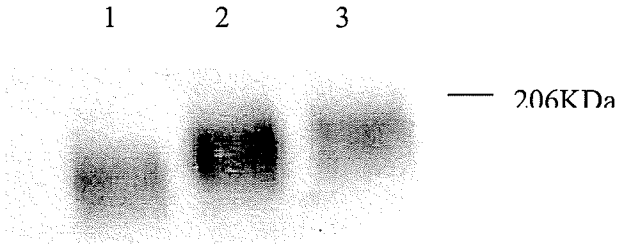


Figure 4. Western blotting analysis for laminin on total lysates of canine airway smooth muscle cell (CASMC) cultures. Samples were analyzed on a 5% polyacrylamide gel in reducing conditions. **(A)** The representative blot shown was stained with a polyclonal antibody directed against laminin (Table2). The total protein loaded in each lane was 10μg. *Lane 1*, 7-day serum-deprived CASMC culture; *Lane 2*, 100% confluent serum-fed CASMC culture; *Lane 3*, 70% confluent serum-fed CASMC culture. Positions of molecular mass standards (kDa) are indicated. **(B)** Densitometry data from 3 independent experiments were normalized to the serum-fed, 70% confluent sample, and then analyzed by one-way ANOVA. Data are expressed as mean ± SEM.

A:


1
2
3
206KDa

B:

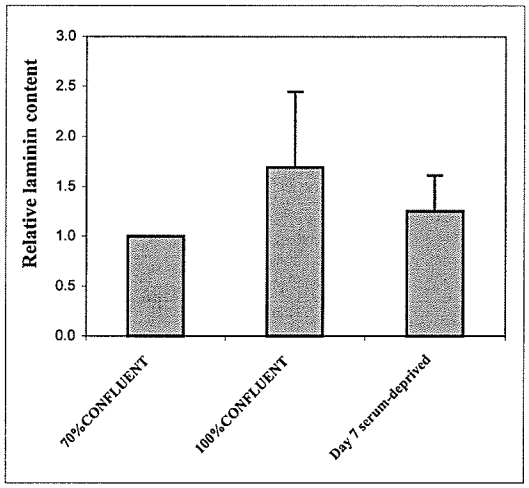
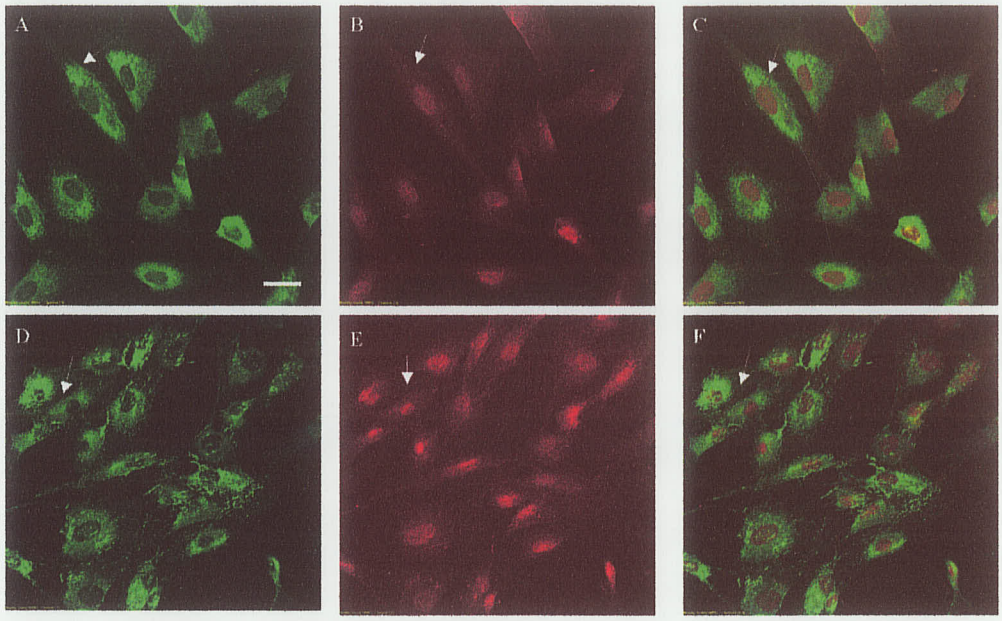

Relative laminin content
3.0
2.5
2.0
1.5
1.0
0.5
0.0
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

Figure 5. Confocal immunocytochemical analysis of smMHC, laminin and fibronectin in proliferating and contractile CASMCs.

70% confluent serum-fed CASMCs **(A-F)** were stained for smMHC **(A, D)** and laminin **(B)**, or fibronectin **(E)**. Merged images of the relative distribution of smMHC with laminin **(C)** and fibronectin **(F)** are also shown. Nuclei were counterstained with Toto-3. Arrows designate single proliferating myocytes in each panel.

7 days serum-deprived CASMCs **(G-L)** were stained for smMHC **(G, J)** and laminin **(H)**, or fibronectin **(K)**. Merged images showing the relative distribution of smMHC with laminin **(I)** and fibronectin **(L)** are also shown. Nuclei were counterstained with Toto-3. Arrows donate single elongated myocytes in each panel. Bar, 50μm


A
B
C
D
E
F

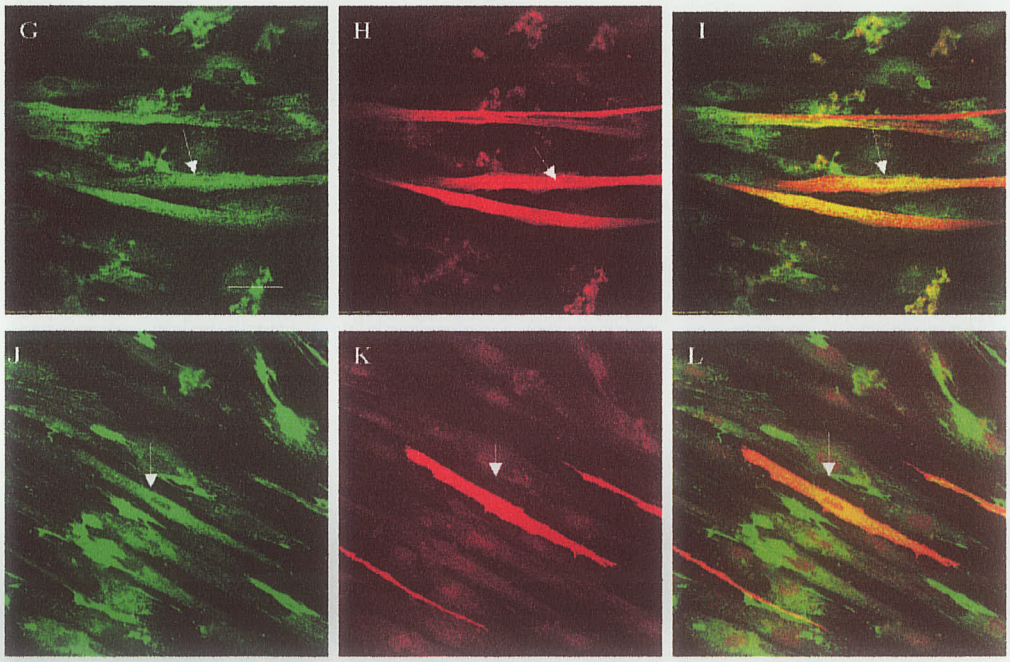

G
H
I
J
K
L

Figure 6. Confocal immunohistochemical analysis of laminin and fibronectin in cryosections of canine bronchial smooth muscle tissue. Tissue sections from 3-4th generation canine bronchi were labeled with a polyclonal antibody against laminin. Nuclear staining was performed with Toto-3. Bar in panel A, 50μm; Bar in panel B, 120μm.

(A) Submucosal area. Vascular smooth muscle (thin arrows) expressed laminin. Thick arrow designates basement membrane.

(B) Bronchial smooth muscle (arrowhead) reacted with anti-laminin polyclonal antibody.

(C) Negative control. Epi: Epithelium; SubM: Submucosa; SM: Smooth muscle; Av: Adventitia.

(D) Bronchial smooth muscle (arrowhead) reacted with anti-fibronectin polyclonal antibody.

(E) Negative control. Epi: Epithelium; SubM: Submucosa; SM: Smooth muscle; Av: Adventitia.

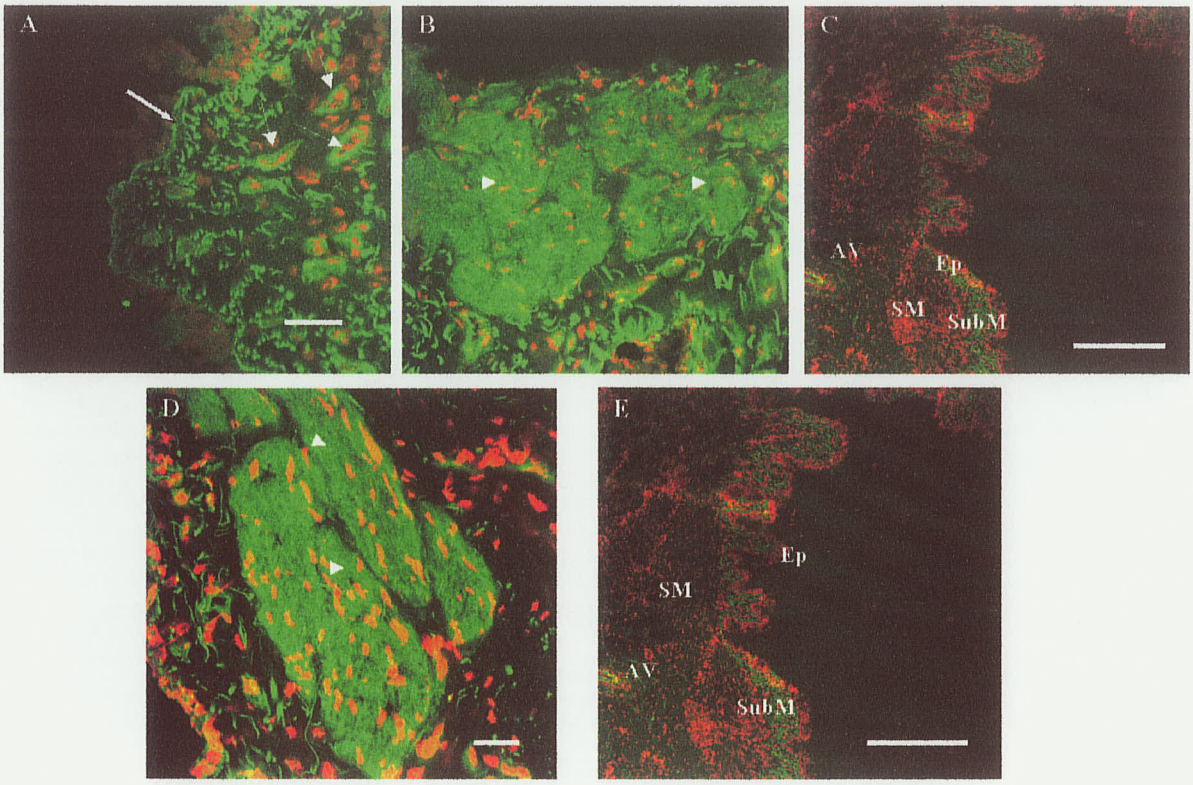

A
B
C
AV
Ep
SM
SubM
D
E
Ep
SM
AV
SubM

Figure 7. Western blot analysis for fibronectin in cultured serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures.

(A) The gel was 5% polyacrylamide. Each lane was loaded with equal amounts of total protein (10 μg). The blot was stained with a polyclonal antibody against fibronectin. *Lane 1*, 7-day serum-deprived CASM culture; *Lane 2*, 100% confluent serum-fed CASM culture; *Lane 3*, 70% confluent serum-fed CASM culture. Positions of molecular mass standards (kDa) are indicated. One band of about 220 kDa was identified. Positions of molecular weight marker are indicated. Three independent experiments were completed. (B) Densitometry data from 3 independent experiments were normalized and analyzed by one-way ANOVA. Data were expressed as mean ± SEM.

A:


1
2
3
200KDa

B:

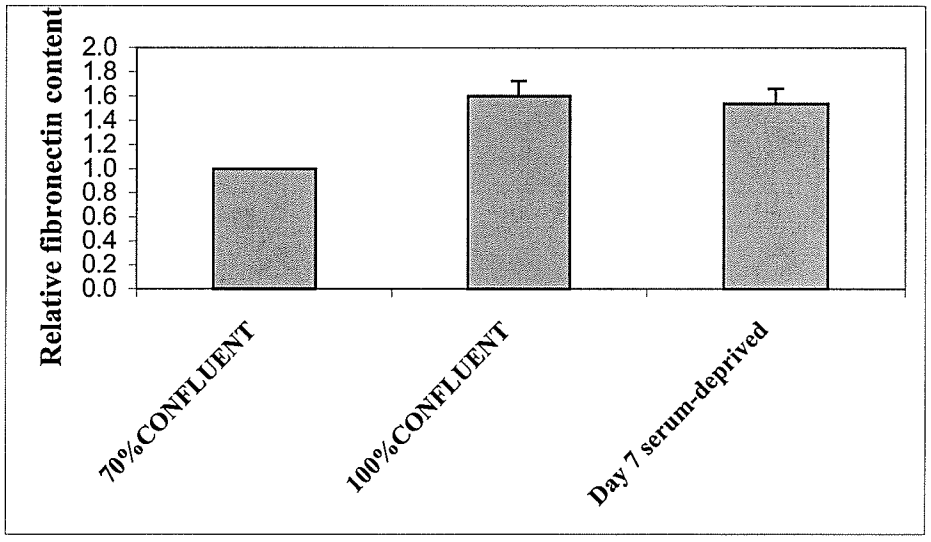

Relative fibronectin content
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

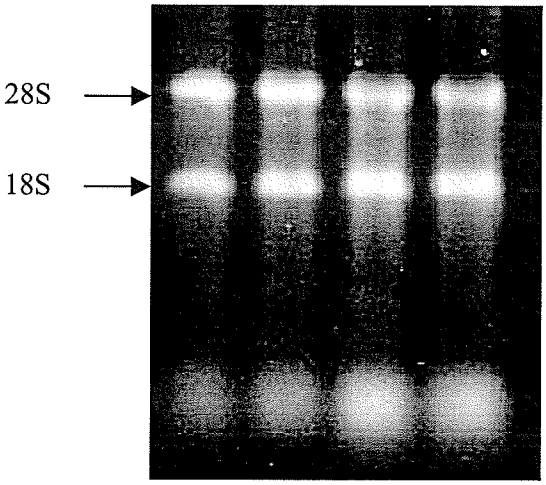
Figure 8. RT-PCR analysis of β-actin and GAPDH mRNA expression on serum-fed and serum-deprived CASMC cultures. We estimated the expression level of GAPDH and β-actin in the cDNA prepared by RT that was used in all studies. A typical example is shown. RT-PCR amplification was performed on 2μg total RNA extracted from serum-fed, 70% confluent canine airway smooth muscle cell (CASMC) cultures *(Lanes 1and 2)*, 7-day serum-deprived canine airway smooth muscle cell (CASMC) cultures *(Lanes 3 and 4)* as described in Materials and Methods.

(A) Analysis of RNA quality and integrity. Representative gels showing the pattern of total RNA (1μg) from serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures after electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. 28s and 18s rRNA bands were visualized by ultraviolet light.

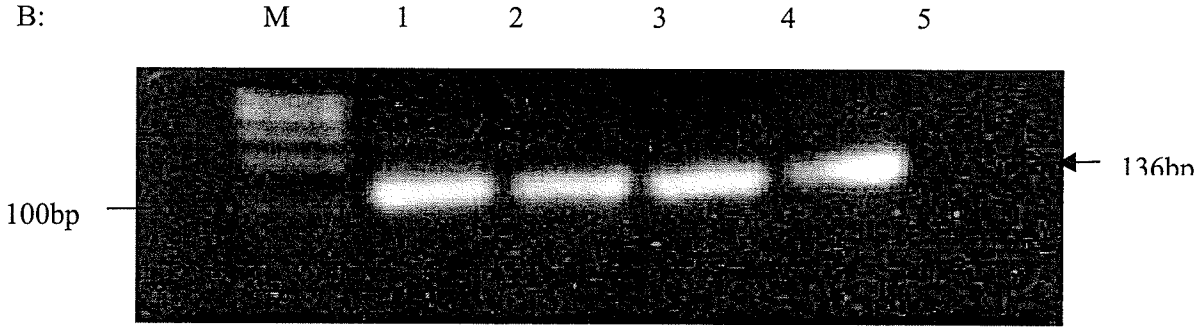
(B) The position of the 136bp band representing GADPH mRNA is indicated by DNA molecular marker shown in *Lane M.* Negative control shown in *Lane 5*.

(C) The position of the 294bp band representing β-actin mRNA, as indicated by DNA molecular marker shown in *lane M.*

A:



B:



C:

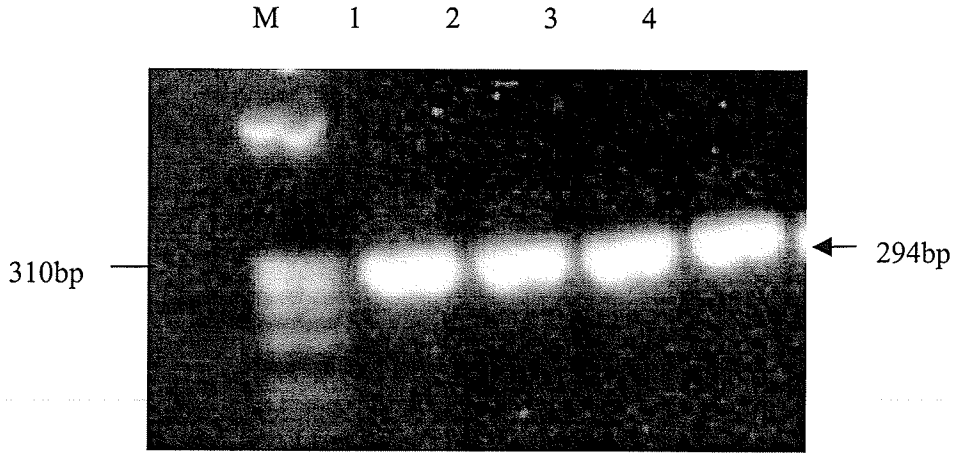


Figure 9. Comparison of β1 integrin mRNA abundance in serum-fed and serum-deprived canine airway smooth muscle cell cultures. Representative gel showing results of PCR amplification (see primers, Table 4) was performed on cDNA prepared using 2 μg total RNA extracted from serum-fed, 70% confluent canine airway smooth muscle cell (CASMC) cultures *(Lanes 1 and 2)*, 7-day serum-deprived canine airway smooth muscle cell (CASMC) cultures *(Lanes 3 and 4)*, intact canine bronchi tissue *(Lane 5)*, intact human bronchi tissue *(Lane 6)* as described in Materials and Methods. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The reaction product corresponding to integrin β1 appears as a band of 159 base pair (bp). A negative control (no RNA) is shown in *Lane 7*. DNA molecular markers are shown in *Lane M*. A total of 3 individual canine cell cultures, and canine and human bronchial smooth muscle tissue samples were analyzed.

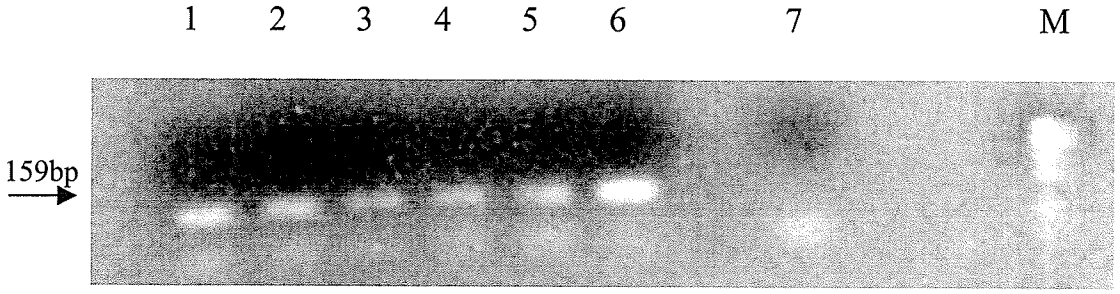
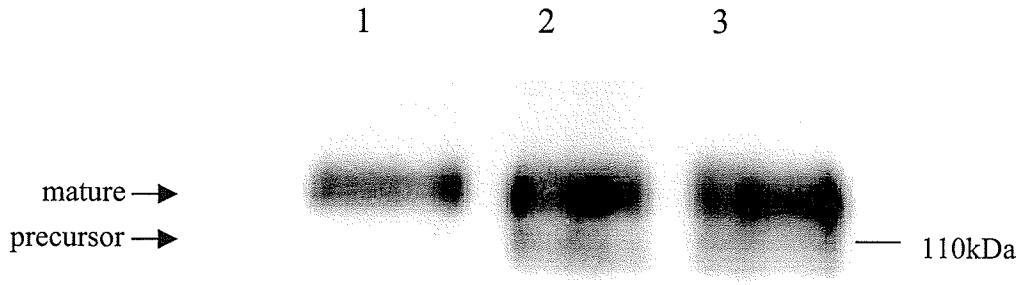


Figure 10. Western blot analysis of β1 integrin expression in serum-fed and serum deprived canine airway smooth muscle cell (CASM) cultures.

(A) Proteins were separated by 7.5% SDS polyacrylamide gel under reducing condition. The blot was stained with monoclonal antibody directed against β1 (clone JB1A) (Table 2) followed by incubation with HRP-conjugated goat anti-mouse IgG. Protein content of each lane was 20μg. *(Lane 1)* 7 days serum-deprived CASMC; *(Lane 2)* 100% confluent serum-fed CASMC; *(Lane 3)* 70% confluent serum-fed CASMC. Positions of molecular mass standards (kDa) are indicated. Two bands are seen corresponding to a precursor isoform (115 kDa) and the mature form (130 kDa) of the subunit in lane 2 and lane 3. Only the mature form of β1 integrin (130 kDa) was detected in 7-day serum deprived cells (lane 1). The result was representative of three independent experiments in three different cell cultures.

(B) Densitometry data from 3 independent experiments were normalized and analyzed by one-way ANOVA. Data were expressed as mean ± SEM.

A:


1
2
3
mature
precursor
110kDa

B:

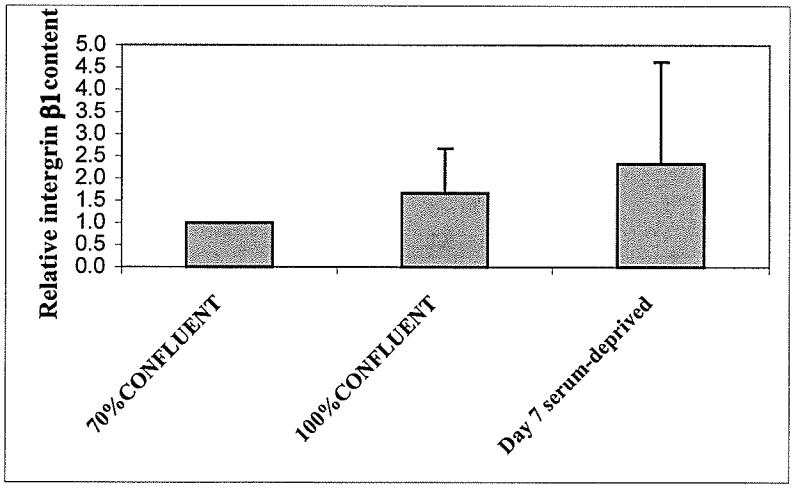

Relative intergrin β1content
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

Figure 11. Confocal immunocytochemical analysis of integrin β1 and α5 in proliferating and contractile CASMCs. An antibody that detects α5β1 heterodimers was used (see Table 2).

70% confluent serum-fed CASMCs were fixed, permeablized and stained for integrin α5β1 (red) **(A, D, G)** and smMHC (green)**(B)**, laminin (green)**(E)**, or fibronectin **(H)**. Merged images showing the relative distribution of integrin β1 with smMHC **(C)** laminin **(F),** and fibronectin **(I)** are also shown. Yellow labeling indicates colocalization. Nuclei were counterstained with Toto-3. Arrows indicate single proliferating myocytes in each panel. Bar = 50μm

7 days serum-deprived CASMCs were fixed, permeablized and stained for integrin α5β1 **(A', D', G')** and smMHC **(B')**, or laminin **(E')**, or fibronectin **(H')**. Merged images showing the relative distribution of integrin β1 with smMHC **(C')**, laminin **(F')** and fibronectin **(I')** are also shown. Nuclei were counterstained with Toto-3. Arrows indicate single elongated myocytes in each panel. Bar, 50μm.

Figure 11, A-I.

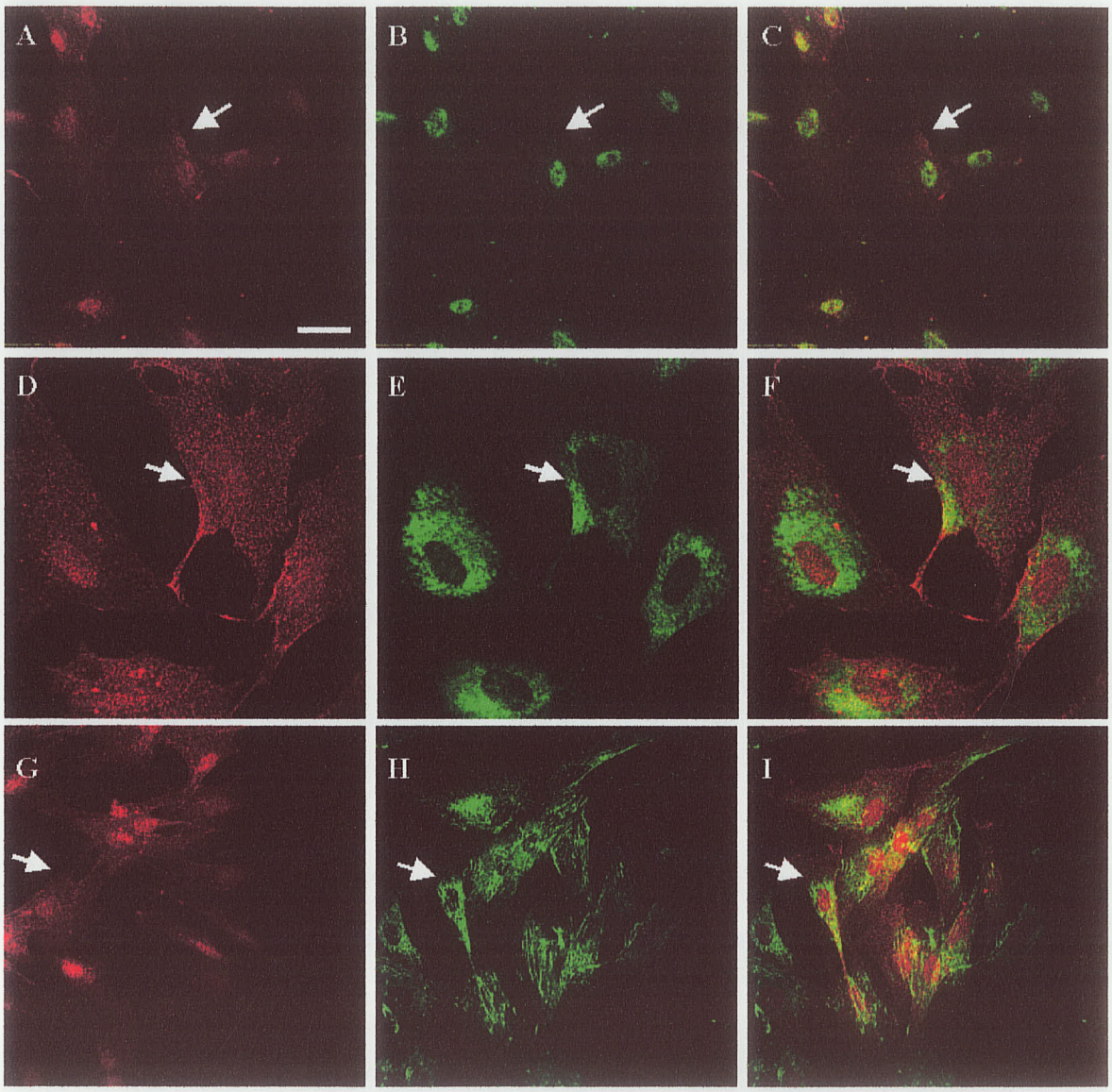


Figure 11, A'-I'.

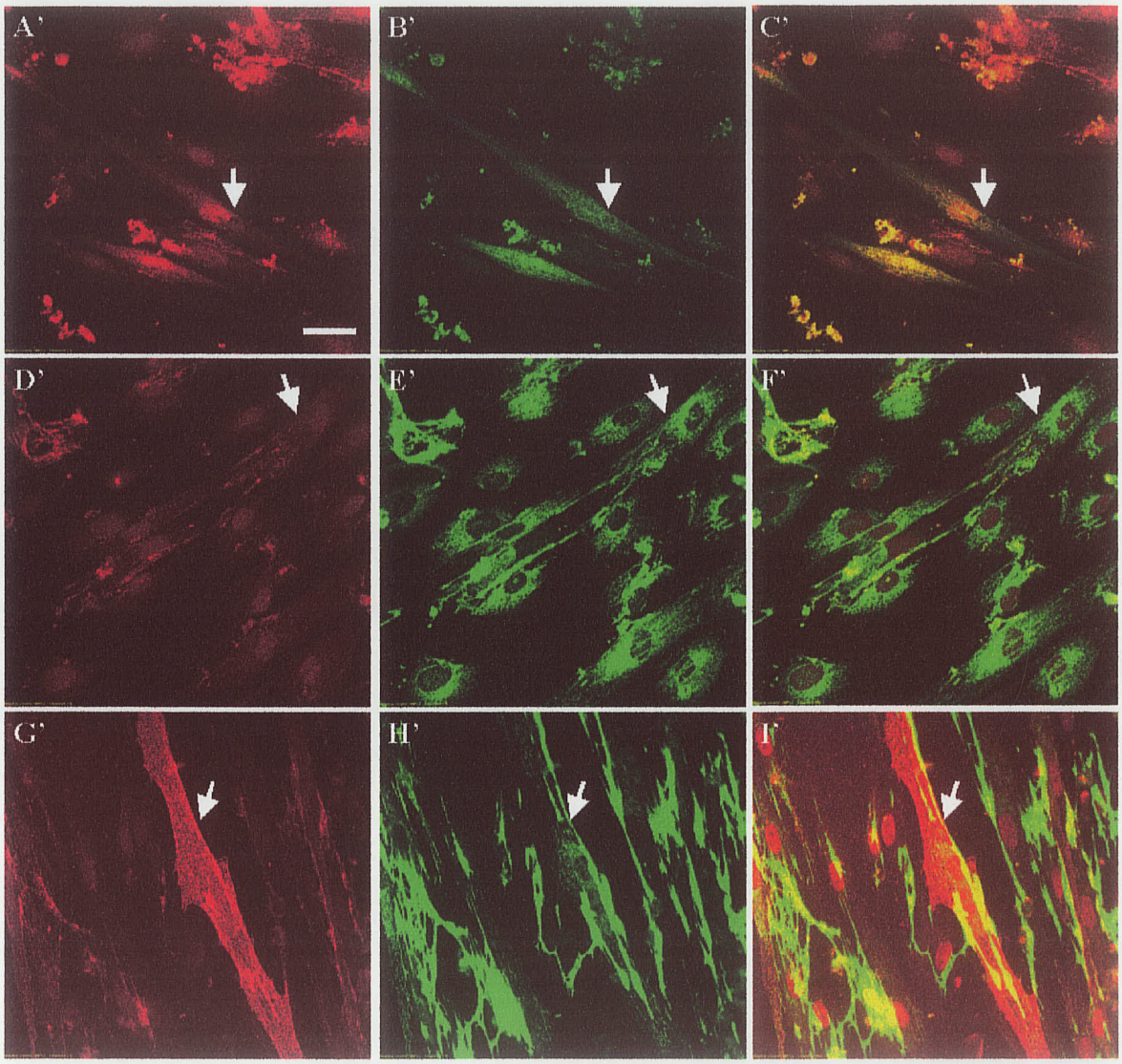


Figure 12. Confocal immunohistochemical analysis of β1 integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeablized and immunostained with a mouse monoclonal antibody against β1 integrin (Table 2) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with Toto-3 (red). Bar, 50μm.

A. β1-integrin staining (green). Bronchial smooth muscle (arrowhead) exhibits general diffuse labeling pattern. Autofluorescence (green) of collagen fibres in submucosa are seen in top right hand corner. Bar = 50μm.

B. Negative control. Note absence of labeling in smooth muscle layer. Autofluorescence (green) of collagen fibres in the submucosa are clearly visible. Bar = 200μm. Epi: Epithelium; SubM: Submucosa; SM: smooth muscle; Av: adventitia.

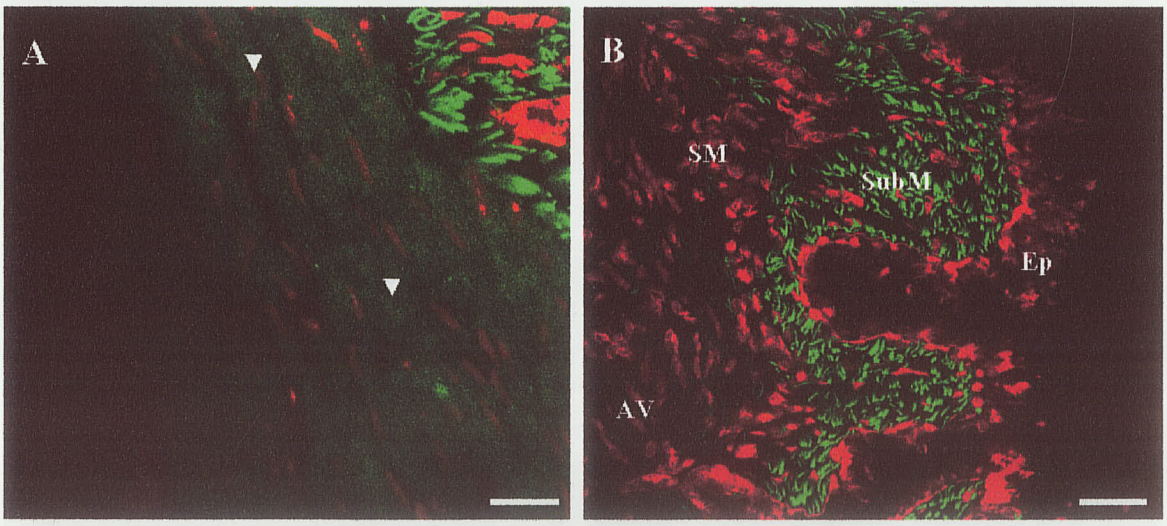


Figure 13. RT-PCR analysis of α3 integrin in serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures, and intact human and canine bronchial smooth muscle. RT-PCR was performed as describe din Materials and Methods using mRNA from intact human bronchi *(Lane 2)*, intact canine bronchi *(Lane 3)*, 7-day serum-deprived canine airway smooth muscle cells *(Lanes 4 and 5)*, and serum-fed, 70% confluent canine airway smooth muscle cells cultures *(Lanes 6 and 7)*.

Serum-fed and serum-deprived canine airway smooth muscle cells expressed only the α3B isoform (516bp). Canine bronchi (lane 3) showed the α3A (660bp) and α3B (516bp) isoforms, while human bronchi (lane 2) displayed only the α3A isoform (660bp). DNA molecular markers are in *Lane M*, and negative control (no RNA) is in *Lane 1*.

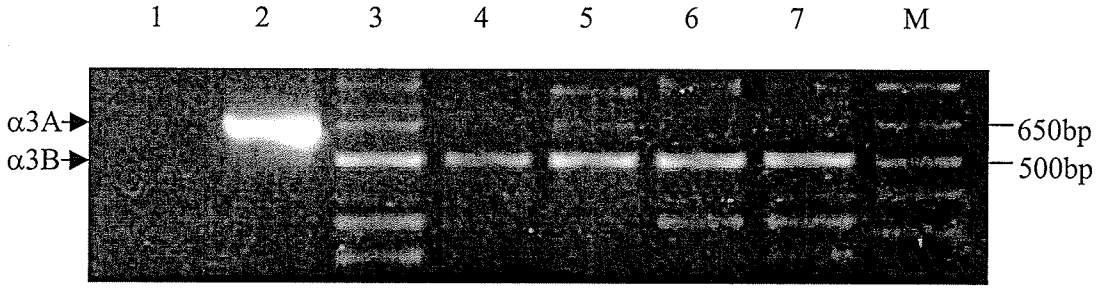
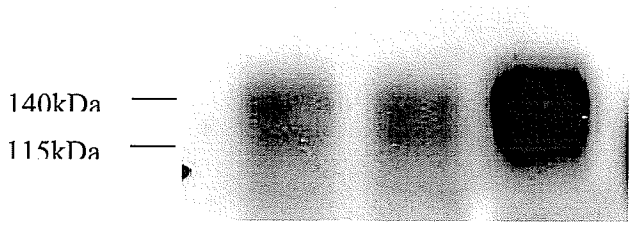


Figure 14. Western blot analysis of integrin α3 expression in serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures.

(A) Proteins were separated by 7.5% SDS polyacrylamide gel under reducing conditions and transferred to nitrocellulose. Protein corresponding to α3 integrin was identified with a polyclonal antibody that detects both A and B isoforms (Table 2). *(Lane 1)* 70% confluent serum-fed CASMC; *(Lane 2)* 100% confluent serum-fed CASMC; *(Lane 3)* 7 days serum-deprived CASMC. Two bands were detected at approximately 115 kDa and 140 kDa. The result shown is representative of three experiments.

(B) Densitometry data from 3 independent experiments were normalized and analyzed by one-way ANOVA. Data were expressed as mean ± SEM. * $p<0.05$ compared to 70% and 100% confluent cultures.

A:


1
2
3
140kDa
115kDa

B:

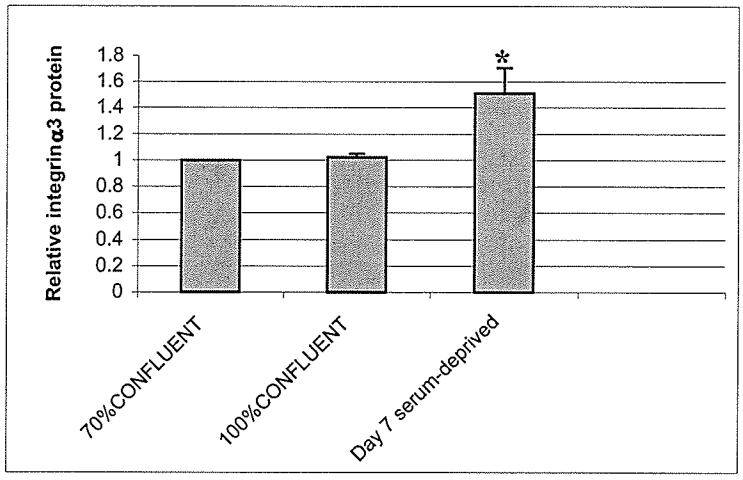

Relative integrinα3 protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

Figure 15. Confocal immunocytochemical analysis of integrin α3 in proliferating and contractile CASMCs. Subconfluent, serum-fed CASMCs were fixed, permeablized and stained for integrin α3 **(A)** and smMHC **(B)**. Nuclei were counterstained with Toto-3 (red). Arrows indicate a representative myocytes in each panel. Seven-day serum-deprived CASMCs were fixed, permeabilized and stained for integrin α3 **(C)** and smMHC **(D)**. A merged image showing the relative distribution of integrin α3 with smMHC **(E)** is also shown. Arrows indicate, an elongate, contractile phenotype myocyte in each panel. Bar, 50μm.

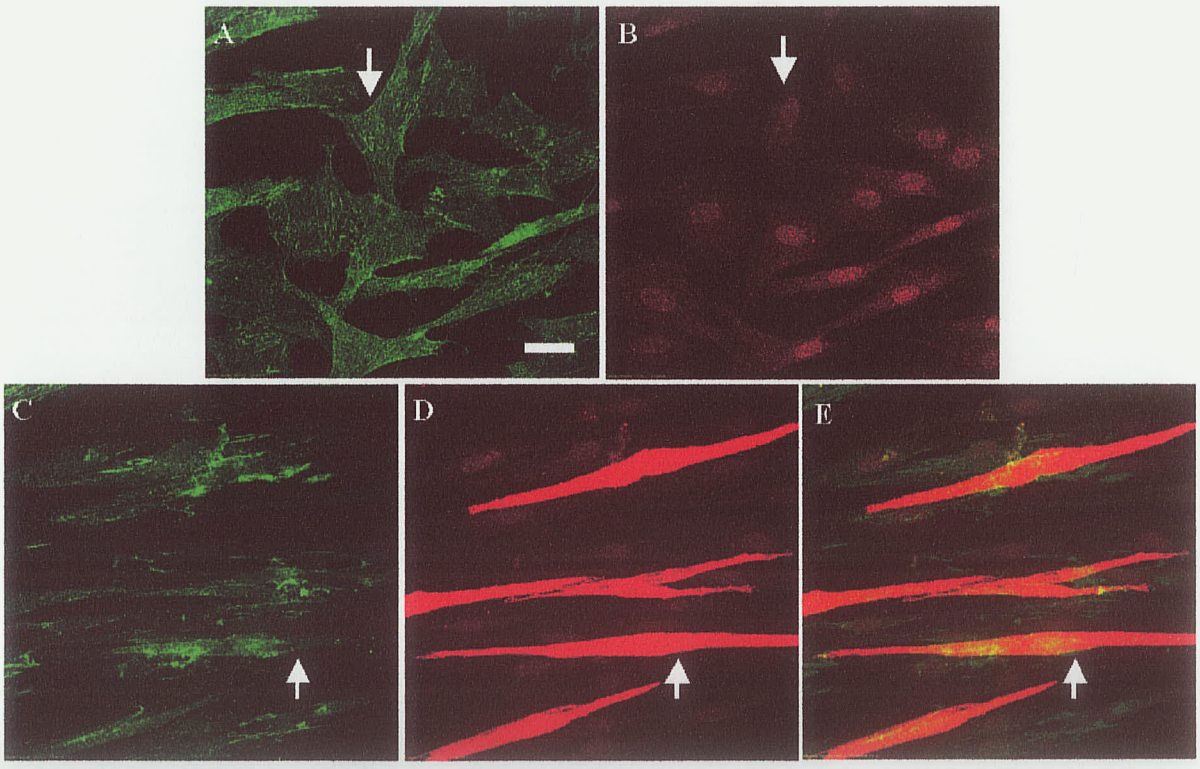


Figure 16. Confocal immunohistochemical analysis of α3 integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed and incubated with a rabbit polyclonal antibody against α3 integrin (Table 1) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with Toto-3 (red). Bar = 50μm.

(A) Submucosa area. Vascular smooth muscle (arrows) expressed α3 integrin.

(B) Bronchial smooth muscle (arrowhead) was positive for anti-α3 integrin polyclonal antibody.

(C) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle; Av: adventitia.

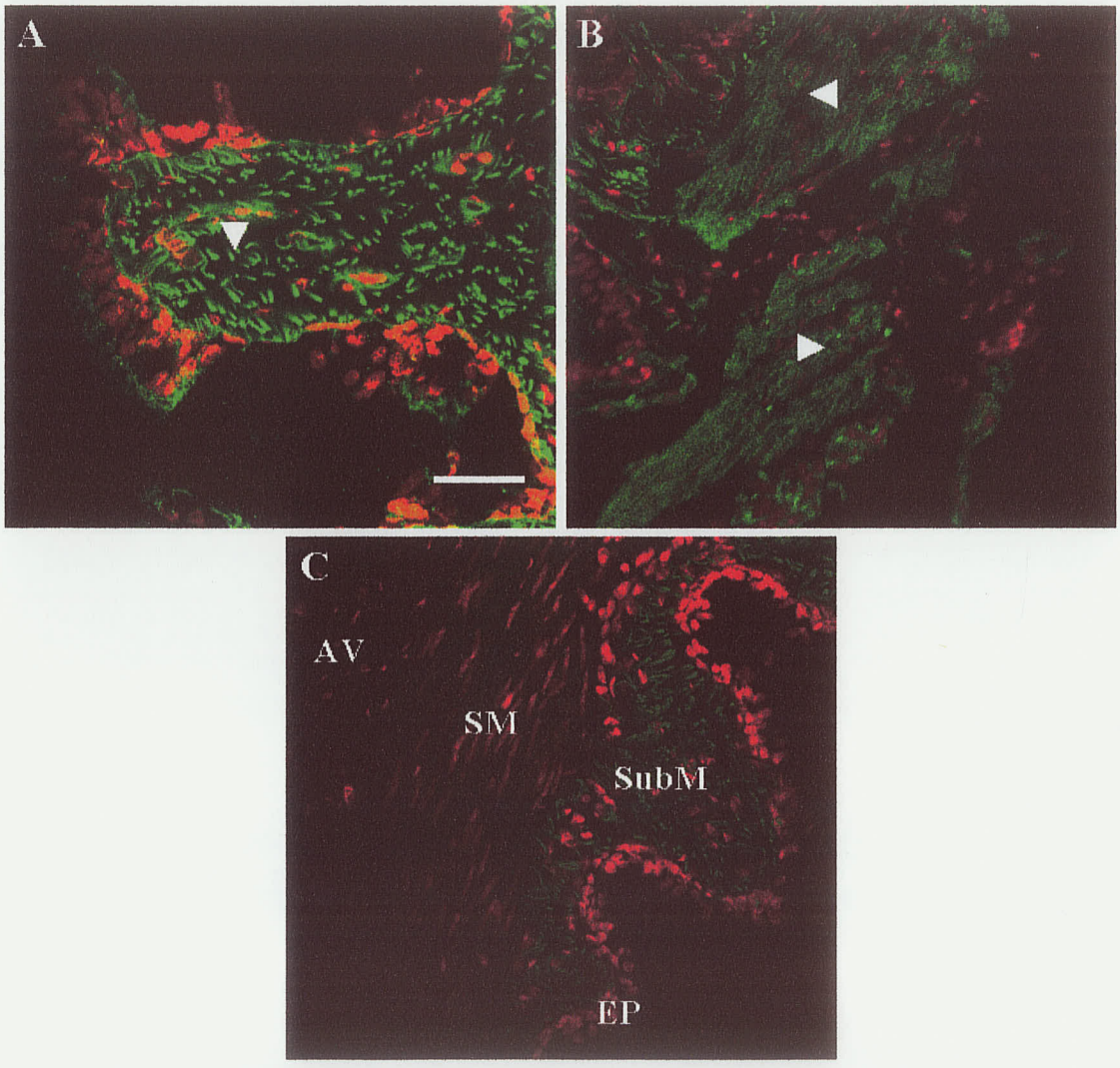

A
B
C
AV
SM
SubM
EP

Figure 17. RT-PCR analysis of α5 integrin mRNA in serum-fed and serum-deprived CASMC cultures, and in human and canine bronchial smooth muscle tissue. PCR (see primers, Table 4) was performed as described in Materials and Methods for total RNA extracted from serum-fed, 70% confluent CASMC cultures *(Lanes 1 and 2)*, 7-day serum-deprived CASMC cultures *(Lanes 3 and 4)*, canine bronchial tissue *(Lane 5)*, and human bronchial tissue *(Lane 6)*. The expected position of the 166bp band representing integrin α5 is indicated by DNA molecular marker shown in Lane M. An additional band of ~700 bp was also detected in intact human bronchial samples.

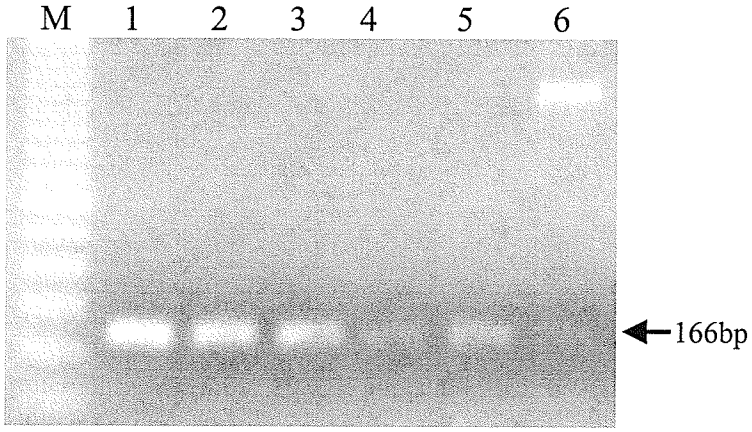
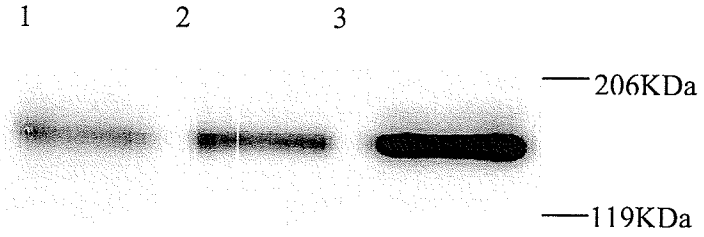


Figure 18. Western blot analysis of α5 integrin protein in serum-fed and serum-deprived canine airway smooth muscle cell (CASMC) cultures. (A) Samples were fractionated by 7.5% SDS-PAGE under reducing condition, transferred to nitrocellulose membranes, and then a rabbit polyclonal antibody against integrin α5 was used for immunodetection. Total protein loaded per lane was 20μg. *(Lane 1)* 7-day serum-deprived CASMC; *(Lane 2)* 100% confluent serum-fed CASMC; *(Lane 3)* 70% confluent serum-fed CASMC. The positions of molecular-weight markers are shown. A band of ~145 kDa corresponding to that predicted for the α5 subunit was visualized in all samples. The result is representative of three independent experiments with three different cell cultures. (B) Densitometry data from 3 independent experiments were normalized and analyzed by one-way ANOVA. Data were expressed as mean ± SEM.

A:


1
2
3
206KDa
119KDa

B:

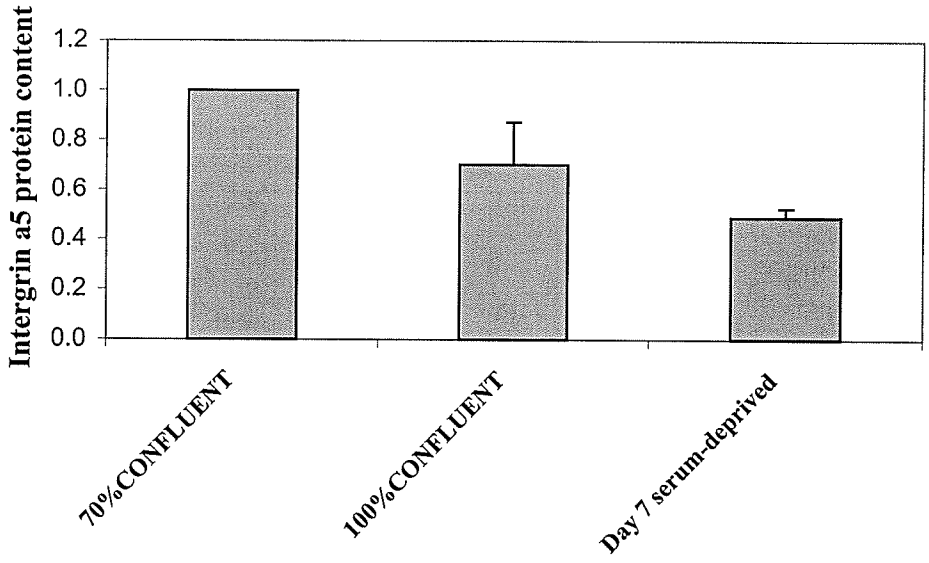

Intergrin a5 protein content
1.2
1.0
0.8
0.6
0.4
0.2
0.0
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

Figure 19. Confocal immunohistochemistry of α5 integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeabilized and incubated with a rabbit polyclonal antibody against α5 integrin (Table 2) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with Toto-3. Bar in Panel A = 50μm; Bar in Panel B = 120μm

(A) Bronchial smooth muscle (arrowhead) stained with anti-α5 integrin polyclonal antibody.

(B) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle; AV: adventitia.

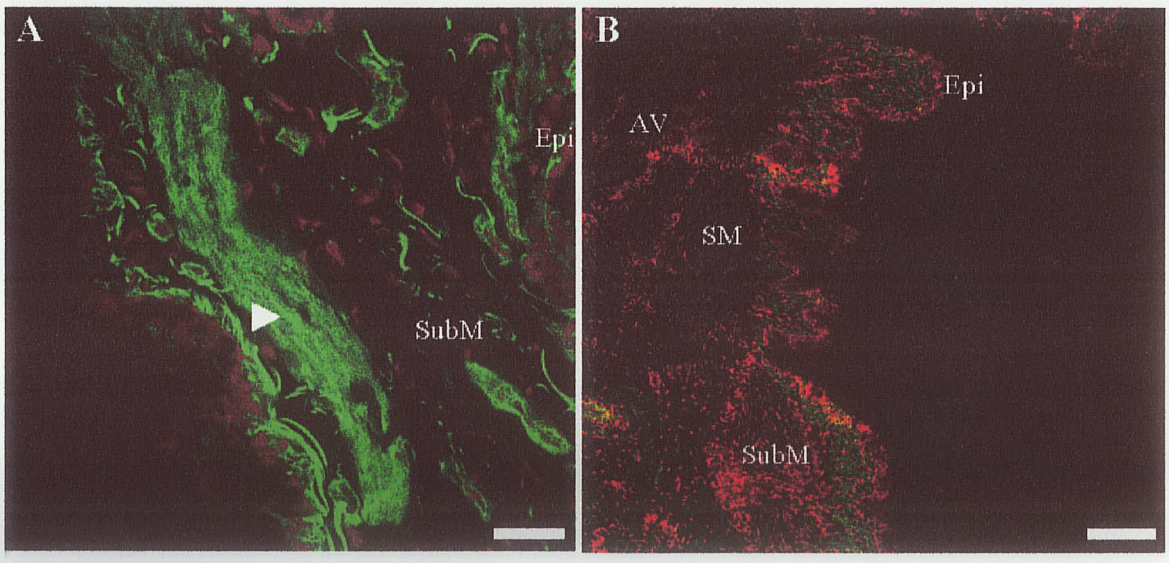


Figure 20. Identification of α6A and α6B integrin mRNA by RT-PCR in serum-fed and serum-deprived CASMC cultures. PCR was performed as described in Materials and Methods using total RNA from serum-fed, 70% confluent CASMC cultures *(Lanes 1 and 3)*, 7-day serum-deprived CASMC cultures *(Lanes 2 and 4)*, canine bronchial smooth muscle *(Lane 5)* and human bronchial smooth muscle *(Lane 6)*. A 540bp fragment corresponding to the α6A isoform was seen in all samples. A 410 bp fragment corresponding to the α6B isoform was expressed by subconfluent CASMC and in tissues, but was not detected in serum-deprived CASMC cultures. A band visible at approximately 520bp is likely a hybrid resulting from re-annealing single strands of the 540bp and 410bp products. Negative control (no RNA) is shown in *Lane 7* and molecular markers in *Lane M.*

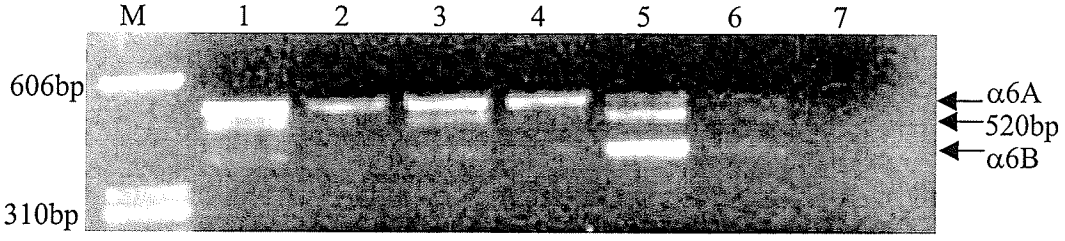


Figure 21. Confocal immunocytochemical analysis of integrin α6A in proliferating and contractile CASMCs.

Subconfluent, serum-fed CASMCs were fixed, permeablized and stained for integrin α6A **(A)** and smMHC **(B)**. A merged image showing the relative distribution of integrin α6A with smMHC **(C)** is also shown. Nuclei were counterstained with TOTO-3. Arrows indicate a representative, non-contractile myocytes in each panel. Bar = 50μm.

Seven-day serum-deprived CASMCs were fixed, permeabilized and stained for integrin α6A **(D)** and smMHC **(E)**. A merged image showing the relative distribution of integrin α6A with smMHC **(F)** is also shown. Nuclei were counterstained with Toto-3. Arrows indicate a representative elongate, contractile phenotype myocyte in each panel. Bar = 50μm.

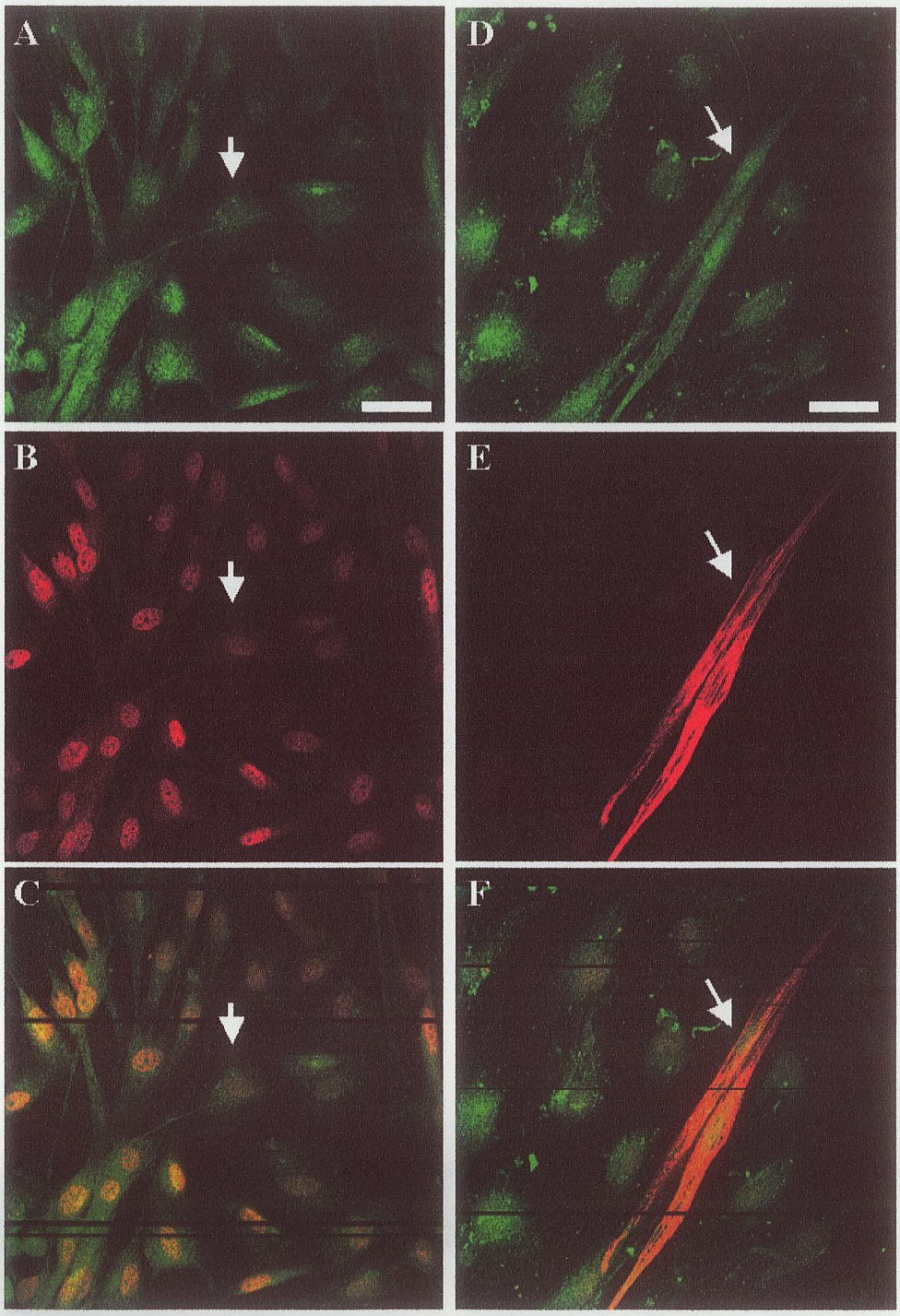

A
D
B
E
C
F

Figure 22. Confocal immunocytochemical analysis of integrin α6B in proliferating and contractile CASMCs. Subconfluent (70%), serum-fed **(A)** and 7 day serum-deprived **(C)** CASMCs were fixed, permeablized, and stained for integrin α6B. A negative control is shown in **(B)** for serum-fed CASMCs, and in **(D)** for serum-deprived CASMCs. Nuclei were counterstained with TOTO-3. Bar = 50μm.

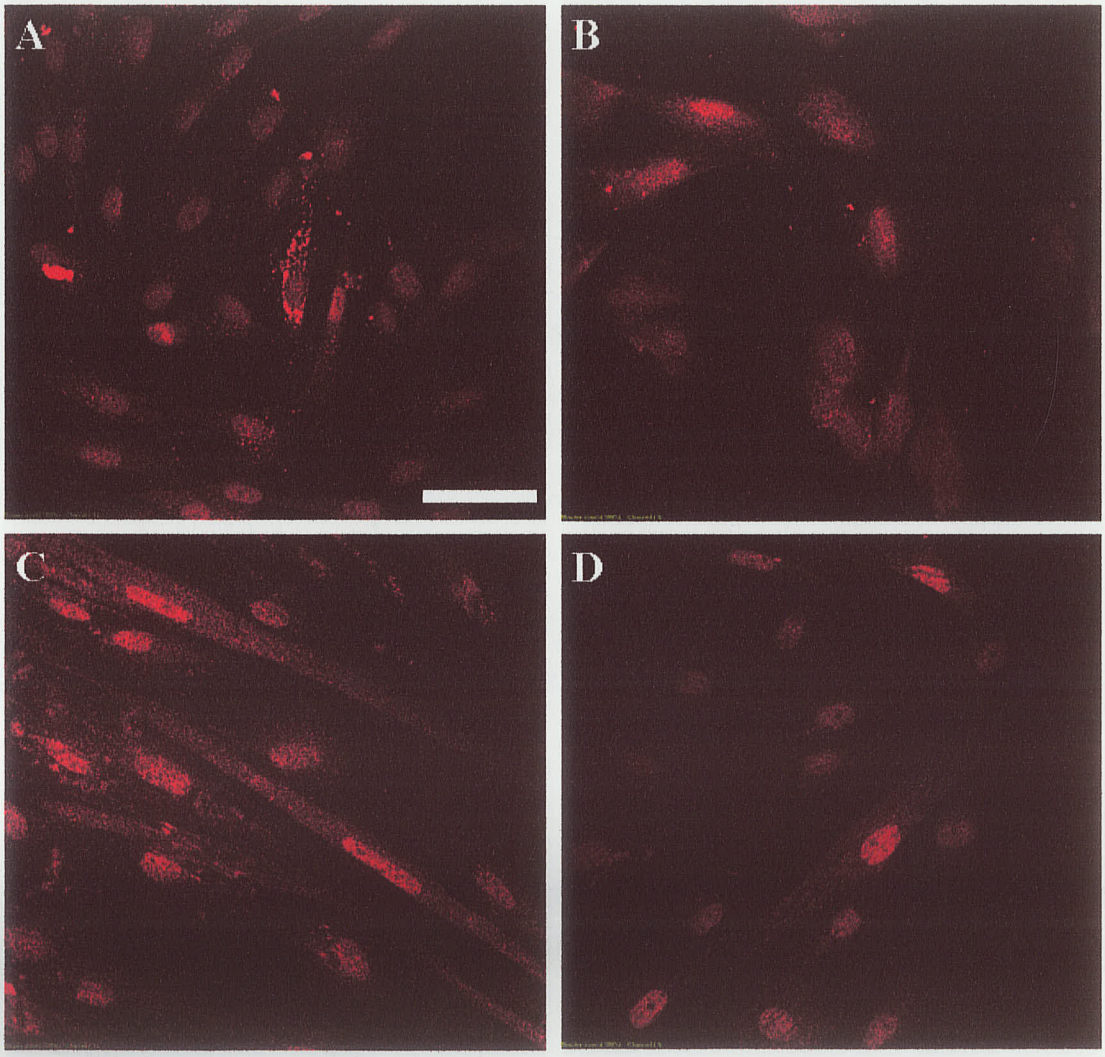


Figure 23. Confocal immunohistochemical analysis of α6A integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeabilized and incubated with a rabbit polyclonal antibody against α6A integrin (Table 1) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with Toto-3. Bar in Panel A = 50μm; Bar in Panel B = 120μm.

(A) Bronchial smooth muscle (arrows) was positive for anti-α6A integrin polyclonal antibody.

(B) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle; AV: adventitial; * = a large piece of cartilage.

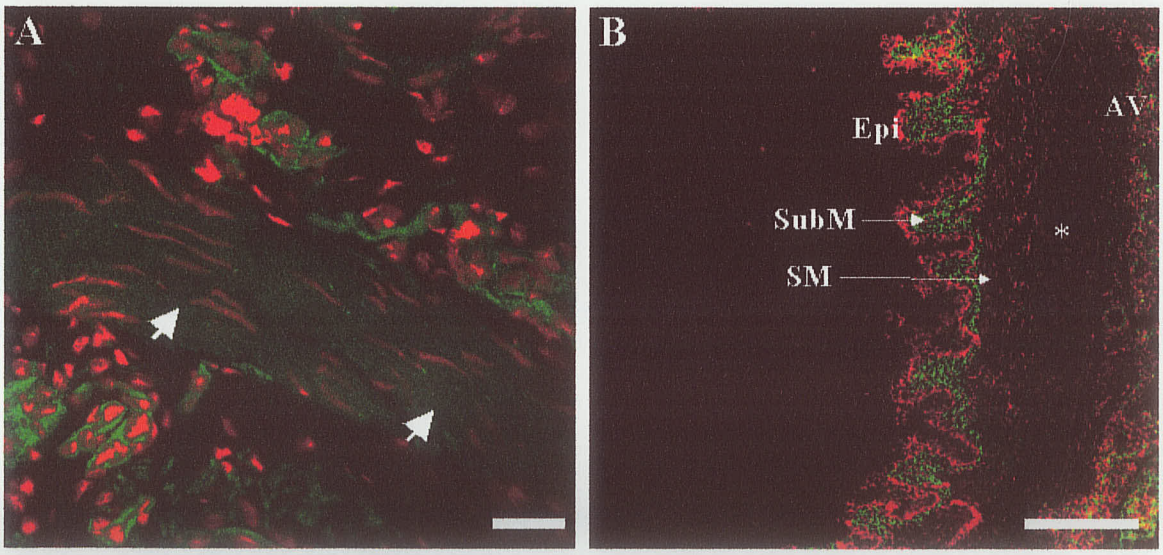


Figure 24. Confocal immunohistochemistry of α6B integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeablized and incubated with a rabbit polyclonal antibody against α6B integrin (Table 2) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with TOTO-3. Bar = 20μm

(A) Bronchial smooth muscle (arrowhead) was negative for anti-α6B integrin polyclonal antibody.

(B) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle; AV: adventitial; * - designates a piece of cartilage.

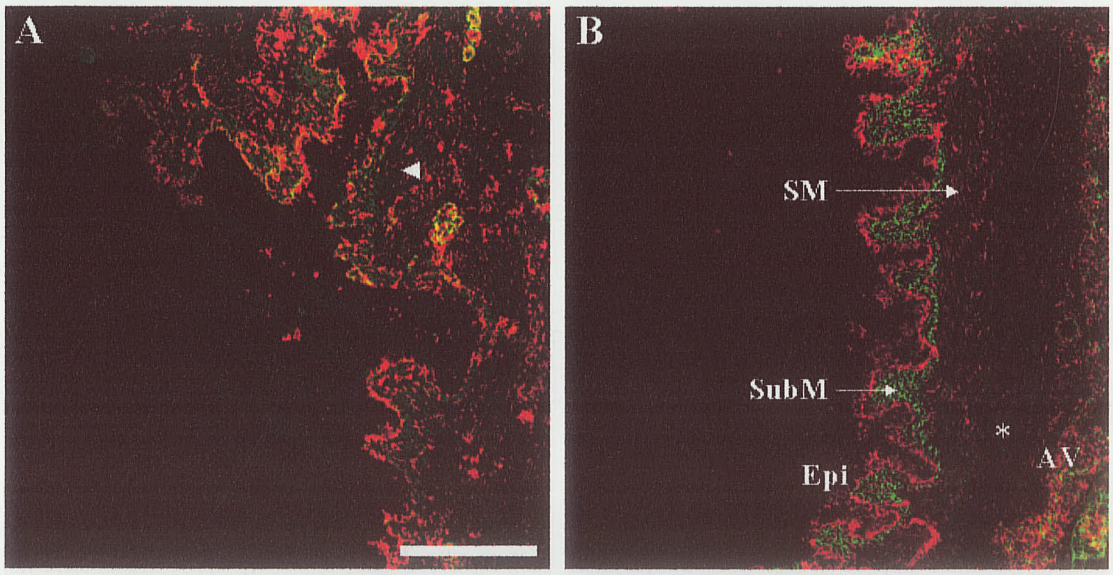


Figure 25. Detection of α7A and α7B integrin mRNA. RT-PCR was performed using with specific primer pairs (Figure 5) as described in Materials and Methods using mRNA isolated from subconfluent (70%), serum-fed CASMC cultures *(Lanes 1 and 2)*, 7-day serum-deprived CASMC cultures *(Lanes 3 and 4)*, intact canine bronchial smooth muscle *(Lane 5)* and intact human bronchial smooth muscle *(Lane 6)*. A 170bp band corresponding to the α7B isoform was visible in all samples tested while the α7A isoform (predicted PCR product 280 bp) was not detected in any sample. Negative control (no RNA) is shown in *Lane 7* and molecular weight markers in *Lane M.*

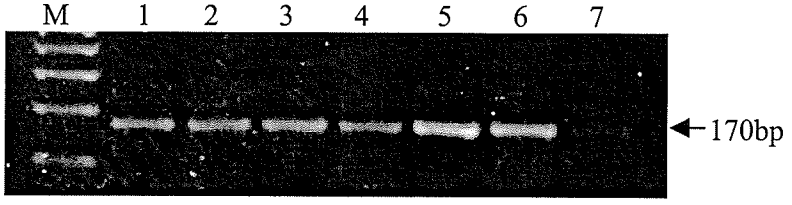


Figure 26. Confocal immunocytochemical analysis of integrin α7A in proliferating and contractile CASMCs. Subconfluent serum-fed **(A)** and 7-day serum deprived **(B)** CASMCs were fixed, permeablized, and stained for integrin α7A. A negative control is shown in **(C)**. Nuclei were counterstained with TOTO-3. Arrows indicate representative non-contractile and elongate, contractile phenotype myocytes in **(A)** (serum-fed) and **(B)** (serum deprived), respectively. No obvious labeling for α7A integrin was evident in any field of cells investigated. Bar=50μm.

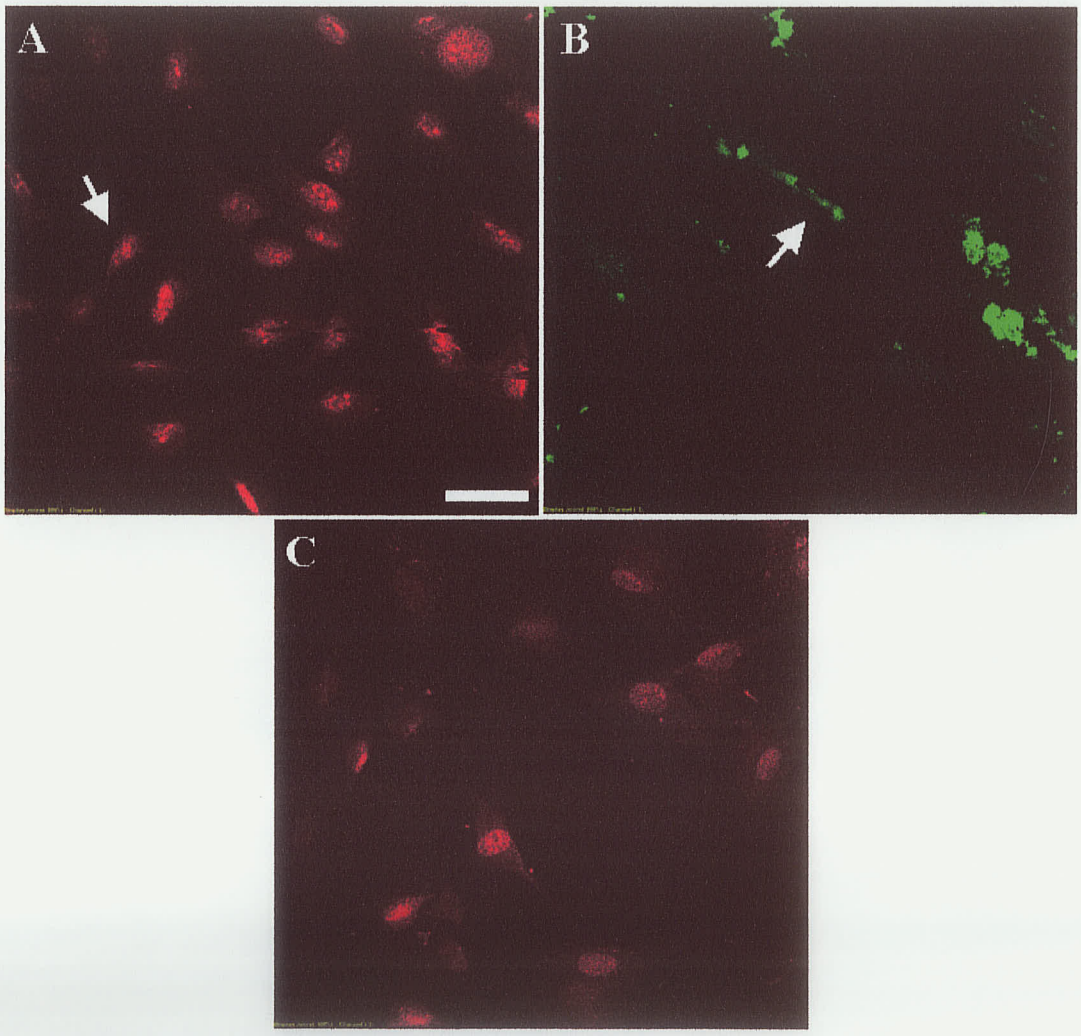


Figure 27. Confocal immunocytochemistry of integrin α7B in proliferating and contractile CASMCs. Subconfluent (70%), serum-fed CASMCs (left column) were stained for integrin α7B **(A)** and smMHC **(B)**; a merged image is shown in **(C)**. Seven-day serum-deprived CASMCs were stained for integrin α7B **(D)** and smMHC **(E);** a merged image is shown in **(F)**. Nuclei in A-C were counterstained with TOTO-3. Arrows indicate representative non-contractile myocytes (Panels A-C) and elongate, contractile phenotype myocytes (Panels D-F) in each panel. Bar=50μm.

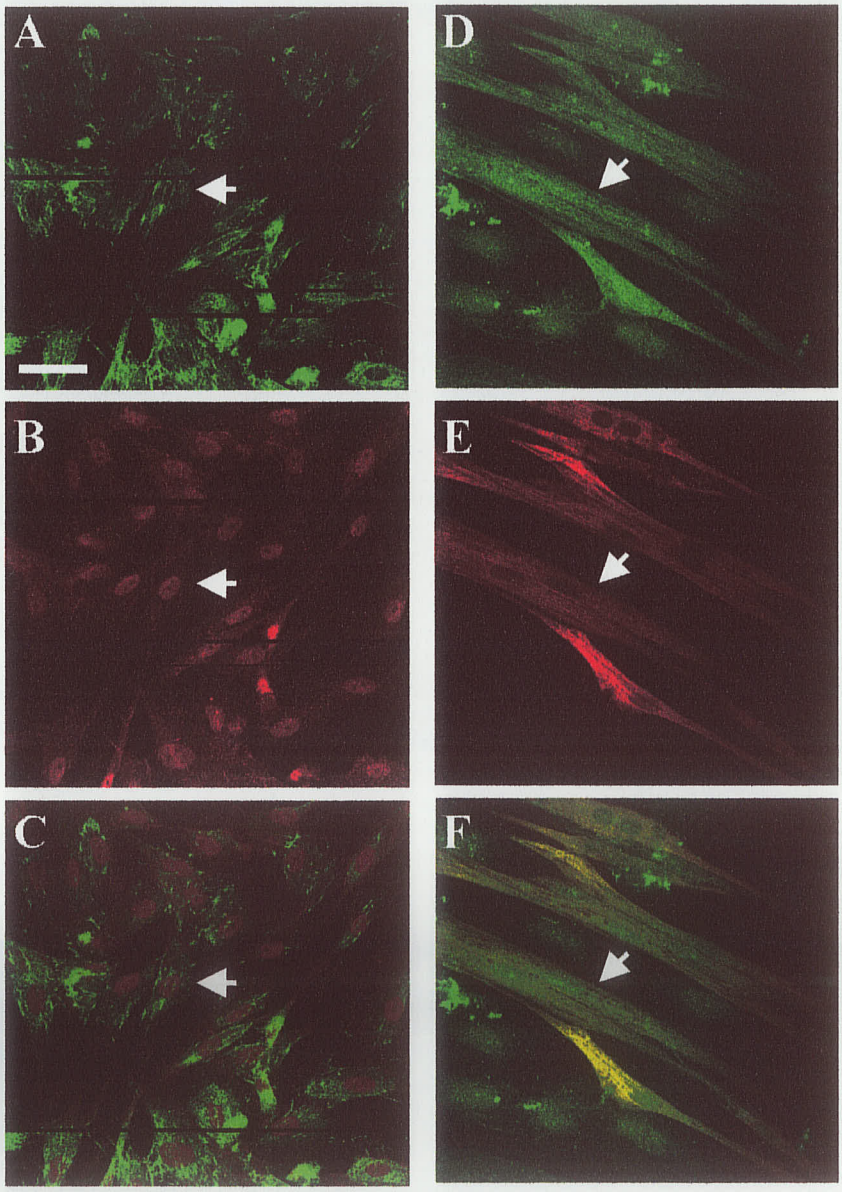


Figure 28. Confocal immunohistochemical analysis of α7A integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeabilized and incubated with a rabbit polyclonal antibody against α7A integrin (Table 1) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with TOTO-3. Bar = 120μm.

(A) Bronchial smooth muscle (arrowhead) was negative for anti-α7A integrin polyclonal antibody.

(B) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle.

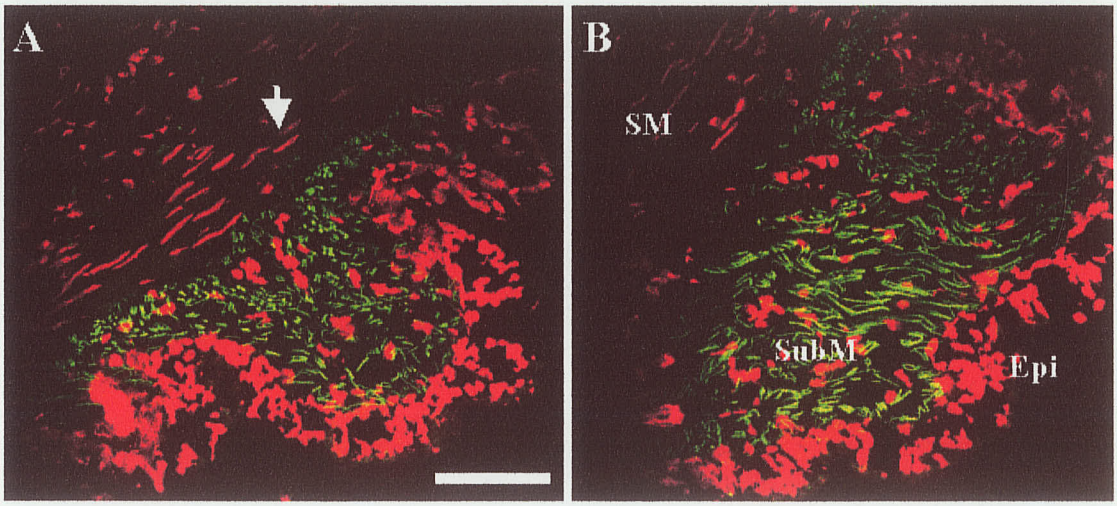


Figure 29. Confocal immunohistochemistry of α7B integrin in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeabilized and incubated with a rabbit polyclonal antibody against α7B integrin (Table 2) followed by secondary antibody as described in Materials and Methods. Nuclear staining was performed with TOTO-3. Bar, 50μm

(A) Bronchial smooth muscle (arrows) reacted with anti-α7B integrin polyclonal antibody.

(B) Negative control.

Legend: Epi: epithelium; SubM: submucosa; SM: smooth muscle.

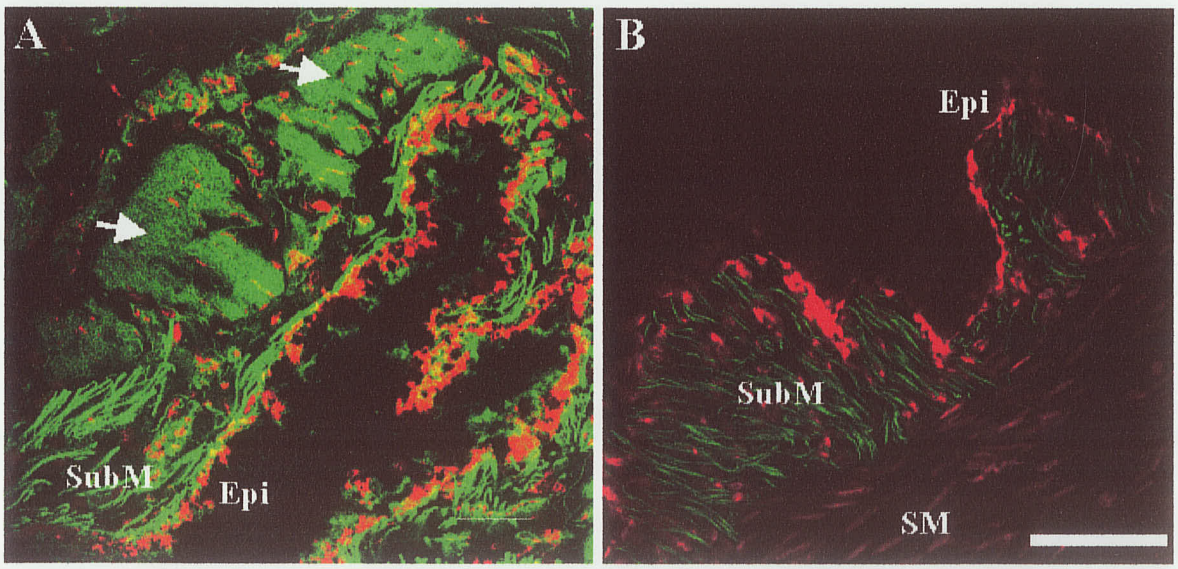
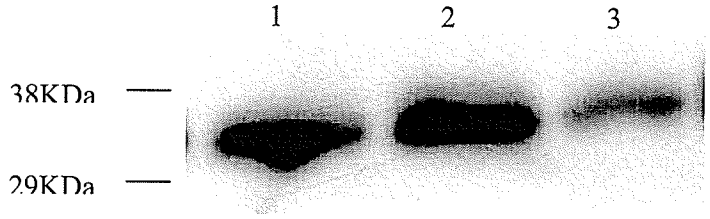


Figure 30. Western blot analysis of CD151 in total protein lysates from canine airway smooth muscle cell (CASMC) cultures. **(A)** Proteins in total cell lysates were separated by non-reduced SDS-PAGE and blotted to PVDF membrane. Blots were incubated with mouse monoclonal antibody against CD151 (Table 2). *Lane 1,* subconfluent (70%), serum-fed CASMC culture; *Lane 2,* 100% confluent serum-fed CASMC culture; *Lane 3*, 7-day serum deprived CASMC culture. Each lane was loaded with 40μg of total protein. Positions of molecular weight marker are indicated. The blot is representative of results obtained from three different cell cultures. **(B)** Densitometry data from 3 independent experiments were normalized and analyzed by one-way ANOVA. Data were expressed as mean ± SEM. * $p<0.05$ compared to deprived CASM cultures versus 100% confluent CASM cultures (One-way ANOVA).

A:


1
2
3
38KDa
29KDa

B:

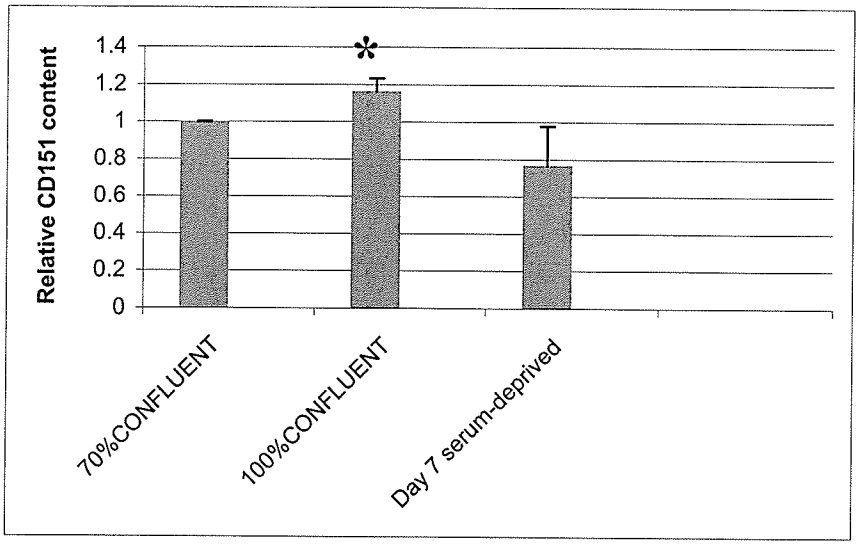

Relative CD151 content
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
70%CONFLUENT
100%CONFLUENT
Day 7 serum-deprived

Figure 31. Confocal immunocytochemical analysis of CD151 in proliferating and contractile CASMCs. Subconfluent (70%), serum-fed CASMCs were fixed, permeablized and stained for CD151 **(A)**. The arrowhead indicates a representative non-contractile myocyte. Seven-day serum deprived CASMCs were fixed, permeablized and stained for CD151 **(B)** and smMHC **(C)**. A merged image showing the relative distribution of CD151 and smMHC is included **(D)**. Arrows in B-D indicate a representative elongate, contractile phenotype myocyte in each panel. Nuclei were counterstained with TOTO-3. Bar = 50μm.

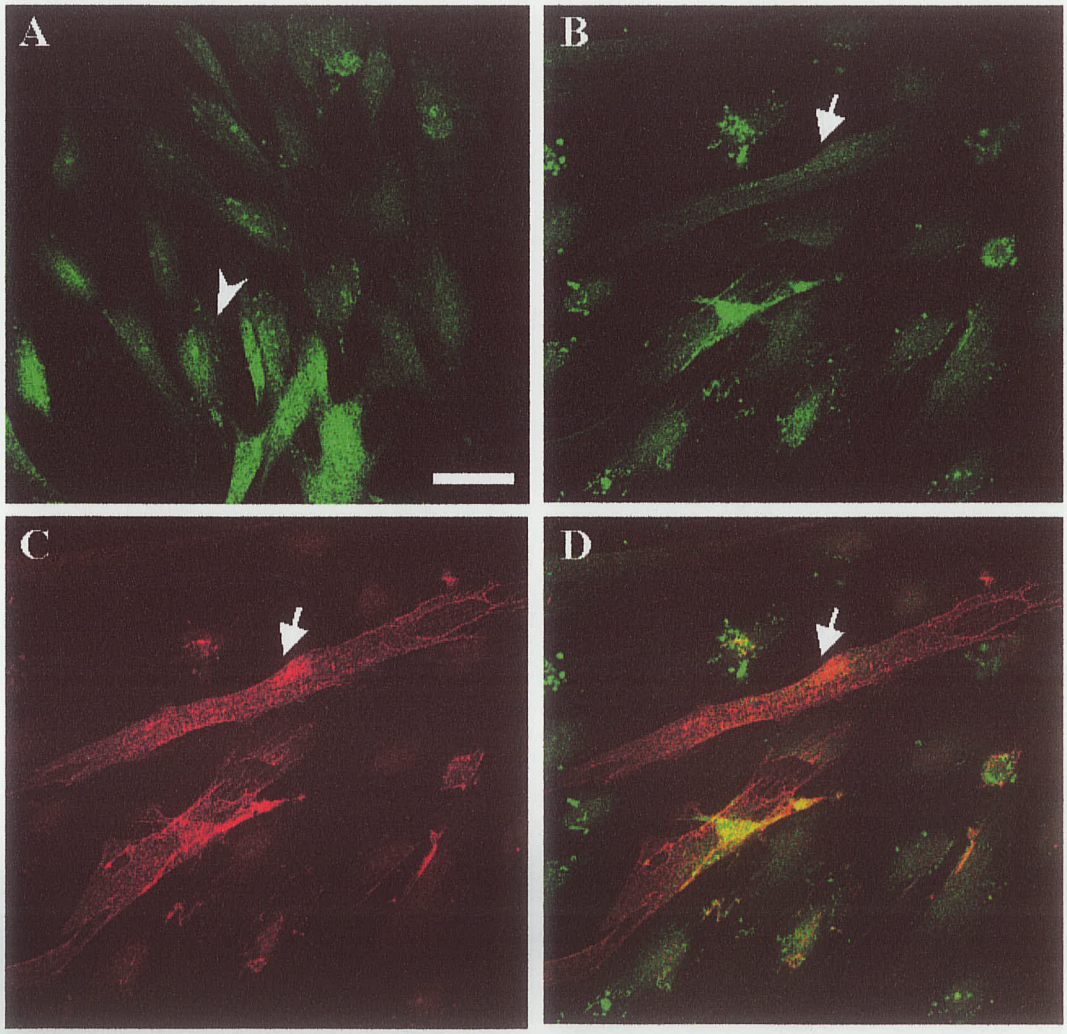


Figure 32. Confocal immunohistochemistry of CD151 in cryosections of canine bronchi. Tissue sections from 3-4th generation canine bronchi were fixed, permeablized and incubated with a mouse monoclonal antibody against CD151 (11B1) (see Table 2) and fluorochrome conjugated secondary antibody as described in Materials and Methods. Nuclei were stained with TOTO-3.

(A) Bronchial smooth muscle (arrowhead) reacted with anti-CD151 monoclonal antibody. Bar = 50μm

(B) Negative control. Epi: epithelium; SubM: submucosa; SM: smooth muscle; AV: adventitia. ; Bar =120μm.

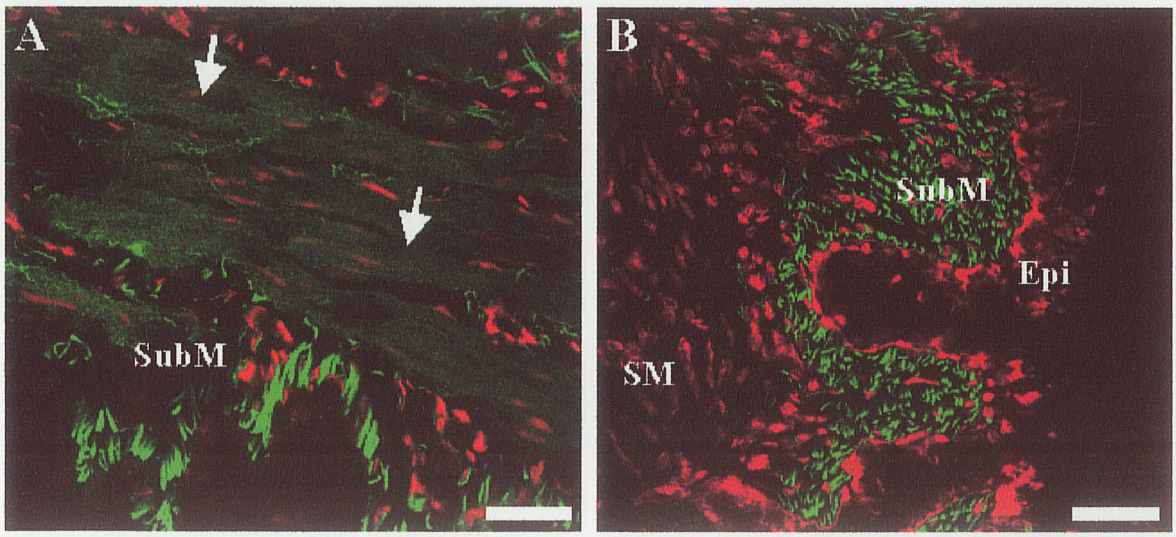
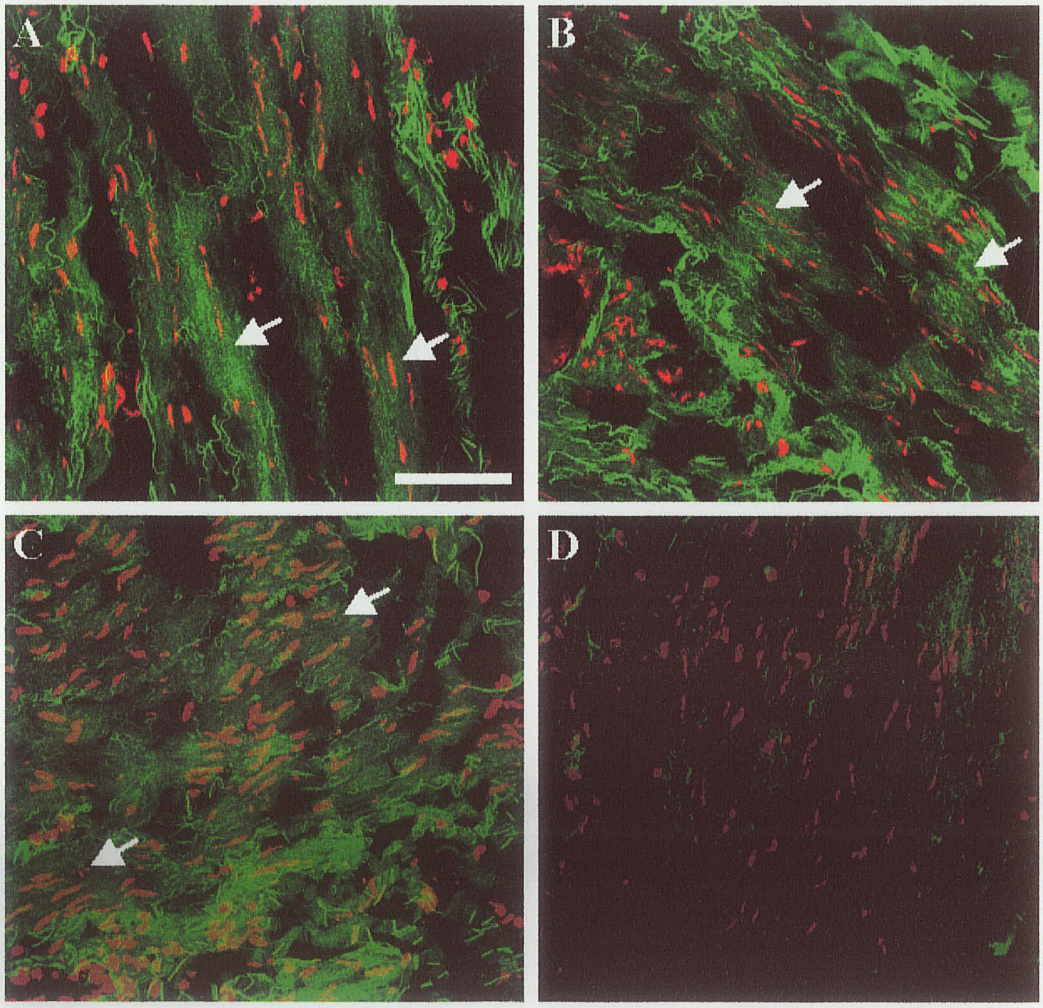


Figure 33. Confocal immunohistochemistry of α3, α5, α6A, α6B integrin in cryosections of human bronchi. Segments from 3rd-5th generation mainstem bronchi from healthy portions of human lungs resected from surgical patients were studied. Tissue sections were fixed, permeablized and incubated with primary antibodies against **(A)** α3, **(B)** α5, **(C)** α6A, or **(D)** α6B integrin and appropriate fluorochrome conjugated secondary antibodies as described in Materials and Methods (see Table 2). Nuclei were stained with TOTO-3. High magnification images that show the bronchial smooth muscle compartment in individual airways are shown. Arrows indicate regions of positive staining for integrins α3, α5, α6A, and α6B integrin, respectively. Bar = 50μm.



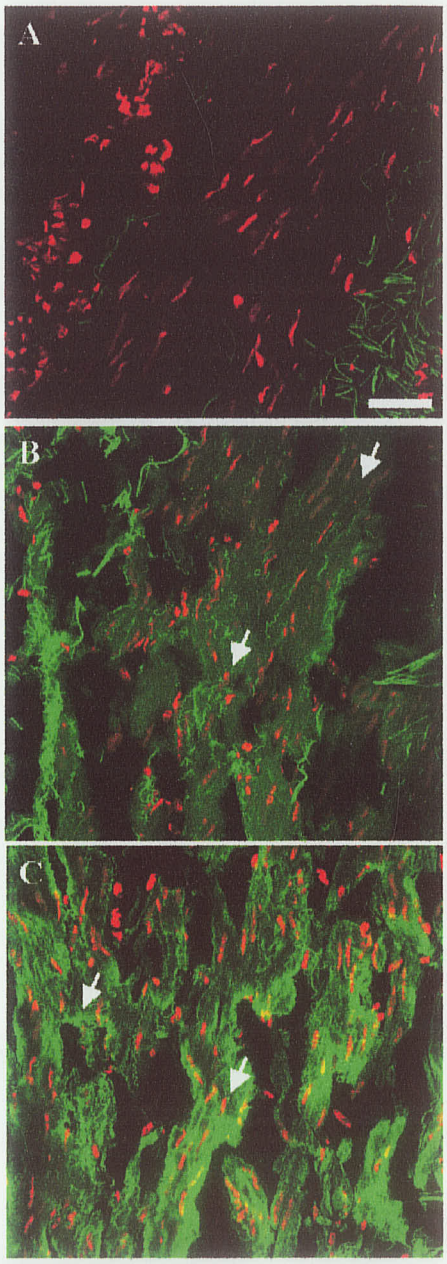


Figure 34. Confocal immunohistochemistry of β1, α7A, and α7B integrin in cryosections of human bronchi. Segments from 3rd-5th generation mainstem bronchi from healthy portions of human lungs resected from surgical patients were studied. Tissue sections were fixed, permeablized and incubated with primary antibodies against **(A)** α7A, **(B)** α7B, or **(C)** β1 integrin and appropriate fluorochrome conjugated secondary antibodies as described in Materials and Methods (see Table 2). Nuclei were stained with TOTO-3. High magnification images that show the bronchial smooth muscle compartment in individual airways are shown. Arrows indicate regions of positive staining for integrins α7B and β1 integrin, respectively. Note that, consistent with data from primary cultured canine myocytes and canine bronchial tissues, little or no staining for α7A was apparent. Bar = 50μm.

Figure 35. Confocal immunohistochemistry of smMHC, laminin, and fibronectin in cryosections of human bronchi. Segments from 3rd-5th generation mainstem bronchi from healthy portions of human lungs resected from surgical patients were studied. Tissue sections were fixed, permeablized and incubated with primary antibodies against **(A)** smMHC, **(B)** laminin, or **(C)** fibronectin and appropriate fluorochrome conjugated secondary antibodies as described in Materials and Methods (see Table 2). Nuclei were stained with TOTO-3. High magnification images that show the bronchial smooth muscle compartment in individual airways are shown. Arrows indicate regions of positive staining for integrins α7B and β1 integrin, respectively. Note that little or no staining for α7A was apparent (A). Bar = 50μm.

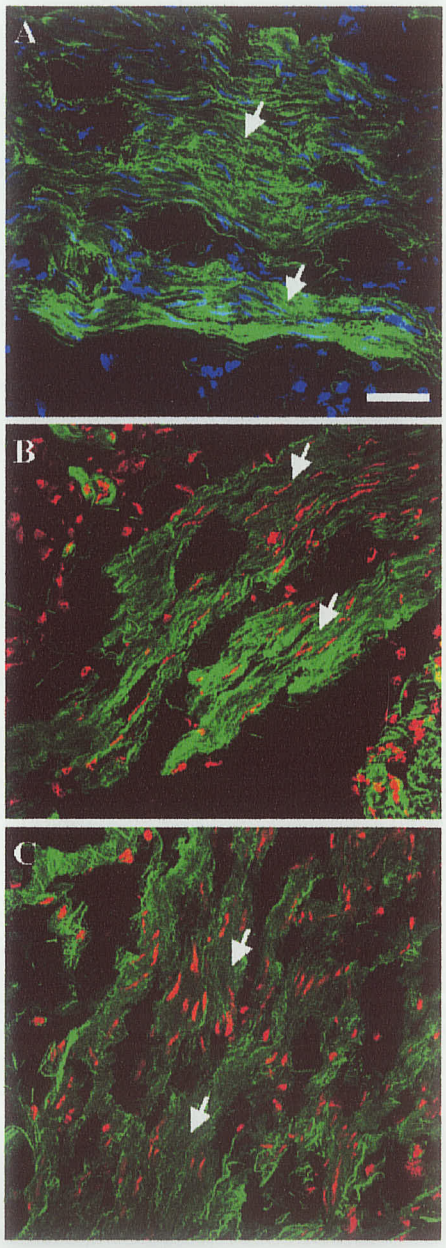
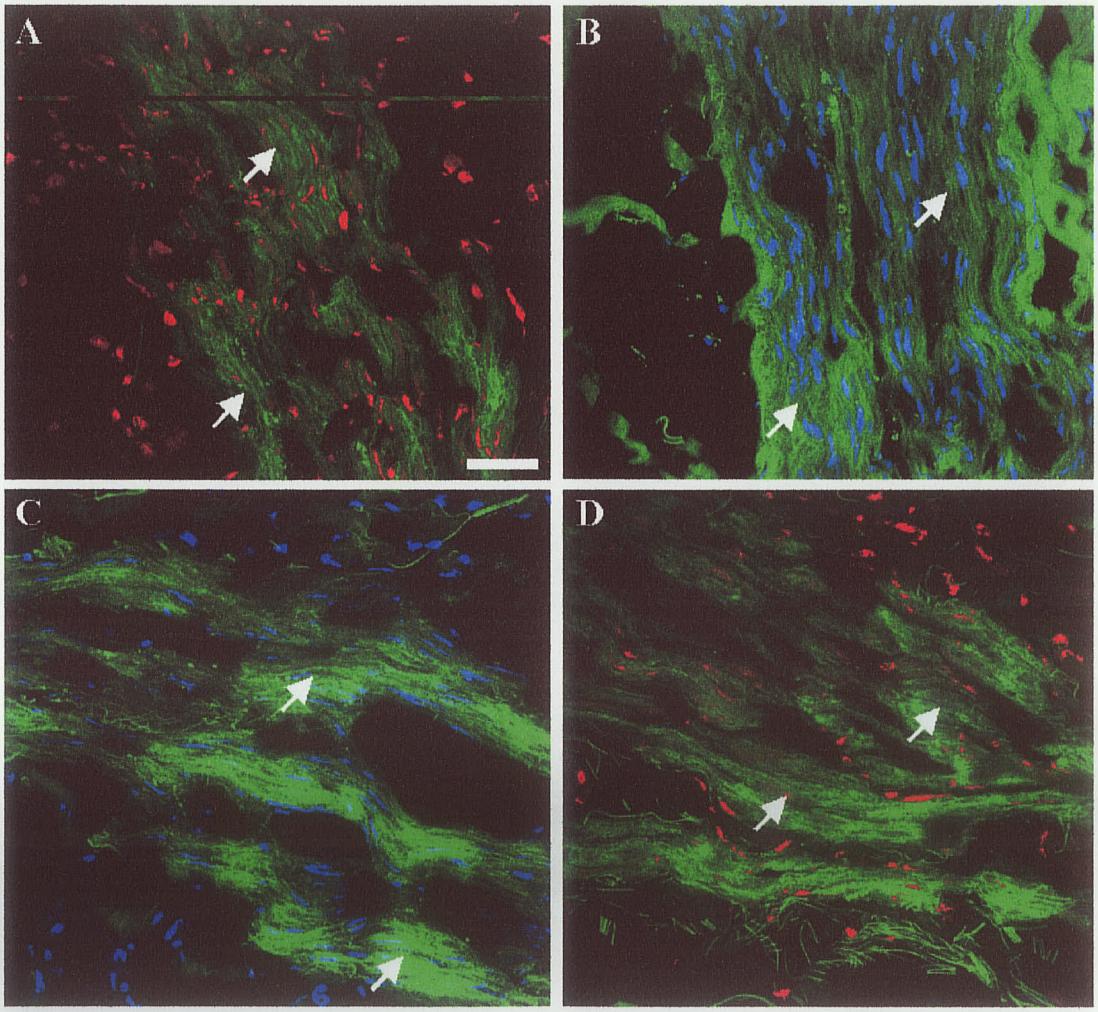


Figure 36. Confocal immunohistochemistry of CD151 in cryosections of human bronchi. Segments from healthy portions 3rd-5th generation mainstem bronchi of human lungs resected from surgical patients were studied. Tissue sections were fixed, permeablized, and incubated with different primary antibodies against CD151 (see Table 2); **(A)** clone 11B1; **(B)** clone 14A2; **(C)** clone 14B5; or **(D)** clone 5C11. Nuclei were stained with TOTO-3. Arrows indicate regions of positive CD151 in each panel, which show high magnification images of the bronchial smooth muscle compartment in individual airways. Bar = 50μm.



CHAPTER 4. DISCUSSION

The basis for this study originates from previous published work that suggests ECM components play an important role in determining the phenotype expressed by airway smooth muscle cells (Hirst et al, 2000). Laminin and collagen IV appear to maintain airway myocytes in a contractile phenotype and *prevent* phenotype modulation, whereas fibronectin and collagen I and III *promote* phenotype modulation and proliferation. In asthma, fibrosis of the airway wall develops and includes accumulation of collagen I and III and fibronectin in the submucosal and the smooth muscle layers. During lung development, formation of a laminin-rich basement membrane and its accumulation around mesenchymal cells is an essential feature of airway modeling and smooth muscle cell differentiation. Thus the control of airway smooth muscle phenotype modulation by external factors such as the ECM is an important component of normal airway biology and in disease, such as during asthma pathogenesis.

The response of smooth muscle cells to ECM proteins is largely mediated via the integrin family of cell surface receptors. We therefore wanted to compare the expression profile of integrins with affinity for laminin in airway smooth muscle cells expressing divergent phenotype states (i.e. proliferative/synthetic and contractile) in vitro, and to assess whether these receptors are expressed by airway myocytes in vivo. We examined the expression of a number of α integrin subunits, which when heterodimerized with β1 subunits have different ligand binding specificity, including: (i) α3 integrin, which binds to fibronectin, laminin, and collagen; (ii) α5 integrin, which binds RGD-containing proteins such as fibronectin; (iii) α6 integrin isoforms, which exclusively bind laminin;

and, (iv) α7 integrin isoforms, which exclusively bind laminin. A general summary of the results from our experiments is included in Table 6, and discussion of specific aspects of this data follows.

Studies using primary cultures of airway smooth muscle (ASM) cells have revealed that airway myocytes develop an immature, so-called "synthetic" phenotype when placed in primary cell culture using media supplemented with growth factor-rich serum (Halayko et al, 1996; 1999). Characteristics of the synthetic phenotype include an increased proliferative and synthetic capacity (Johnson & Knox, 1997), and decreased contractile responsiveness due to the loss of contractile apparatus-associated proteins and cell surface receptors, such as the muscarinic M3-receptor, for contractile agonists (Halayko et al, 1996; Halayko & Solway, 2001; Mitchell et al, 2000). Conversely, contractile phenotype myocytes express an abundance of contractile apparatus associated proteins (eg. smMHC, calponin, sm-α-actin) and receptors for contractile agonists, and have limited capacity for proliferation. It is now well established that mature ASM cells retain the capacity for phenotype plasticity, thus contractile state myocytes can be induced to modulate to a synthetic phenotype, and vice versa, in response to changes in an array of environmental cues, including the ECM (Halayko and Solway, 2001).

Changes toward the synthetic phenotype that occur in primary culture are reversible, thus cultured cells are used as an effective model to investigate control of phenotype switching of ASM cells. Long-term maintenance of confluent primary cultures in serum-free media re-induces a contractile phenotype (Halayko et al, 1999). Of note, in primary

culture only a discrete subpopulation (~20%) of ASM cells reacquire a contractile phenotype during prolonged serum deprivation, whereas the remaining cells exhibit a non-contractile phenotype devoid of smooth muscle-specific contractile proteins. Contractile cells induced by prolonged serum deprivation exhibit an large, elongate morphology, re-accumulate contractile proteins, and re-express functional cell surface receptors such as the muscarinic M3-receptor (Halayko & Solway, 2001; Mitchell et al, 2000). Some studies suggest that serum deprivation may induce a functionally "hypercontractile" phenotype in canine ASM cells (Ma et al., 1998). These observations highlight an additional key feature of smooth muscle cells; they appear to be comprised of discrete sublineages of mesenchymal cells with divergent capacity for phenotype responses to exogenous cues. This feature may be key to understanding the role of myocytes in airway remodelling and hyperresponsiveness associated with asthma. The factors that mediate smooth muscle phenotypic changes only in specific subsets of myocyte are poorly understood. The role for extracellular matrix components and SMC-matrix interactions in regulating of these molecular processes are not defined, though it seems clear that the ECM is likely an important factor in regulating airway smooth muscle phenotype.

In order to analyze the relationship between ECM and integrin expression and smooth muscle phenotype, we used primary cultured cells to induce a contractile phenotype in ASM cells as has been well described by our group (Halayko et al, 1996, 1999). As expected the long term serum deprivation conditions we employed for our current studies induced a significant accumulation of contractile proteins, including smMHC, sm-α-

actin, calponin, and SM22 (Figure 4); these contractile apparatus-associated proteins are all markers of a functional contractile state and are expressed in abundance by mature ASM cells in intact tissue. Confocal immunocytochemistry confirmed that contractile protein accumulation occurred exclusively in ~$1/6^{th}$ of myocytes in serum deprived cultures, and that these cells were easily distinguished on the basis of their large, elongate morphology (Figure 5). Within the same cultures, we also observed abundant numbers of myocytes that did not accumulate contractile proteins and could be distinguished based on their unique flattened, oval morphology.

A new and important observation we made in the course of our studies was that elongate, contractile phenotype myocytes in serum-free cultures were distinct in that they expressed an abundance of laminin that decorated the cell surface (Figure 5). Immunohistochemistry of intact contractile canine and human bronchial smooth muscle tissue also revealed an abundance of laminin (Figures 6 & 35). In contrast, non-elongate myocytes deficient in contractile protein expressed very little laminin protein, which, when visible, appeared to be intracellular in distribution. Of note, though serum-fed, subconfluent myocytes clearly expressed laminin in the absence of abundant contractile proteins, like non-contractile oval cells in serum-free culture, the laminin in these cells also appeared to remain intracellular. This contrasts with fibronectin distribution, which appeared to be homogeneously expressed and secreted to the extracellular space by all cells in all conditions studied. Collectively these data suggest a strong correlation between the capacity for a myocyte to acquire a contractile phenotype and its ability to express laminin and orchestrate its accumulation in the surrounding basal lamina.

We next compared the expression of laminin receptors α3β1, α6β1, and α7β1, and the fibronectin receptor, α5β1, in contractile and non-contractile ASM cells in primary culture and in intact human and canine bronchial smooth muscle tissue. Our approach was to assess, where possible, relative abundance of mRNA and protein for isoform-specific slice variants using unique sets of primers for RT-PCR, and monoclonal antibodies for immunocytochemistry, immunohistochemistry, and immunoblotting. By confocal analyses we were able to determine the relative distribution of proteins in individual cells, and the fraction of cells within a population (i.e. culture dish or intact tissue) that express the protein of interest. Moreover, where possible we used a dual antibody labelling approach to detect ECM (laminin or fibronectin) or contractile protein (smMHC), and integrins by microscopy analyses and enable us to confirm whether ASM phenotype-specific expression of integrin subunits existed. Our studies were specifically designed to determine whether a correlation exists between ECM and integrin expression and ASM phenotype, thus, we are unable to confirm any cause-and-effect relationship. Nonetheless, out studies do provide significant new insight that will form the basis of future projects to dissect specific signal transduction mechanisms associated with ECM-mediated control of myocyte phenotype and function.

It's necessary to recognize that our studies did not examine avidity of integrins. It is recognized that α integrin subunit function is influenced both by their abundance on the cell surface, by their conformation, and the identity of the β subunit with which they form heterodimers (Springer et al, 1990). Thus, failure to demonstrate changes in the amount

of any given receptor does not necessarily indicate that there are no changes in its binding avidity or the signaling pathways that it may induce. Nonetheless, our data do provide solid new rationale for future studies of integrin avidity and signal transduction to better discern biological factors that determine airway myocyte phenotype in health and disease.

It is well known that ASM cells express an array of the integrins, including α1β1, α2β1, α3β1, α6β1, α8β1, α9β1, αvβ3, αvβ5, and α9β1 (Table 1). Using flow cytometry to study primary cultured human airway smooth muscle cells, Freyer et al (2001) reported that α5, αv, and β1 integrin subunits are universally expressed, whereas only about half of airway myocytes express cell surface α6-integrin. In addition, less than a third of cells have detectable surface α1, α3 or α4 integrin. Nguyen et al (2002) reported that human ASM cells have high levels of α2, α5, β1 and β2 integrins, low levels of α1 and α6 integrin, and intermediate levels of α4 and α5 integrin. Our studies using canine smooth muscle cells and canine and human bronchial tissues are consistent with and extend these reports. We demonstrated that both β1 and α5 integrin are more-or-less ubiquitously expressed by airway myocytes in vitro and in vivo, independent of myocyte phenotype (Figures 9-12, 17-19, 33 & 34). The α5β1 integrin is thought to mediate, in part, strong survival signals provided by fibronectin, collagens I and IV, and laminin in cultured ASM cells (Freyer et al, 2001); the homogeneous expression we observed for these two subunits is consistent with this concept.

Our immunohistochemical data (Figures 12, 16, 19, 23, 24, 28, 29, & 32-36) are the first direct evidence for α3β1, α5β1, α6Aβ1, and α7Bβ1 in intact canine and human

bronchial smooth muscle. Previously, Damjanovich et al (1992) reported immunohistochemistry studies that revealed the expression of α1, α2 and α3 but an absence of α5 and α6 in human bronchial tissues. The differences with our data likely are due to the fact we used different antibodies for our studies, and we used cryostat tissue sections whereas former studies were performed using formalin-fixed, paraffin-embedded tissues.

Our studies of α3 integrin expression revealed that the protein is expressed in abundance, but not exclusively, by contractile phenotype canine ASM cells both in vitro and vivo, and by myocytes in contractile bronchial smooth muscle from human airways (Figures 13-16 & 33). This receptor subunit belongs to the laminin-binding integrin family (see Figure 2), and forms heterodimers with both β1 and β4 integrin to create receptors for laminin, fibronectin, and collagens. It is likely that ASM cells express the α3β1 dimer, as our experiments using RT-PCR, immunoblotting and immunomicroscopy have been unable to detect expression of β4 integrin (not shown). Our RT-PCR analyses revealed that canine ASM cells predominantly express the α3B isoform. Myocytes of a contractile phenotype may also express low levels of the α3A isoform, as a transcript became apparent in myocytes subjected to prolonged serum deprivation and was detected in intact canine bronchial smooth muscle. Interestingly, RT-PCR analyses suggested that species-specific expression of α3 isoforms might exist, as only the α3A isoform was detected in human tissues (Figure 13). The mechanisms that might confer distinct species-specific expression profiles of α3 integrin isoforms are not clear. Immunohistochemistry confirmed that an abundance of α3 integrin is present in both intact canine and human

ASM tissue; however the antibody we used did not distinguish between the A and B isoforms. Immunocytochemistry revealed that in cultures subjected to prolonged serum withdrawal, α3 integrin protein was predominantly localized to elongate, contractile phenotype myocytes. These observations are consistent with the concept that ligation of myocytes to the ECM via a repertoire of laminin-binding receptors such as α3β1 is a key determinant of ASM cell phenotype expression.

Our studies have demonstrated for the first time that contractile phenotype ASM cells exclusively express both α6 and α7 integrin in vitro and in vivo (Figures 20-29, 33 & 34). Both integrin subunits belong to the laminin-binding integrin family (see Figure 2), and form selective receptors for laminin when dimerized with β1 integrin. The α6 subunits also form laminin-selective receptor dimers with β4 integrin, but as noted above we have been unable to acquire any evidence that β4 integrin is expressed by ASM cells. Importantly, α7 integrin is selectively expressed by elongate, contractile phenotype myocytes in serum free culture, whereas, α6 integrin, though clearly expressed abundantly in these myocytes, is also present at moderate levels in oval shaped, non-contractile myocytes. Thus, it appears that myocytes expressing a contractile phenotype are uniquely marked by the capacity to express an abundance of *both* α6 and α7 integrin subunits. Collectively the data presented suggest that a unique repertoire of laminin binding subunits is expressed by contractile phenotype myocytes, and these include, but may not be limited to, α3, α6, and α7 integrins. These observations are clearly consistent with the hypothesis that myocyte binding to laminin via a specific set of selective receptors is a key determinant of ASM cell phenotype expression.

We examined the abundance of mRNA for α6 and α7 integrin subunits by analyzing cDNA derived from total RNA of canine ASM cells in serum-fed and serum-deprived cultures. The gene transcripts detected for α6 and α7 subunits indicate that both the α6A and α6B isoforms are expressed. Transcript for the α6A isoform was expressed in both proliferating and contractile (serum deprived) canine airway myocyte cultures, whereas α6B isoform mRNA was only detected in proliferating canine airway myocytes. RT-PCR did not reveal the presence of α7A mRNA in any cell or tissue sample, thus α7B appears to be the only α7 isoform expressed by ASM cells.

RT-PCR results were confirmed by studies using isoform-specific antibodies to investigate the abundance of the corresponding proteins for individual α6 and α7 isoforms. Immunocytochemistry showed that α6A is expressed by all cells in serum fed and serum deprived culture, however, only a moderate degree of labeling was apparent in individual myocytes of subconfluent, serum fed cultures, and in oval, non-contractile myocytes in serum free cultures. In contrast, labeling for α6A was greatest in elongate, contractile myocytes in primary culture, and was present in abundance in all cells of intact canine and human bronchial smooth muscle tissue. Little or no labeling for α6B integrin was seen in tissue, and despite the presence of mRNA in cultured cells, little or no protein labeling was evident in these cells. Similarly, as expected no evidence of α7A integrin protein was seen for cultured cells or intact tissue. Immunocytochemistry did reveal significant labeling for α7B integrin in elongate, contractile phenotype myocytes in culture and in ASM cells of intact tissues. Furthermore, consistent with RT-PCR, low

levels of α7B integrin protein was seen in subconfluent, serum fed cultures. Collectively these data reveal that the mRNA and protein for α6A and α7B integrin are associated with expression of a mature, contractile phenotype by ASM cells.

Integrin isoforms differing in cytoplasmic domains that share common extracellular and transmembrane domains may trigger different biological functions when cells interact with ECM proteins such as laminin (Yao et al, 1996 & 1997). While the findings from our studies do not provide direct evidence of a role for α3, α6 and α7 integrin chain diversity in determining ASM phenotype and function, they do suggest the need for further investigation of the role of myocyte binding via these receptors to laminin in determining cell phenotype. In light of previous and present findings, we speculate that structural differences in the cytoplasmic domain of α3, α6, and/or α7 chain isoforms could be key in regulating ASM cell responses to the ECM.

The integrin extracellular domains form the ligand-binding sites, while the cytoplasmic domains interact with the intracellular environment and are likely to be critical mediators of integrin function. Diversity in expression of isoforms of α subunit cytoplasmic domain variants is a determinant of integrin-mediated cell signaling, and thus, may be key to functional responses of cells. Deletion or replacement of an α-chain cytoplasmic domain can alter the binding affinity of the heterodimer to its ligand (O'Toole et al, 1991) and alter its capacity to promote collagen gel contraction (Chan et al, 1992). In addition to the contribution the diversity in cytoplasmic domains of α-chains of integrins may make to altered signal transduction, integrin diversity also plays a key role in "inside out

signaling" (Song et al, 1993). These observations imply that the cytoplasmic domain may confer specific functions to the integrins and perhaps participate in signal transduction, both into the cell and to the extracellular ligand-binding regions of the molecule. A study from Shaw et al (1993) demonstrated that sequence differences between the α6A and α6B variants accounted for differences in adhesive strength, morphology and migration in cells transfected with α6 integrin isoforms. Collectively this evidence provides a functional rationale for the existence of multiple cytoplasmic domain variants of a specific integrin subunit that are likely in effect in ASM cells.

Song et al (1993) suggested that diversity in conformation and changes in conformation upon ligand binding exist in alternate forms of α7 integrin, and these differences likely underlie diversity in the role of α7 integrin chains in skeletal muscle biology. For example, unique tyrosine and serine/threonine phosphorylation sites in the cytoplasmic domains of α7 integrin isoforms appear to direct diverse signal transduction cascades at different stages of muscle development (Song et al, 1993). These signaling events appear to have a profound effect of skeletal muscle development and growth. In our studies we found a strong correlation between the expression of laminin, and members of the laminin-binding integrin family, with expression of smMHC, the most rigorous marker of a functionally contractile state in smooth muscle cells (Halayko et al, 1996, 1999; Halayko and Solway, 2001). This relationship suggests the possibility that the expression of specific laminin receptors and their ligation with the ECM may be associated with mechanisms controlling the differentiation of ASM cells.

Members of the tetraspanin family (TM4SF) of proteins have been implicated in diverse cellular functions, including regulation of integrin associated cell growth and differentiation in many cell types. The association of CD151 and CD81 with α3β1 alters receptor affinity and signal transduction in response to binding ligand (Sterk et al, 2002). The TM4SF protein, CD151 appears to associate selectively with integrins that bind laminin such as α3β1, α6β1, and α7β1, and to coordinate association with other integrins such as α2β1, α4β1, α4β7, α5β1, α6β4, and αIIbβ3 (Sterk et al, 2002). TM4SF proteins form diverse yet specific complexes with integrins and this association likely modulates integrin function because tetraspanins provide specific anchoring sites for an array of signalling effectors associated with important transduction cascades such as those involving MEK/ERK, FAK, and PI(3) kinase (Berditchevski et al, 1995). The role of TM4SF proteins themselves or of integrin-TM4SF complexes in SMC phenotype and function has not been investigated. Our studies have, for the first time, shown that canine and human ASM cells in vitro and in vivo express CD151 protein. Moreover, we have shown that CD151 is exclusively expressed on elongated smooth muscle cells of a contractile phenotype. We used different monoclonal antibodies against CD151 in our studies. Though all the antibodies used recognized CD151 in contractile smooth muscle cells, only one (monoclonal, 11B1) was suitable for immunoblot experiments and required the use of non-reducing conditions. This is consistent with a previous study in which the 11B1 clone was identified as the most reliable for detecting CD151 in different cells and tissues (Geary et al, 2001).

Our findings concerning CD151 expression are unique and significant, as they provide strong circumstantial evidence that molecules associated with signal transduction induced via laminin binding integrins may be key to the unique developmental and functional responses of some airway myocyte sublineages. Furthermore, this implicates CD151 as a unique marker of mesenchymal cells that are capable of acquiring a mature smooth muscle contractile phenotype. Thus, it is conceivable that the association of CD151 and/or other TM4SF proteins with laminin-bonding integrins could play a significant role in processes that control SMC phenotype and function.

In summary, we have found that laminin is exclusively expressed on elongate, smMHC-rich myocytes whereas fibronectin is widely distributed among contractile and non-contractile myocytes. Specific laminin receptor α6A and α7B are exclusively expressed on contractile phenotype cells, whereas the fibronectin receptor subunit, α5 integrin, is expressed homogenously. Moreover, we report the first evidence that ASM cells of a contractile phenotype uniquely express CD151, which associates with integrins that bind laminin and coordinates signal cascades induced from different integrins. Our findings are consistent with observations that laminin delays spontaneous ASM phenotype modulation in primary culture, whereas fibronectin is essential for adherence and survival of airway myocytes (Schuger et al, 1997; Yang et al, 1998; Freyer et al, 2001). Vascular smooth muscle phenotype correlates with integrin expression; α1 integrin is significantly down regulated upon phenotype modulation (Belkin et al, 1990), α7 integrin expression is associated with the differentiated phenotype (Yao et al, 1997), αv and α5 integrin are increased upon phenotype modulation (Mechtersheimer et al, 1994), and α5β1

expression is strongly associated with "synthetic" myocytes in injured arterial intima (Pickering et al, 2000). Given that the ligation of integrins triggers multiple signaling events, and that signals transduced from laminin and fibronectin through $\alpha 6\beta 1$, $\alpha 7\beta 1$, and $\alpha 5\beta 1$ integrins are likely to antagonistically regulate cell proliferation and differentiation, future studies that unravel the coordination of concomitant signal transduction from these receptors, and the specific role of CD151 in this process are warranted. These studies may provide new insight of molecular mechanisms that control ASM phenotype and function during lung development, normal physiology, and during pathogenesis of airway diseases such as asthma.

CHAPTER 5. SUMMARY OF MAIN FINDINGS

1. Gene transcripts for β1, α3, α5, α6, and α7 integrin were seen in canine ASM cells. The mRNA for α6A subunit was present in serum-fed and serum-deprived myocytes, whereas α6B subunit was apparent in low abundance in serum-fed myocytes. The α7B subunit was present in both serum-fed and serum-deprived myocytes but α7A was absent. Canine and human bronchial smooth muscle expressed mRNA for β1, α3, α5, α6A and α7B integrin.
2. Laminin was selectively expressed by contractile phenotype myocytes, and fibronectin expression was associated with all cultured myocytes. Staining for both α6A and α7B integrin proteins appeared exclusively in contractile phenotype myocytes. Significant labelling for both isoforms was also seen in intact smooth muscle from canine and human bronchi. The α5 integrin subunit was widely distributed in cell culture, and was expressed by myocytes in smooth muscle of canine and human bronchi. Though α3 integrin was expressed by myocytes in all cell culture conditions, it was increased significantly in contractile phenotype myocytes, and was abundant smooth muscle of canine and human bronchi.
3. CD151 protein was expressed exclusively by contractile phenotype myocytes in primary culture and by canine and human contractile bronchial smooth muscle cells in intact tissue.

CHAPTER 6. REFERENCES

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