PHENOTYPIC MODULATIONS OF CULTURED CANINE AIRWAY SMOOTH MUSCLE CELLS AND GROWTH ARRESTED CELLS

BY

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A Thesis

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MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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LIST OF ABBREVIATIONS

Cy3 -	indocarbocyanine
FITC -	fluorescein isothiocyanate
kDa -	kilodalton
MLC -	myosin light chain
MLCK -	myosin light chain kinase
nm -	non muscle
nmMHC -	non-muscle myosin heavy chain
PAGE -	polyacrylamide gel electrophoresis
RLC -	regulatory light chain
SDS -	sodium dodecyl sulphate
sm -	smooth muscle
SMC -	smooth muscle cell
SMCs -	smooth muscle cells
smMHC -	smooth muscle myosin heavy chain

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1. ABSTRACT

Vascular SMCs are known to exhibit phenotypic switching in culture. There is a paucity of data relating to the development of the type of switching in airway smooth muscle. This was the rationale for the current study the objective of which was to determine whether airway SMCs in culture modulate from a contractile to a synthetic type. As SMCs modulate from a contractile to a proliferative state, there is enhanced expression of "non-muscle" (nm)-isoforms and a concomitant reduction in smooth muscle (sm-) isoforms of various proteins. MLCK regulates acto-myosin interactions and plays a role in modulating contraction, cell motility and cytokinesis. In this study the abundance of MLCK isoforms in freshly isolated and cultured canine tracheal SMCs was determined by Western blot analysis using isoform-specific antibodies. Cellular sm-MLCK (138kDa) content decreased (>75%) after 5 days in primary cultures and increased (25%) post-confluence. Less than 20% of the sm-MLCK in freshly isolated SMCs remained after 7 passages. Conversely the content of a nm-MLCK isoform (206kDa) rapidly increased (>40-fold) in primary culture and remained elevated after several passages. Subcellular localization of sm- and nm-MLCK was assessed by fluorescent immunocytochemistry in unpassaged primary cultures. Both nm- and sm-MLCK co-localized with α-actin-positive stress fibers. Intense nuclear and perinuclear staining were seen with anti-nm-MLCK antibodies. Confocal microscopic analysis confirmed that the nm-MLCK was present within interphase nuclei but did not appear to be associated with condensed chromatin in mitotic cells. In contrast, sm-MLCK appeared to be restricted to cytoplasmic domains in

interphase cells and was absent in myocytes undergoing mitosis. Our data suggest that isoforms of MLCK are differentially expressed and localized in airway SMCs of different phenotypic states and that disparate regulatory roles may exist for nm- and sm-MLCK isoforms. The second hypothesis tested was that cells in culture change into a unique phenotype with serum deprivation. To study the effect of serum deprivation on phenotypic modulation in post-confluent cultured cells, long term serum deprivation (growth arrest) was used. Two phenotypically distinct groups of cells were identified in primary airway SMC cultures. One group of cells, which were elongated and spindle-shaped with refractile sarcolemma, appeared after the second day of arrest and their number increased as the deprivation was prolonged. After 15 days of deprivation they comprised about 30% of all the cells present, but occupied almost 40% of the total area of the culture dish. The other group of cells were smaller, flat and stellate. Western blot analysis showed that the expression of contractile and regulatory proteins, such as sm-α-actin, sm-MLCK and sm-MHC in spindle-shaped cells was increased significantly. Further immunocytochemical study showed that the spindle-shaped cells stained strongly for sm-MHC, sm-α-actin and sm-MLCK, while the flat cells stained weakly or negatively for sm-MLCK, sm-α-actin and sm-MHC. In addition these cells also stained strongly for nm-MLCK and β-tubulin. A dramatic change of the distribution of sm-MLCK in the flat cells was also noticed. The spindle-shaped cells were also stained with desmin early at day 1 of arrest which progressed up to day 15, but these two types of cells stained evenly with vimentin during the whole period of deprivation. Our results demonstrated that prolonged serum deprivation could induce functional contractile, spindle-shaped, elongated cells with higher

expression of contractile protein and regulatory proteins. These experiments demonstrated that serum deprivation can cause phenotypic modulation of SMCs in culture. This model may be useful for the study of SMC differentiation and contraction. (This study was supported by grants from the Medical Research Council of Canada and Inspiraplex, Canada)

Key Words: smooth muscle cells, confocal microscopy, immunocytochemistry, cell division, serum deprivation, phenotype modulation.

2. BACKGROUND AND LITERATURE REVIEW

2.1. Smooth Muscle Cell Phenotypes

SMCs are multi-functional mesenchymal cells capable of modulating their phenotype *in vivo* and *in vitro* (1). In general, mature SMCs exist in a "contractile" state, though in response to pathological stimuli they can modulate to a matrix-secreting "synthetic" phenotype. There are many factors which can influence smooth muscle phenotype and the hypertrophic response, such as chalones, trophic factors from nerves, growth factors and hormones. These modulated SMCs are predisposed to enter a "proliferative" state in response to exogenous growth stimuli. Cells of these phenotypic states are characterized by differential expression of sm- and nm-isoforms of cytostructural, contractile and regulatory proteins (2).

Fully differentiated, mature SMCs differ fundamentally from striated muscle cells in their ability to respond to environmental stress by modulating their phenotype from that of a mitotically quiescent contractile cell, to one which is mitotically active, shows expression of non-muscle type contractile and regulatory proteins typically seen in replicating cells, and secretes extracellular matrix, cytokines and growth factors (3). The capacity of the mature smooth muscle cell for phenotypic plasticity allows a given myocyte to express a broad range of different phenotypes in response to different physiological and pathological factors (4, 1, 5, 3). At present, assessment of the differentiation state of a SMC depends upon several criteria, including its anatomic

location, cellular and subcellular morphology, functional characteristics of the cell and expression of genes encoding SMC-specific marker proteins (2, 3).

Individual SMC demonstrate striking dissimilarities, proliferative responsiveness and ability to synthesize extracellular matrix components (6, 7, 8, 9, 10). Differentiated SMC demonstrate a range of phenotypes which fall between two extreme states which are classically refered to as the contractile and the synthetic phenotypes (11, 1). The contractile phenotype is associated with fully mature SMCs, whereas the synthetic phenotype, although still considered to be differentiated, appears to be common to SMCs in developing foetal tissues in proliferative states and pathophysiologic conditions(12, 13, 14).

Differentiation is the event during which embryonic cells, that have been committed to a particular lineage, i.e. undergone determination, begin to demonstrate fundamental biochemical, morphological and/or physiological characteristics that are particular for a specific mature cell type. Maturation is a late stage of differentiation during which SMCs that have differentiated to an irreversible foetal phenotype, develop the predominant characteristics of adult SMCs in fully developed organisms. Various cell types can alter their character when the environment changes. These alterations in character are called modulations of the differentiated state and involve reversible interconversions between phenotypes. Mudulation in cell phenotype may occur as a result of cell interactions, alterations of extracellular matrix, or in response to other signals such

as hormones. Modulation of phenotype does not deliver a cell to the multipotent, undifferentiated state, and is not considered to be dedifferentiation. Morphological and biochemical changes accompanying phenotypic modulation have been best characterized in primary cultures where contractile SMCs undergo rapid, reversible modulation after seeding (11, 15, 16).

Several characteristics, including, cellular morphology and function, anatomic location and the abundance SMC-specific proteins and their isoforms, are recognized as markers for discriminating SMCs of different phenotypes. The contractile and synthetic phenotypic states have been described to represent idealized extremes indicative of the multi-functional nature of SMCs (11). Most SMCs likely exist in a native, intermediate state which falls within an existent phenotypic continuum particular to specific organs and species (17). It is also well established in primary culture that native, contractile SMCs from a variety of organs, rapidly modulate to a mitotically active, synthetic phenotype in the presence of serum (11, 15, 16).

Mature SMCs generally approximate a contractile state characterized by the presence of a high volume fraction of myofilaments and the expression of numerous smooth muscle-specific genes encoding contractile proteins and proteins which regulate contraction (1, 15, 16). Thin myofilaments, composed of actin and other actin-binding proteins such as calponin, caldesmon and tropomyosin, and thick myofilaments, composed of smooth muscle-specific myosin, can occupy 80-90% of total cytoplasmic volume in

contractile SMCs (18). The presence of a large number of membrane-asssociated and cytoplasmic dense bodies, which act as anchor points for thin and intermediate filaments, is another feature characterizing mature SMCs (19, 20). Intermediate filaments in mature SMCs are composed of desmin and vimentin whereas in immature SMCs the filaments are almost exclusively composed of vimentin (21). Organelles associated with protein synthesis (eg. Golgi apparatus, rough endoplasmic reticulum, free ribosomes) are located near the nuclear poles but comprise only a small fraction of total cell volume (1).

Synthetic SMCs have an abundance of organelles for protein processing and synthesis, including rough endoplasmic reticulum and Golgi apparatus, and a limited number of myofilaments (1). They synthesize and secrete extracellular matrix proteins and proteases abundantly, and express genes for growth factors, cytokines and their requisite receptors (5, 15). The phenomenon of phenotypic modulation in culture has been exploited for vascular myocytes, with relevance to atherosclerosis and hypertension, to identify protein markers of SMC phenotype which correlate with the physiological function of vessel wall SMCs (15, 2).

2.2. Smooth Muscle Cell Contractile, Regulatory and Cytoskeletal Proteins

Loss of contractility by proliferative vascular SMCs in sub-confluent primary culture is a manifestation of changes in contractile and cytoskeletal protein content.

Proliferating cells exhibit a reduction in smooth muscle α- and γ-actins (15), sm-MHC isoforms (22), smooth muscle regulatory 20KD MLC isoform (23) and *h*-caldesmon (26), the sarcoplasmic reticulum membrane-associated protein, phospholamban (15), and the cytoskeletal proteins, desmin (21) and meta-vinculin (24). Furthermore, phenotypically modulated cultured cells are known to re-express foetal and non-muscle isoforms of several proteins including β-actin(25), nmMHC (22), the non-muscle regulatory myosin light chain isoform (23), *l*-caldesmon (26), the intermediate filament associated protein, vimentin (21) and several secreted proteins including collagen and elastin (27).

A number of adult smooth muscle-specific protein markers have been identified, including sm-α-actin (28), sm-γ-actin (29), sm-MHC (30), calponin (31), *h*-caldesmon (32), SM22 (31), and most recently, a cytoskeletal protein called smoothelin (33). These findings suggest that the relative abundance of these particular markers may be the most useful molecular indices of the contractile and/or secretory function of SMCs.

2.3. Asthmatic Airway

Asthma, one of the most common diseases in industrialized countries, is a clinical state characterized by episodes of reversible dyspnea and wheezing. It is a disease of the airway, characterized symptomatically by persistent airway hyper-responsiveness to allergic and non-allergic stimuli (34). At present, asthma is generally recognized as a chronic inflammatory disease highlighted by excessive airway narrowing in response to

various stimuli (35). Acute exacerbations of the disease are the result of airway obstruction resulting from spasm of smooth muscle and luminal plugging with excessive mucous secretion (34). Severe inflammation is characteristic of patients who die of asthma (36). It now appears that remodelling and thickening of the airway, which occur as a result of chronic inflammation, may represent the basic mechanisms underlying irreversible airway hyper-reactivity (37). Sub-epithelial and adventitial fibrosis (38) and increased airway smooth muscle mass are characteristic pathological features of chronic asthma that contribute to airway wall thickening (39, 40).

Structural changes associated with airway remodelling have been identified. These changes include infiltration with eosinophils and macrophages (36), oedema, epithelial denudation (41), hyperplasia of mucus secreting glands, thickening of the basement membrane (38, 40), subepithelial collagen deposition (42), subepithelial accumulation of myoblasts (43), and substantial thickening of the medial smooth muscle layers (37, 44, 45). Regional disparity in airway myocyte growth suggests that: 1) different mechanisms and/or stimuli might trigger smooth muscle growth in different areas of the lung; and 2) heterogeneous populations of airway myocytes may be differentially distributed along the bronchial tree.

Cultured airway myocytes provide a convenient model system to study: 1) the effects of specific mediators on SMC proliferation and differentiation; 2) secondary signaling pathways which regulate contraction and proliferation; 3) membrane receptor

and ion channel properties; 4) regulation of gene expression and post-translational processing of functionally important smooth muscle proteins; and 5) the effects of therapeutic interventions on the properties of airway SMCs. Primary cultures of airway myocytes have been used as a model system for the proliferative response of airway SMCs associated with airway remodelling in chronic asthma. Cells from human airway smooth muscle have beeen used by several investigators (46, 47, 48). In addition, numerous studies using airway myocyte primary cultures of canine (49, 50, 16), guinea pig (51, 52,), ovine (53), bovine (54) and lepine (55) origin have been reported.

It is now well established that in primary culture, serum-stimulated SMCs undergo spontaneous, reversible modulation from the contractile to the synthetic phenotype similar to the changes seen during injury repair and proliferative response *in vivo* (11, 56). Data obtained using immunoblotting techniques (16) have provided the most thorough and quantitative analysis of the changes occuring in protein expression of primary cultured myocytes. Assessment of the temporal changes occuring in the content of proteins which compose and regulate the contractile apparatus in canine tracheal myocytes (16) indicates that the phenotype of cultured cells modulates rapidly when cells are cultured in the presence of serum. The cells also demonstrate phenotypic plasticity as the levels of contractile proteins increase again post-confluence.

2.4. Myosin Light Chain Kinase

A fundamental role for changes in phenotypic expression of airway SMCs is as a determinant of intrinsic contractility of airways which may be related to hyper-responsiveness (57, 16, 58). In addition increased activity and content of the 138kDa sm-MLCK isoform is the primary mechanism for increased shortening velocity of sensitized airway smooth muscle (59).

MLCK is a key enzyme in the regulation of smooth muscle contraction. In smooth muscle, phosphorylation of the regulatory light chain of myosin by the Ca^{2+}/calmodulin-dependent MLCK is a well-characterized event in the initiation of contraction. Ca^{2+} binds to calmodulin and the Ca^{2+}-camodulin complex then binds to MLCK and activates the enzyme. Activation of MLCK results in phosphorylation of the 20kDa regulatory light chain subunit of myosin and stimulation of the actin-activated myosin Mg^{2+}ATPase activity. These events lead to force generation or shortening of the muscle. Decrease in the cytosolic Ca^{2+} concentration results in the dissociation of calmodulin from MLCK and conversion of the kinase to an inactive enzyme. With kinase inactivation, RLC is dephosphorylated by a MLC phosphatase localized to the contractile elements (60).

*Scheme for activation of MLCK in cells (Biochemistry of Smooth Muscle Contraction, edited by Michael Barany, 1996, ACADEMIC PRESS, INC.)

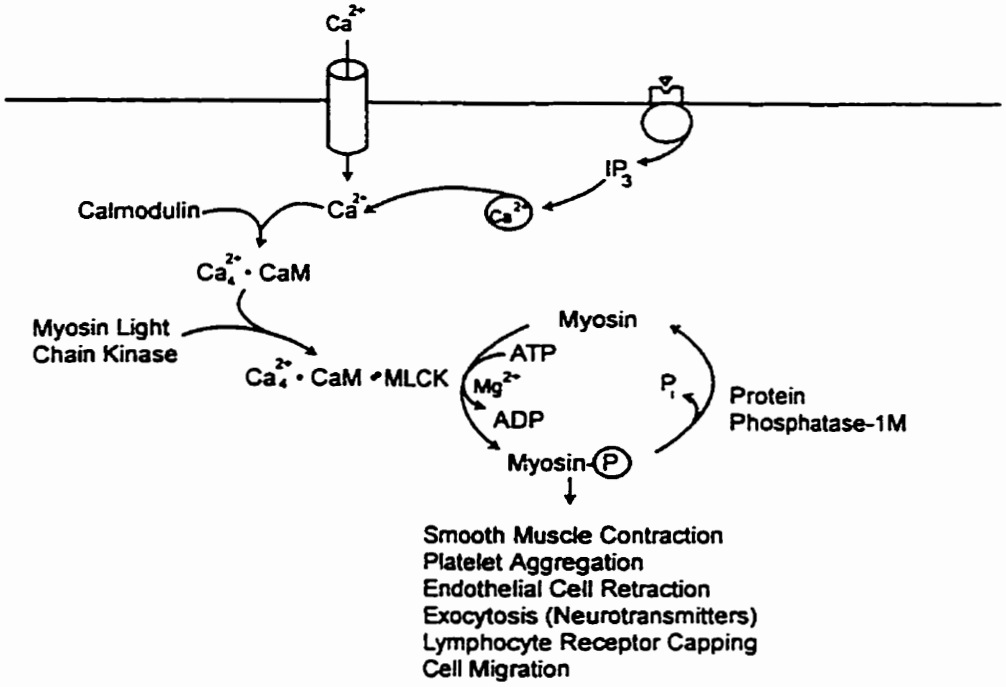


FIGURE 1 Scheme for activation of MLCK in cells.

Ca^{2+}/calmodulin-dependent MLCKs have been cloned and sequenced from chicken (61), rabbit (62), and bovine (63) smooth muscles, in addition to rabbit (64) and chicken (65) skeletal muscles. There are at least two distinct genes for MLCK, represented by the smooth/ non-muscle versus the skeletal muscle MLCK. The domain organization of the sm-MLCK is similar among different isoforms as shown in figure 2. The N-terminal first 80 residues are highly conserved in all three smooth muscle kinases and contain an actin-binding domain. Following the N-terminal 80 residues, there is a tandem repeat of 12 amino acids in the rabbit and bovine MLCKs that is not present in chicken smooth muscle MLCK. The identity of the amino acid residues in this region is about 70%, which is the

least conserved portion in smooth muscle MLCKs. Following the tandem repeat sequences, four unc structural motifs are present in all three smooth muscle MLCKs but not in the skeletal muscle enzymes. Motif I is related to the type III module of fibronectin and is N-terminal of the catalytic core. Motif II belongs to the C II set of the immunoglobulin superfamily. The third motif II is located at the C- terminus of MLCK, and it is expressed as an independent protein, telokin, in some smooth muscle tissues. The expression of telokin is due to an alternate promotor in the MLCK gene. MLCK contains a catalytic core in the central portion of the enzyme that is highly homologous to other protein kinases. The C-terminal of the catalytic core is the autoregulatory domain which contains a sequence connecting the catalytic core to the calmodulin binding domain.

*Domain organization of sm/nm-MLCK (from "Biochemistry of Smooth Muscle Contraction", edited by Michael Barany, 1996, ACADEMIC PRESS, INC.)

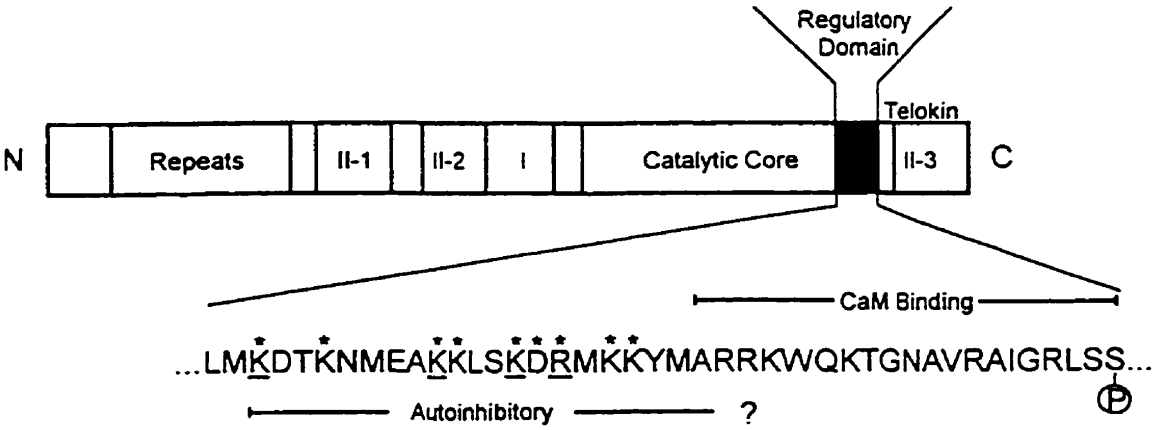


FIGURE 2 Domain organization of mammalian smooth/nonmuscle MLCK. Residues in the regulatory domain have been examined individually by mutagenesis for involvement in autoinhibition (asterisk). Four basic residues (underlined) may contribute to autoinhibition by binding to acidic residues in the catalytic core. The sequence of the calmodulin binding domain is noted. The original pseudosubstrate sequence (. . . RRKWQKTGNAV . . .) is in the N terminus of the calmodulin binding domain. The serine phosphorylated *in vivo* by the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II is identified with the circled "P." The phosphorylation desensitizes MLCK to activation by Ca^{2+}/calmodulin.

2. 5. Isoforms of Myosin Light Chain Kinase

Several isoforms of MLCK have been identified in a variety of striated and smooth muscle and non-muscle tissues, two of the most important of which are sm-MLCK and nm-MLCK. The important role of sm-MLCK stems from its being a serine/threonine kinase which specifically phosphorylates residues (Serine[19], Threonine[18]) on the 20kDa MLC_{20} of myosin molecules. This phosphorylation activates actin-activated myosin Mg^{2-} ATPase resulting in cross-bridge cycling. Another example of MLCK function is incorporated in a suggestion of Sweeney (66). He has adduced evidence to suggest that in skeletal muscle phosphorylation of the RLC leads to contraction-induced potentiation of isometric tension production by conformational change of the myosin head. Additionally Fishkind et al. (67) reported that in normal rat kidney cells (NRK-52E; American Type Culture Collection, Rockville, MD) activity of MLCK can affect mitosis and cellular cortical activities, however additional control mechanisms are likely involved in the regulation of cytokinesis.

The above evidence shows the presence of MLCK in both muscle cells and in non-muscle contractile cells. In the latter cell type, non-muscle MLCK is the predominant isoform while in muscles, sm-MLCK is predominant. In the muscle cells a small amount of nm-MLCK is also present. In fully contractile cells, sm-MLCK clearly regulates contractility, while nm-MLCK seems to regulate cell replication, migration, receptor capping and platelet or endothelial cell contraction (68, 69, 70, 71, 72, 73, 74, 75).

While nm-MLCK has been reported (76) in vascular and intestinal smooth muscle, it is transcribed from the same gene as the sm-MLCK isoform, either by alternative splicing or through a transcriptional initiation at a site distinct from that of the sm-isoform (62, 61). However, no studies have yet demonstrated whether nm-MLCK is present in adult airway SMCs.

The nm-isoform (206kDa) possesses a larger molecular weight than the sm-isoform (138kDa) and is expressed in high abundance in embryonic SMCs and some non-muscle contractile cells. No data regarding the activity, substrate specificity and cellular localization of the nm-isoform exist. Interestingly the nm-MLCK isoform possesses a larger amino-terminal domain which is a portion of the kinase thought to interact with structural arrays of myosin and actin filaments (77).

To complete the tally of known isoforms to date mention must be made of the embryonic isoform identified by Gallagher et al. (78). This novel 208kDa MLCK is distinct from sm- and nm-MLCK and has been identified by cross-reaction to two antibodies raised against sm-MLCK. Additional antibodies against the NH_2^+ and COO^- - terminals of the sm-MLCK do not react with the 208kDa MLCK, suggesting these regions are distinct. Nevertheless the embryonic isoform of MLCK does phosphorylate the 20kDa myosin light chain in a Ca^{2+}-calmodulin dependent manner, supporting its place in the MLCK family. The unique feature of this embryonic isoform, and from which it derives its name, is that it is most abundant during early development and declines at birth.

Fisher and Ikebe (76) reported the presence of a 206kDa MLCK isoform which is expressed in developing smooth muscle-containing tissues from chicken embryos. They named it embryonic/non-muscle MLCK isoform because its higher level of expression occurred in early development in chicken embryos.

Gallagher and Garcia et al. (78) recently described yet another isoform of MLCK in cultured endothelium. Its molecular weight was 214kDa and it exhibited the ability to phosphorylate the 20kDa myosin light chain. Augmentation of intracellular cAMP levels markedly enhanced MLCK phosphorylation and reduced kinase activity by 4-fold in MLCK immunoprecipitates. These data suggest novel mechanisms of endothelial cell contraction and barrier regulation.

Finally, the extreme COOH terminus of smooth muscle MLCK is a highly acidic region of unknown function that is also expressed as an independent protein, telokin (62). Telokin, and by analogy, the COOH terminus of smooth muscle MLCK have been proposed to be involved in myosin binding and myosin filament assembly (79). In closing this section it must be mentioned that while the function of sm-MLCK in regulating contraction is well understood, that of the other isoforms has not yet been clearly elucidated.

2.6. Differentiation of Smooth Muscle Cells

It is well known that smooth muscle cells exhibit a high degree of plasticity and do not undergo terminal differentiation (3, 80). The principal function of mature SMCs is contraction which depends on expression of a large number of different contractile and regulatory proteins in precisely controlled concentrations (81). These cells undergo a rapid phenotypic modulation in primary culture which is manifested by marked decreases in the content of smooth muscle specific contractile and regulatory proteins and increases of non-muscle proteins (15, 16). Retention of differentiated contractile and regulatory proteins and functional surface receptors were reported in cultured SMCs (82), and re-expression of smooth muscle marker proteins was identified in post-confluent cultures (16), but contraction of SMCs in culture has not been shown. Studies using magnetic twisting cytometry and atomic force microscopy suggested that cultured SMCs retain the ability to stiffen in response to contractile agonists (83, 84). Ca^{2-} transients were also reported in cultured SMCs in response to a variety of contractile agonists (46, 85). However this indirect evidence does not prove the existence of contraction in cultured SMCs. Presently it is believed that cells in non-confluent proliferative culture undergo dedifferentiation which only partially reverses at confluence. Functionally they remain very poorly contractile. In contrast with striated muscle cells, relatively little is currently known about molecular mechanisms that control smooth muscle differentiation, due in part to the extreme plasticity of this type of cells, and to limitations with respect to the inducibility and/or retention of the differentiated phenotype in cultures. Many factors can

cause the phenotypic modulation of SMCs among which growth factors such as platelet-derived growth factor (PDGF), endothelial-derived growth factor (EDGF), monocyte/macrophage-derived growth factor (MDGF) and lipoprotein, are very important in the maintenance of smooth muscle integrity and proliferation in culture. Absence of these factors, as in serum deprivation, can cause growth arrest, and might affect the phenotypic modulation and the function of the SMCs.

3. HYPOTHESIS AND SPECIFIC OBJEVTIVES

3.1. Hypothesis:

1) Airway SMCs show phenotypic switching in culture from contractile synthetic type.

2) In serum deprivation cultured cells modulate to a contractile phenotype.

3.2. Specific Objevtives:

1) To show changes in phenotypic structure of the cultured cells;

2) To show changes in contractile, regulatory and cytoskeletal protein phenotype of the cells in culture.

4. MATERIALS AND METHODS

4.1. MATERIALS

All media, antibiotics, sera and other reagents used for cell culture experiments were obtained from Life Technologies-Gibco/BRL (Burlington, ON) unless otherwise noted. Plastic tissue culture plates used were from Corning Costar (Cambridge,MA). Collagenase used for the isolation of cells from tissue for primary culture was purchased from Life Technologies-Gibco/BRL (Burlington, ON). Elastase and nagarase protease were obtained from Sigma Chemicals (St. Louis, MO). All reagents for protein electrophoresis were obtained from Bio-Rad (Mississauga, ON). Nitrocellulose membrane (0.22μm pore size) was manufactured by Micron Separation Inc. (Westboro, MA). The Enhanced Chemiluminescence reagents (Amersham Life Science Inc., Oakville, ON) were used for chemiluminescence detection of proteins on immunoblots. Chemilumigrams were developed on Hyper Film ECL film (Amersham Life Science, Oakville, ON). Biotinylated secondary antibodies and horseradish peroxidase-conjugated antibodies were also obtained from Amersham Life Science Inc.

Paraformaldehyde used for immunocytochemistry was purchased from TAAB Laboratories Equipment Ltd. (Reading, Berks, England). FITC-conjugated mouse, and rabbit antibodies were purchased from Becton Dickinson (San Jose, CA). All other FITC- and Cy^3-conjugated antibodies used in fluorescence immunocytochemistry study were

obtained from Jackson and ImmunoResearch Labs Inc. (Bio Can Scientific, Mississauga, ON). Nylon filters used in preparation of cells for flow cytometry purchased from BioDesign Inc. (Carmel, New York).

4.2. METHODS

4.2.1. Canine Tracheal SMC Primary Culture

Tracheas were excised from anesthetized, 6-12 month old mongrel dogs and placed into ice-cold calcium-free, aerated Kreb's solution. Trachealis muscle was dissected, cleaned of serosa and epithelia at room temperature, and washed four times under aseptic conditions in Hank's Balanced Salt Solution (HBSS) containing 100mg streptomycin/ml and 100U penicillin/ml. The muscle was minced thoroughly with fine scissors, then cells were isolated by resuspending the tissue slurry in 12 mls of digestion buffer (HBSS containing 600 U collagenase/ml, 8 U type IV elastase/ml, and 1 U type XXVII Nagarse protease/ml). Cells were incubated at 37^{0}C for 45 mins. in a shaking waterbath 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 supernate, containing isolated cells, was removed using a Pasteur pipette. The supernate was immediately diluted 1:1 with DMEM containing 10% foetal bovine serum (FBS) and antibiotics and then the cells were pelletted by centrifugation (600xg, 5 mins.). The resulting supernate was discarded and pelleted cells were resuspended in 5 mls of fresh

DMEM/10%FBS plus antibiotics and stored on ice.

A fresh aliquot (12 mls) of digestion buffer was added to the tube containing the debris that remained after the first digestion. After mixing, the debris was placed back in the shaking water bath for a further 40 mins. to isolate the remaining SMCs. After the second digestion, the tissue pieces were triturated again and the remaining debris allowed to settle. The supernate was carefully removed using a Pasteur pipette and diluted 1:1 with DMEM/10%FBS/antibiotics, centrifuged and resuspended in fresh DMEM in the same manner that the first digestion was handled. The first and second digest fractions were subsequently pooled and filtered through 70μm nylon mesh. The cell suspension was pelletted by centrifugation then diluted in DMEM/10%FBS/antibiotics. Cell number was estimated by counting using a model Z_{BI} Coulter counter or a haemocytometer after which the cells were plated onto 100-mm plastic culture dishes at a density of 5000 cells/cm^2 and allowed to attach for 36 hrs. Cells were grown at 37^{0}C in a humidified atmosphere consisting of 95% air/5% CO_2. The medium was then replaced with fresh medium containing 10%FBS and antibiotics every 72 hrs. Typically each experiment used cells isolated from 1 dog. In general 500mg - 1 gm of crude tracheal muscle from 1 dog (epithelium removed) yielded 8 - 12 x 10^6 SMCs for primary culture.

4.2.2. Long Term Culture

To passage confluent cultures, plates were first washed three times with ice cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) which had been prewarmed to 37^0C. Following the final wash, PBS was aspirated. Cells were lifted by adding a solution containing trypsin (0.05%)-EDTA.4Na(0.53mM) in PBS (2 ml for each 100mm diameter plate). After 1-1.5 min cells were dislodged from the plates by trituration using a Pasteur pipette. The cell suspension was transferred to a conical polystyrene centrifuge tube and diluted with DMEM/10%FBS plus antibiotics then centrifuged (600xg, 5mins) to pellet cells. The supernant was discarded and cells were resuspended in fresh DMEM. A 1 to 4 split was employed to passage the cells.

4.2.3. Growth Arrest

For experiments in which growth-arrested cells were required, the following protocol was employed. When the culture was confluent (day7-9), culture medium was aspirated and the plates were subsequently washed three times with PBS/antibiotics which had been prewarmed to 37^0C. After removing the final PBS wash, the cells were washed once with serum-free arrest media, which was composed of Ham's F-12 Medium containing insulin (5μg/ml), transferrin (5μg/ml), selenium (5μg/ml) and antibiotics. Then the medium was added to a volume of 6 mls/plate and the plates were returned to the cell culture incubator. The medium was replaced every 72 hrs up to 15 days. The cells were

fixed for immunocytochemistry or prepared for protein homogenates at different times in culture by the methods described below.

4.2.4. Preparation of Protein Homogenates

A fraction of the tracheal SMCs isolated for culture was removed prior to plating and used to prepare crude protein homogenates. The isolated cells were washed by resuspension in ice-cold PBS following centrifugation. This step was repeated twice; after the second resuspension step, cells were repelleted by centrifugation and protein extracts were prepared by Tris lysis buffer containing 1.5% Nonidet P-40 (pH 7.6) to which fresh protease inhibitors, such as leupeptin, phenylmethyl sulfonyl fluoride(PMSF), and soybean trypsin inhibitor, were added. Cells were disrupted using a syringe. Samples were stored at -20^{0}C until used for electrophoretic analysis.

To prepare extracts from cultured cells (primary culture, passaged culture, growth-arrested culture), the medium was aspirated, then plates were rinsed three times with cold PBS. Cells were lysed and proteins solubilized by adding 750μl extraction buffer to each 100mm dish. Cells were further disrupted by scraping with a rubber policeman, then the extracts were placed into tubes and stored at -20^{0}C for use.

4.2.5. Protein Assay

The protein concentration of the extracts was estimated spectrophotometrically using the Bio-Rad Protein Assay Kit which is based on the Coomassie dye-binding protocol first described by Bradford (1976). Absorbance of 595 nm light was measured using a Milton Roy Spectronic 1001 Plus spectrophotometer. Bovine serum albumin (BSA), Fraction V (Sigma Chemicals, St. Louis) was used as a relative protein standard for all assays. Assay results were used to calculate the dilution required to bring all samples to a protein concentration of 2 mg/ml prior to storage at -20^{0}C.

4.2.6. Western Blot Analysis of Protein Homogenates

Samples were prepared for SDS-PAGA by diluting into loading buffer. Equal protein aliquots (6μg/well) were loaded into the wells of 6% SDS-PAGE 8cm x 10 cm mini-gels. Proteins were fractionated by running the gels at constant voltage (200V) for 45 mins. at room temperature. Transfer time was 48 mins. using a constant voltage of 100 V. Immediately after transfer, blots were washed for 5 mins in Tris-buffered saline (TBS) (20mM Tris, 500mM NaCl, pH 7.5) containing 0.1% Tween-20. Blots were subsequently stained for total protein using Ponceau S (Sigma Chemicals, St. Louis). After 15 mins. blots were rinsed in distilled water to remove background staining and visualize all bands. Blots were then destained by washing the blots in water untill no color remained, and were incubated in TBS-0.1% Tween-20 (TTBS) for 30 mins. prior to subsequent

immunodetection of protein constituents.

For immunodetection, blots were blocked overnight at 4^0C in 10mM Tris buffered saline, 0.1% Tween (TTBS) containing 3% non-fat dried milk powder. Blots were then incubated in primary antibody (1:1000 mouse anti-sm-MLCK monoclonal antibody, M4907, Sigma; 1:10,000 mouse anti-sm-α-actin, Boehringer Mannheim Canada, Laval, PQ; 1:7500 rabbit anti-nm-MLCK polyclonal antibody, D119, gift from Dr. Gallagher, Indiana U; 1: 5000 rabbit anti-sm-MHC antibody, supplied by Groeschel-Stewart 1976.) diluted in TTBST containing 1% milk for 1 hour at room temperature on a shaker. Blots were then rinsed three times with TTBS/1%milk before being incubated for 40 mins. at room temperature with biotinylated secondary antibodies (Amersham Life Science, Oakville, ON) diluted 1:1000 in TTBS/1% milk powder. Blots were rinsed again with TTBS and finally incubated for 40 mins. at room temperature with streptavidin-horse radish peroxidase conjugate (Amersham Life Sciences, Oakville, ON) diluted in TTBS. Semi-quantitative staining of specific proteins was achieved using chemiluminescence detection; blots were washed in TTBS and subsequently dipped for one min into luminol substrate solution (Amersham Life Sciences, Oakville, ON); Chemilumigrams were developed on Hyperfilm-ECL (Amersham Life Sciences, Oakville, ON); the normal exposure times ranged from 30 secs. to 5 mins.

4.2.7. Laser Densitometry

An LKB Ultroscan XL laser densitometer was employed to scan the developed films for estimation of protein content. All values were normalized to total protein loaded onto the gels to allow for comparison between samples. The entire width of the bands on chemilumigrams was scanned by consecutive parallel and adjacent passes, which covered an 8 μm path width each time. Data for each scan were captured using Gel Scan XL 2400 software; Scanplot (Cunningham Engineering, Calgary) was used to convert the raw data into X- and Y-plane positional data and the Z-axis into absorbance data. After subtraction of background absorbance, integration of the three-dimensional raw data yielded a volume that correlates quantitatively to the amount of stained protein in a particular band. Linearity of the detection of the chemiluminescent signal from the blots by Hyper film ECL was determined prior to any experiments by running blots with a wide range of standards at known protein concentrations. The numbers obtained revealed that the response of the film was linear from 0.08 up to 1.9 AU maximum, hence all scanned bands had to fall into this range in order to be included in subsequent statistical analysis. Data are expressed as relative amount of protein (arbitrary units) on graph.

4.2.8. Fluorescent Immunocytochemistry

For fluorescent immunocytochemistry, freshly isolated cells were plated in 6-well dishes containing 22x22mm Rat Tail Collagen, Type I coated glass coverslips (Becton

Dickinson). At predetermined times (Day1,2,6&7) after cell plating in primary culture, and daily after growth arrest, the coverslips and the attached cells were rinsed with PBS and the attached cells were then fixed in 1% paraformaldehyde/PBS (pH 7.6) for 15 mins. at 4°C. Cells were subsequently permeabilized by replacing the fixing solution with 0.1% TritonX-100/PBS and incubating for 15 mins. at 4°C. Coverslips were then rinsed 3 times with PBS and were used immediately for immunostaining or were stored in PBS/0.05% sodium azide for a maximum of 14 days.

For immunostaining, individual coverslips were transferred into separate 35 mm plastic dishes and then rinsed twice with 2 ml of PBS/0.1% Tween-20. The coverslips were then coated with approximately 100 μl of blocking solution (PBS / 5% normal goat serum / 0.1% Tween-20) and incubated for 2-4 hrs. at 4°C in a humidified chamber. The blocking solution was removed by rinsing the coverslips once with 2ml of PBS/ 1% BSA/ 0.1% Tween-20 in a 35 mm plastic dish. All subsequent antibody incubations were carried out in humidified staining chambers at 4°C unless otherwise stated.

Approximately 100μl of PBS/1%BSA/0.1% Tween-20 containing diluted primary antibody was added onto each coverslip to cover the attached cells and subsequently incubated overnight. The antibodies used were as follows: 1:200 rabbit anti-nmMLCK, D119; 1:25 mouse anti-smMLCK; 1:25 mouse anti-sm-α-actin, Boehringer Mannheim Canada, Laval, PQ; 1:25 rabbit anti-sm-MHC, Groeschel-Stewart; 1:25 mouse anti-β-tubulin, Amersham International plc.1:100 mouse anti-desmin, Boehringer Mannhein,

Germany; and 1:100 rabbit anti-vimentin, Boehringer Mannheim GmbH, Germany. Negative controls were incubated in PBS/1%BSA/0.1%Tween-20 without primary antibody. All subsequent washing and staining steps were identical for stained cells and their matched negative controls. Coverslips were washed three times using 3-4 mls of PBS/1%BSA/0.1% Tween-20 and then secondary antibody solutions were added.

All secondary antibodies were diluted 1:100 in PBS/1%BSA/0.1% Tween-20 according to manufacturer's instructions. The secondary antibodies included: 1) FITC-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, Code Number 515-095-003); 2) FITC-conjugated donkey anti-rabbit IgG (Amersham Life Science, Catalogue Number N1034); 3) Cy^3-conjugated goat anti-mouse IgG and IgM (Jackson ImmunoResearch, Code Number115-165-044); and 4) Cy^3-conjugated sheep anti-rabbit IgG (Jackson ImmunoResearch, Code Number111-165-003). The applied secondary antibody or combination of secondary antibodies was determined by the identity of the specific primary antibody or combination of primary antibodies employed. Approximately 100μl of secondary antibody solution was added to each coverslip and the cells were subsequently incubated in the dark, for 1-2 hrs at 4^0C.

After secondary antibody incubation, coverslips were washed three times with 2ml. of PBS then twice with double-distilled water. Coverslips were subjected to nuclear staining using Hoechst 33342 dye prior to being mounted, cell side down, on glass slides using anti-fade media (85%glycerol/1mM p-phenylenediamine/100mM Tris-HCL, pH 7.4).

To stain cells for DNA, after the final water-wash noted above, the individual coverslips were placed in a 35 mm plastic dish and immersed in 3ml. of Hoechst 33342 dye (20μg/ml bisbenzamide, Catalogue Number H-33342, Sigma Chemicals, St. Louis), for 1 min. Coverslips were then removed, rinsed twice in 2ml. of double-distilled water and subsequently mounted onto microscope slides using mounting media described above. Prepared slides were stored in the dark at -20^{0}C until they were viewed and photographed using a Olympus BHT-2 microscope equipped with epifluorescence optics and a 35mm camera. Confocal microscopic study was carried out on primary cultured cells in cooperation with Dr. Dwight Nance, Department of Pathology, Faculty of Medicine, University of Manitoba.

5. RESULTS

Typically, 8-12 x 10^6 SMCs were isolated from the canine trachealis tissues of each dog used for generating primary cultures in these studies. Cell viability, assessed by trypan blue staining, ranged from 65-85%. The cells started to attach to the bottom of the culture dish about 6 hrs. after seeding. The cell number in each culture dish began to increase approximately 4 days after plating until confluence (covering 90% of dish) was reached 8-10 days after initial seeding. At confluence cultures (cell density = $1x10^7$ per plate) exhibited a "hill-and-valley" pattern as described by Tom-Moy (11) and possessed numerous areas of multi-layered, focal over growth, which is common to SMCs in confluent cultures (11). The SMCs appear spindle- or ribbon-shaped with a large, oval, central nucleus marked by distinct nucleolus or nucleoli and having well-defined perinuclear granules and/or vesicles. Immunofluorescent staining revealed that greater than 95% of the cells in the primary cultures were positive for both sm-α-actin and sm-MHC seven days after initial seeding.

The general profile of proteins in isolated tracheal myocytes varied considerably with time in culture. Western immunoblot assays were used for semi-quantitative measurement of the temporal changes in marker proteins. Laser densitometric analysis of sm-MLCK on 6% SDS-PAGE gels revealed a large and significant ($p<0.05$) decrease (>75%) occured in cellular sm-MLCK content within 5 days of primary culture. Conversely nm-MLCK content increased dramatically (>40 fold) in primary culture

(Fig.3). Temporal patterns of sm- and nm-MLCK expression were also measured in long-term primary cultures. The nm-MLCK content increased abruptly 5 days after cell plating and increased steadily up to 20 days in culture. Figure 4 shows a chemilumigram obtained from a single experiment. We can see a 206kDa band at day 6 that is detected by anti-nm-MLCK antibody, and this band gets thicker and darker with increased time in culture, indicating increased expression of this protein. At day 0, there is almost no visible band at 206kDa. Instead a lower band, with a molecular weight 138kDa was detected by the nm-MLCK antibody, which is the smooth muscle isoform of myosin light chain kinase. Figure 5 shows the results of three experiments. The nm-MLCK content increased significantly within 5 days after culture and remained high until day 20. This change in concentration of MLCK isoforms likely indicates the phenotype switching of airway SMC in primary cultures.

We compared the content of sm-MLCK over several passages in confluent cultures. The protein content appeared to decline with time, however some oscillations were apparent (see passage 1 and 5) as shown in Figure 6. Less than 20% of sm-MLCK in freshly isolated SMCs remained after 7 passages in culture. Non-muscle-MLCK was maintained at very high levels in long term culture(Fig.7). (The standard errors are high; this is due to the biological variability resulting from the pooling of data from different experiments. Comparisons of means were carried out using the Kruskal-Wallis test combined with the Dwass test). At very late passages (after passage7), an additional band with a molecular weight 214kDa, similar to that reported for the endothelial isoform of

MLCK, was visualized with the nm-MLCK antibody. The extra band is seen in P_7D_5 and P_9D_5 (P=Passage number; D=day number) of Figure 8. It is believed to be the recently reported endothelial isoform of MLCK.

Smooth muscle-α-actin is the earliest marker of differentiation and also the most abundant protein in mature SMCs. The expression of this protein in cultured cells was studied. Laser densitometric analysis of sm-α-actin on 10% SDS-PAGE gel indicated an abrupt decrease in cellular sm-α-actin level (Fig.9) consistent with the decrease of cellular sm-MLCK in primary culture. The similar oscillation pattern of sm-α-actin(Fig.10) and sm-MLCK in long term cultures is of interest.

Fluorescent immunocytochemical analysis of protein markers in tracheal SMCs was carried out on cells grown on coverslips as described before. Immunocytochemical staining of primary cultured cells showed a typical "hill and valley" pattern at confluence. The immunostaining with antibodies that recognized either sm-MLCK or nm-MLCK isoforms demonstrated a difference in the subcellular localization pattern of sm-MLCK and nm-MLCK. Staining showed that greater than 95% of attached cells in the primary culture were positive for both sm- and nm-MLCK. Smooth muscle-MLCK was distributed homogenously throughout the cytoplasm as seen in Figure 11A. Most of the nuclei were not stained for sm-MLCK as shown by the transparent area in the center of the cell and the further Hoechst staining of the nuclei (Fig.11B). Non-muscle MLCK staining showed fibers mostly running in parallel and along the long axis of the cell, and an intense

nuclear staining was seen (Fig.12A). This was different from that for sm-MLCK in which no nuclear staining was seen. Figure 12B shows the nucleus of the same cell stained with Hoechst 33342 which is specific for DNA. This suggests that what was identified as staining for nm-MLCK in the nucleus was indeed the nucleus. This was substantiated by confocal microscopy. Figure 13 shows a series of 6 sections at 0.8 μm intervals through a cell. The series show that nm-MLCK was maximally localized to the nucleus, but also is seen along stress fibers. Interestingly, mitotic cells demonstrated intense cytoplasmic staining for nm-MLCK as shown in Figure 14A. Figure 14B shows the same cells with their nuclear DNA stained with Hoechst 33342. Further studies at higher magnification demonstrated that condensed chromatin was not stained with the antibodies. Figure 15A shows localization, in a stress fiber-like arrangement, of nm-MLCK in the cytoplasm. Figure 15B in which FITC-labelled anti-sm-α-actin was used, suggests colocalization of sm-α-actin and nm-MLCK in the cytoplasm along the stress fiberss. Figure 16A shows the localization of nm-MLCK in the cytoplasm along the stress fibers and exclusively in the nucleus. Figure 16B shows localization of β-tubulin visualized with monoclonal specific antibodies. It is present in the cytoplasm homogeneously, but no immunoreactivity can be detected in the nucleus. No evidence of co-localization of β-tubulin and nm-MLCK can be identified.

To study the effect of serum deprivation on cultured SMCs, the cells were cultured in F-12/Redu-serum after confluence. Morphologically, two distinct groups of cells appeared in post-confluent primary SMC culture during long term serum deprivation.One

group consisted of cells that were flat, rounded and similar to most of the cells present before arrest (Fig.17A). The second group showed the normal elongated, spindle shape cell; Most of them were aligned side by side and end to end in bundles. There was a refractile sarcolemma around each cell (Fig.17B). These cells began to appear after two days of growth arrest and their number was increased as deprivation prolonged. After 15 days of deprivation, they comprised about 28.5%±4.6 (SE) of all the cells present and occupied almost 40% of the total area of the culture dish.

Western blot analysis showed that with time, there was an increased number of elongated spindled shaped cells and the expression of contractile proteins increased. After 15 days of arrest, total smooth muscle myosin heavy chain content increased 10.8±1.1 fold, sm-α-actin content increased 5.9±1.0 fold (Fig.18A). Sm-MLCK increased by 62.9±13.5 fold compared with contents of the cells in non-arrested confluent cultures(Fig.18B). The content of sm-MHC reached the same level as in freshly-isolated cells (Fig.19). Surprisingly the content of sm-MLCK and sm-α-actin were significantly higher than those of freshly isolated cells, up to 30 times for sm-MLCK and 2 times for sm-α-actin.

Immunocytochemical study was employed to further study the characteristics of the elongated cells. Specific antibodies were used. The spindled-shaped cells were intensively stained for sm-MHC, sm-α-actin, and sm-MLCK, while the flat cells stained less intensively with sm-MLCK and were almost negative for sm-MHC and sm-α-actin

(Fig.20). In addition, these cells were also strongly stained for nm-MLCK and β-tubulin (Fig.21). Centrally located cigar-shaped nuclei were identified in spindled shaped cells, but round nuclei were seen in flat cells (Fig.20). Before serum deprivation, most of the cells (>90%) stained positively for all the above antibodies.

For sm-MLCK staining, there was a significant change in the cellular distribution of sm-MLCK. Before arrest, the cells were homogeneously stained for sm-MLCK in the cytoplasm. At day 1 of arrest sm-MLCK demonstrated a "spidery" distribution in the cytoplasm. After day 2, the elongated cells appeared which were strongly and homogeneously stained for sm-MLCK. With prolonged serum deprivation, more and more elongated cells appeared and they were arranged like bundles in the "valley" as shown at day12 (Fig.22).

To study the role of desmin in the differentiation and phenotype switching of the SMCs in culture, the cells were stained with anti-mouse-desmin antibody. Before arrest, the cells did not stained for desmin. Then with increasing times of serum deprivation, the number of elongated cells that were intensly desmin-stained decreased (Fig.23). The positively-stained cells appeared early at day 1 of arrest. On the contrary, the cells were stained with vimentin before arrest, during arrest, the vimentin staining of both the flat cells and elongated cells stayed the same. There was no enhanced staining of elongated cells with vimentin relative to round cells (Fig.24).

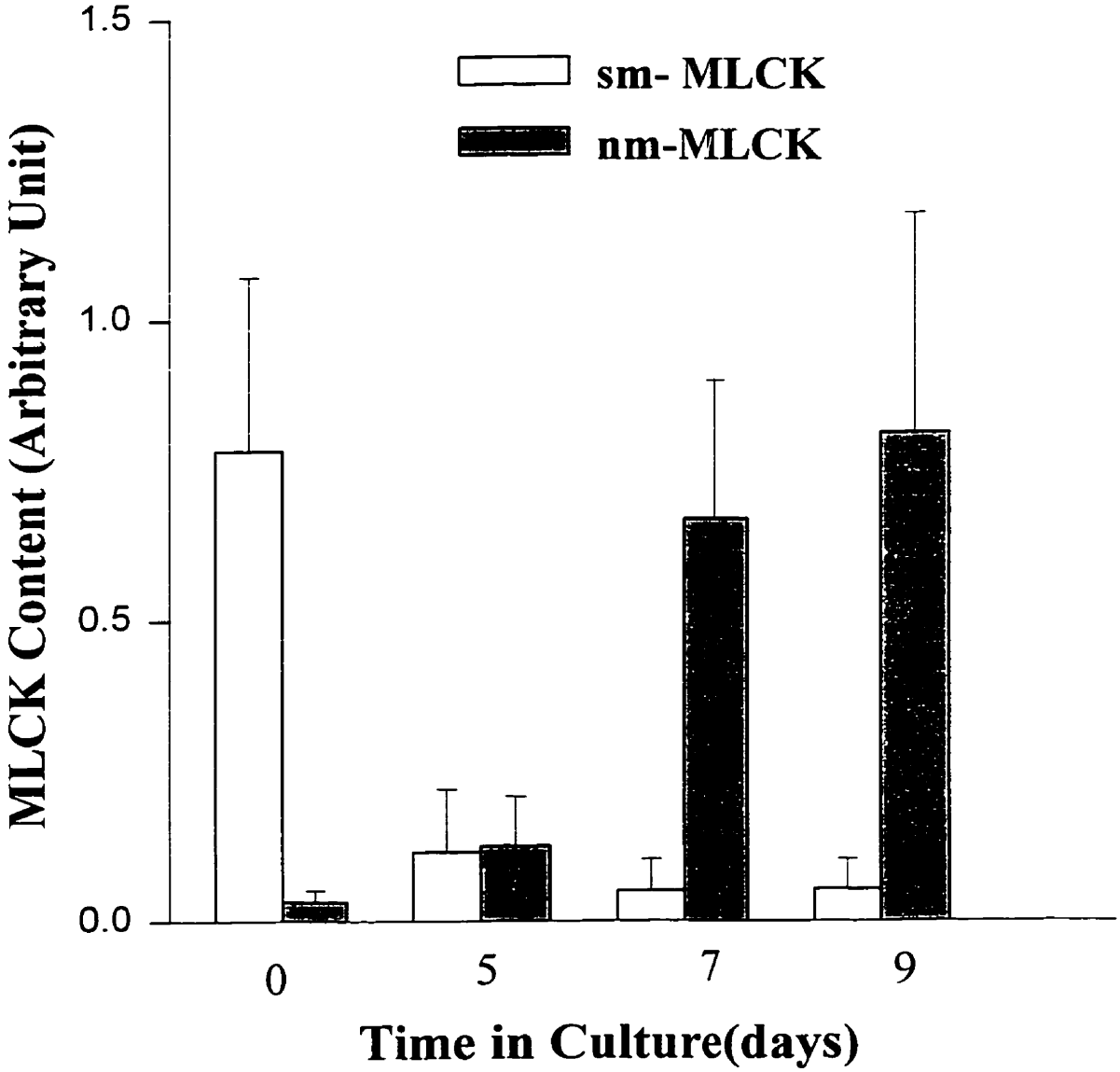
Other studies conducted by Xuefei Ma of our laboratory also showed that these elongated spindle-shaped cells had surprisingly higher shortening capacity and velocity (personal communication). These data suggest that serum deprivation induces super-contractile SMCs and phenotypic switching from "synthetic" state to "contractile" state.

FIGURE 3. Change in content of sm- and nm-MLCK isoforms in primary culture. Laser densitometric analysis of sm-MLCK on 6% SDS-PAGE gel revealed a statistically significant decrease in cellular sm-MLCK content by 5 days of primary culture (open bar). Conversely nm-MLCK content increased dramatically in primary culture (closed bar). Confluence occurred between day 7 to day 9. (N=6, error bars=SE).

MLCK Content

in Canine Tracheal Smooth Muscle Cells

-Primary Culture-



WESTERN BLOT ANALYSIS: NM-MLCK IN CANINE TRACHEAL SMOOTH MUSCLE CELLS IN PRIMARY CULTURE

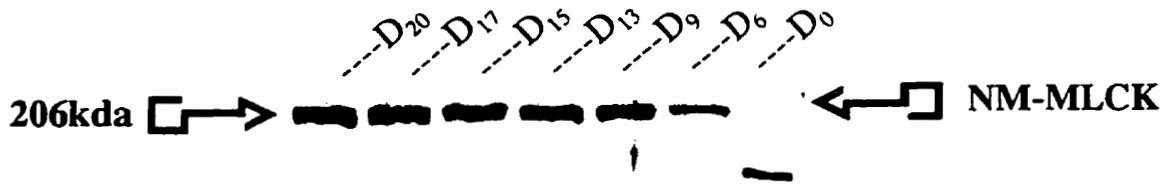


FIGURE 4. Chemilumigram from a single long duration (up to 20 days) primary culture. A 206kDa band is seen at day 6 (D6) which gets darker with time and achieves a maximum at day 20 (D20). At day 0 (D0) this band is not seen at all. However a band is seen lower down at 138kDa molecular weight and probably represents sm-MLCK.

FIGURE 5. Change of nm-MLCK content in long duration primary cultures (up to 20 days). Western blot analysis of nm-MLCK content on 6% SDS-PAGE minigels showed that the nm-MLCK content increased with time of culture. (N=3, error bars=SE).

NM-MLCK Content in Canine Airway Smooth Muscle Cells

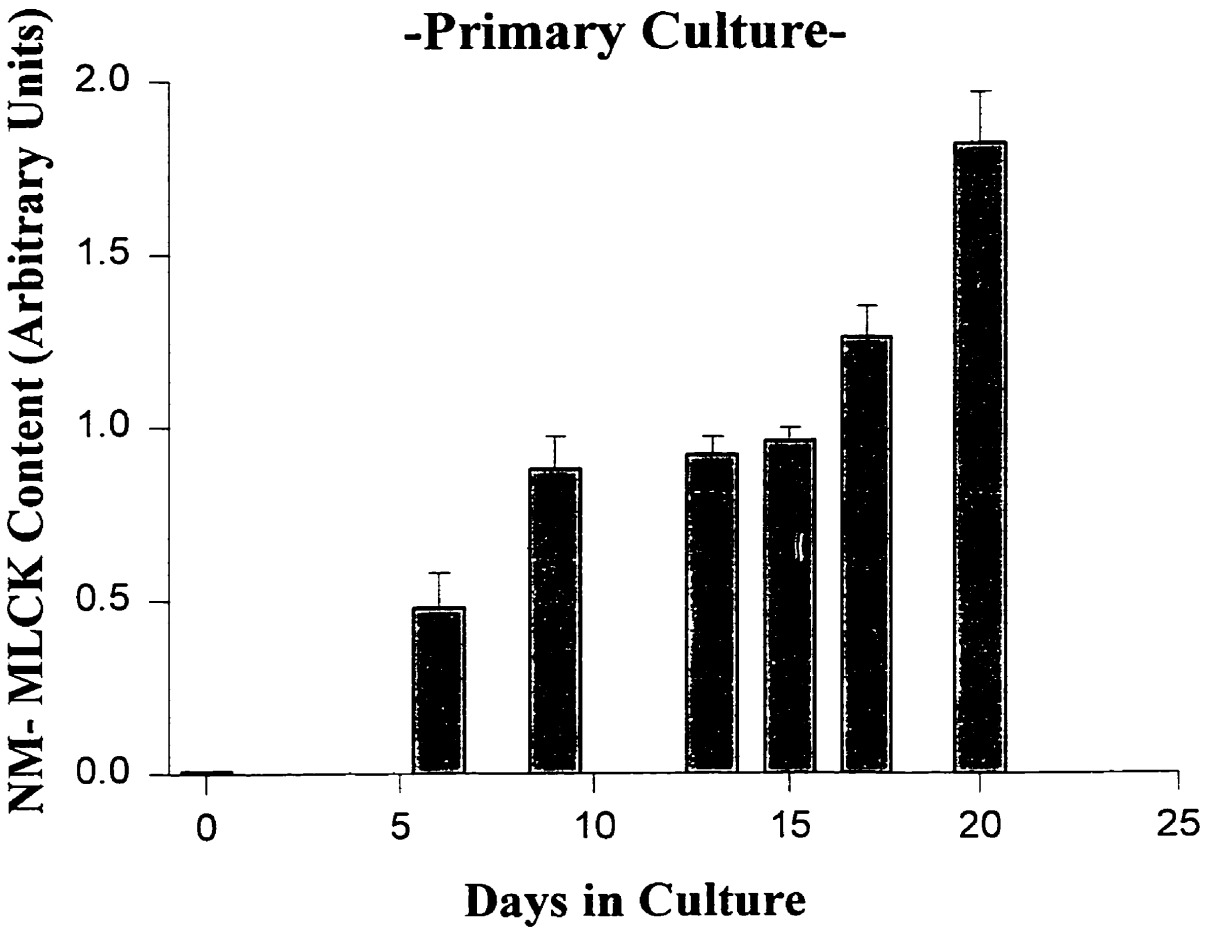


FIGURE 6. Change in sm-MLCK content in long-term culture. Western blot analysis of sm-MLCK content on 6% SDS-PAGE minigels showed that the sm-MLCK content at confluence appeared to decline with each passage, however some oscillations were apparent (see passage 1 and 5). "iso"= freshly isolated cells. Cells were passaged every 5-7 days at confluence. (N=6, error bars=SE)

smMLCK Content
in Canine Tracheal Smooth Muscle Cells
-Long Term Culture-

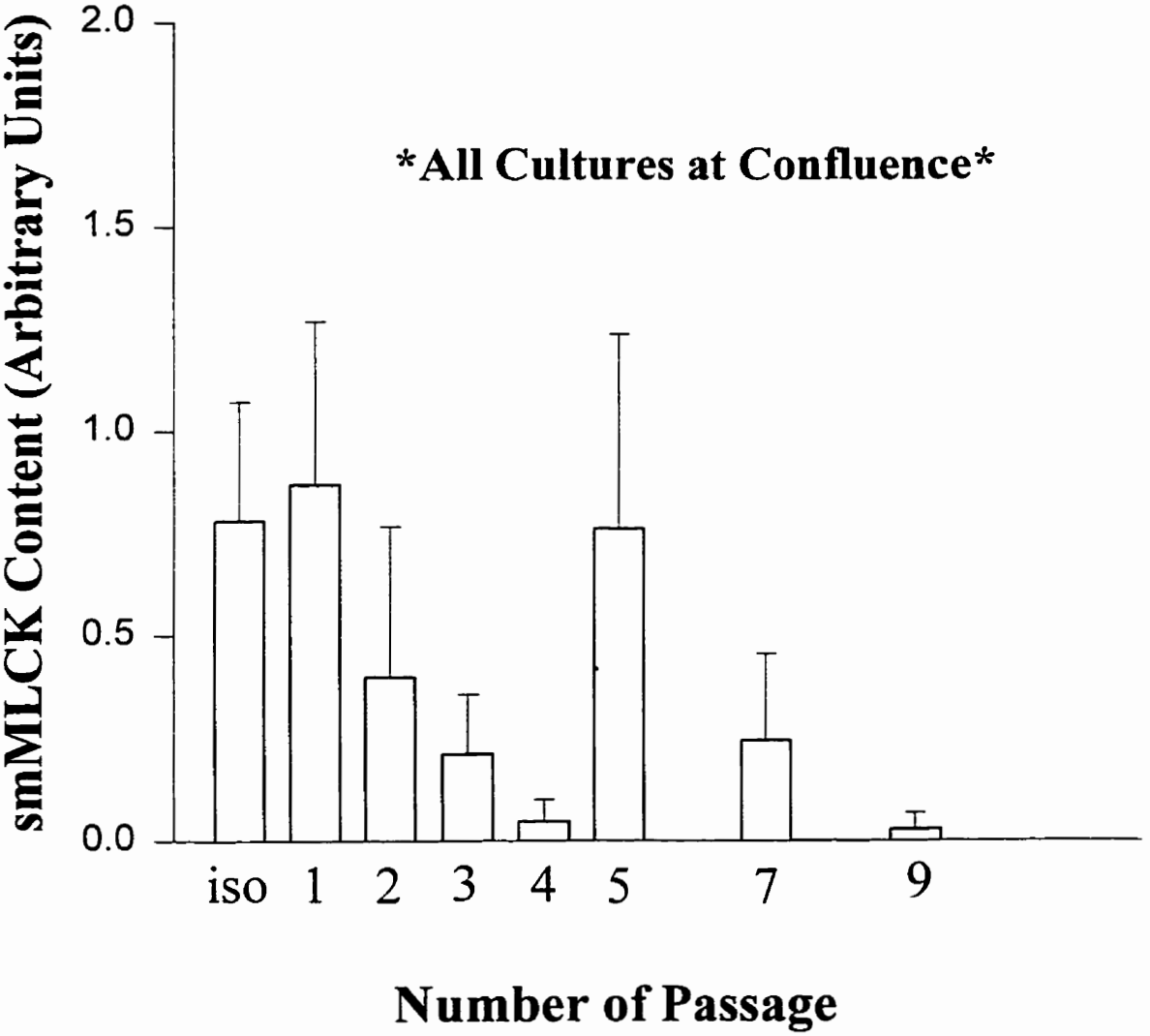
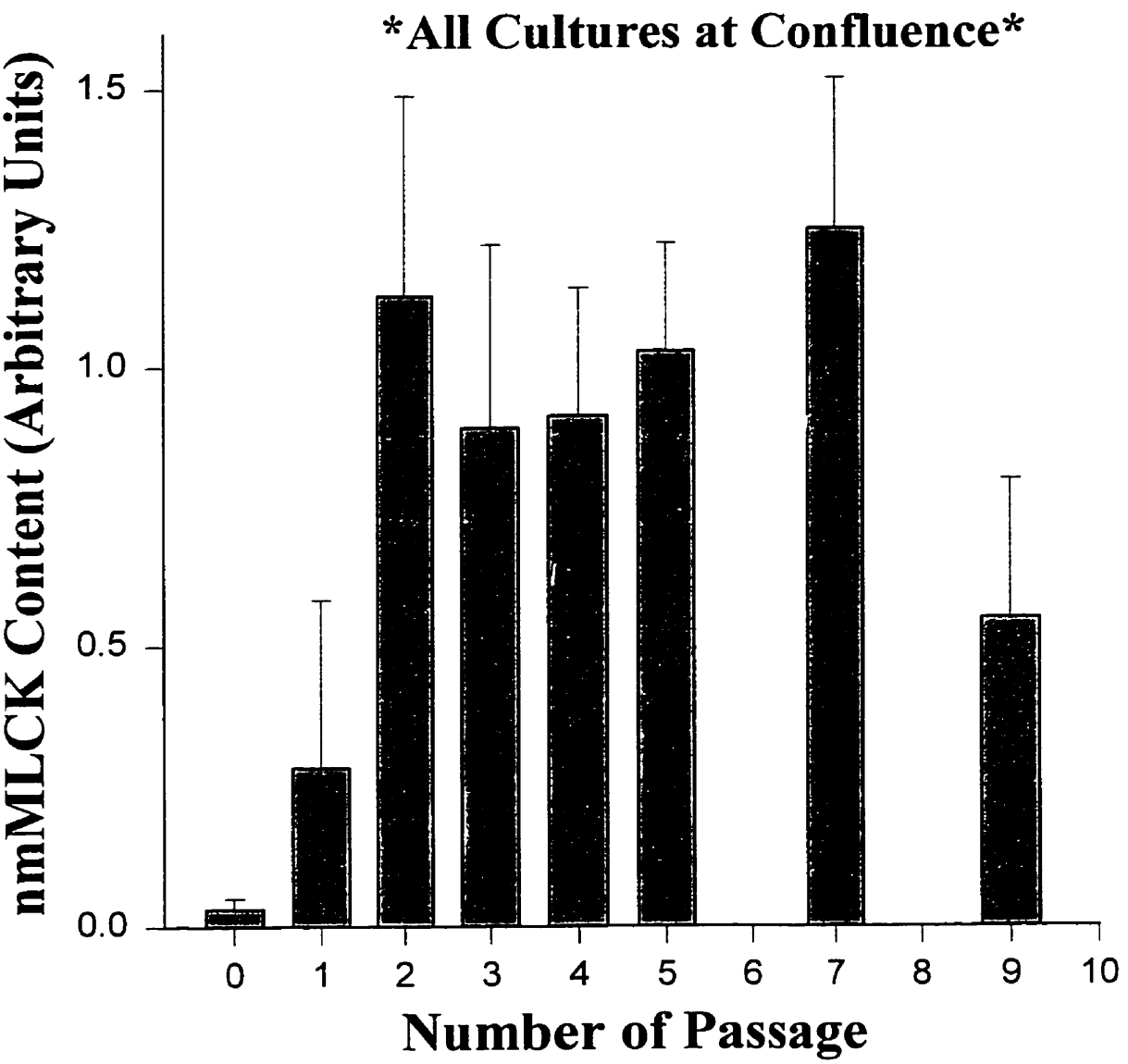


FIGURE 7. Change in nm-MLCK content in long-term culture. Western blot analysis of nm-MLCK content on 6% SDS-PAGE minigels showed that the nm-MLCK content was maintained at very high levels from passage to passage (N=6, error bars=SE). Extracts assayed were prepared from confluent plates. iso=freshly isolated cells.

NM-MLCK Content

in Canine Tracheal Smooth Muscle Cells

-Long Term Culture-



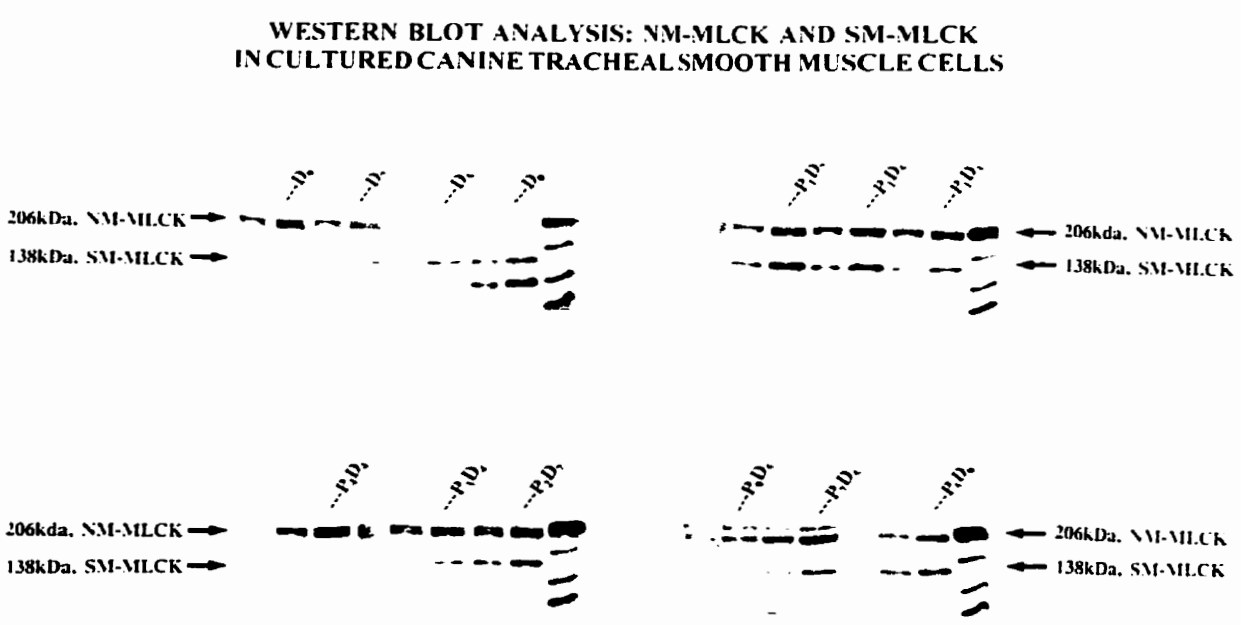


FIGURE 8. Chemilumigram from a single long-term culture. Two bands, MW 206kDa and MW 138kDa, were identified by sm-MLCK and nm-MLCK antibodies. At late passages (passage 7 and 9), an extra band (MW 214kDa) was visualized with nm-MLCK antibody."P" denotes passage and the numerical subscript denotes the number of the passage. "D" refers to day in culture and the subscript denotes the day number, thus for example P_5D_9 refers to the 9th day of culture during the 5th passage.

FIGURE 9. Change of α-actin level in primary culture. Western blot analysis of 6% SDS-PAGE minigels showed that the α-actin content decreased abruptly in primary culture. (N=6, error bars=SE)

α-actin Content

in Canine Airway Smooth Muscle Cells

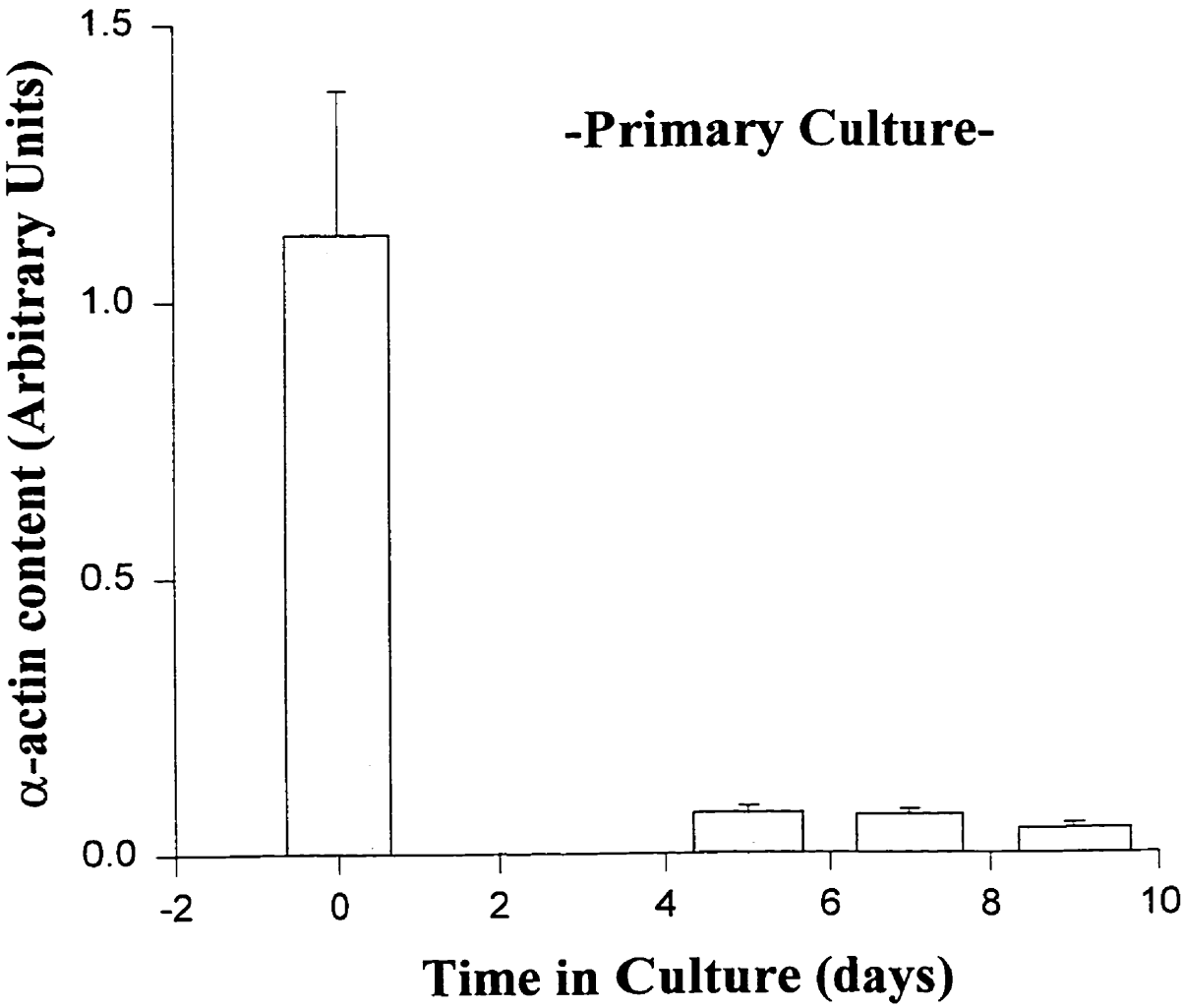


FIGURE 10. Change of α-actin content in long term culture. Western blot analysis of α-actin content on 6% SDS-PAGE minigels showed that the α-actin content decreased within 4 passages, then it came back to 70% of that of the freshly isolated cells. After the 5th passage it decreased again, by passage 9 it showed the similar oscillation pattern as shown for sm-MLCK. (N=6, error bars=SE)

α-actin Content

in Canine Airway Smooth Muscle Cells

-Long Term Culture-

All Cultures at Confluence

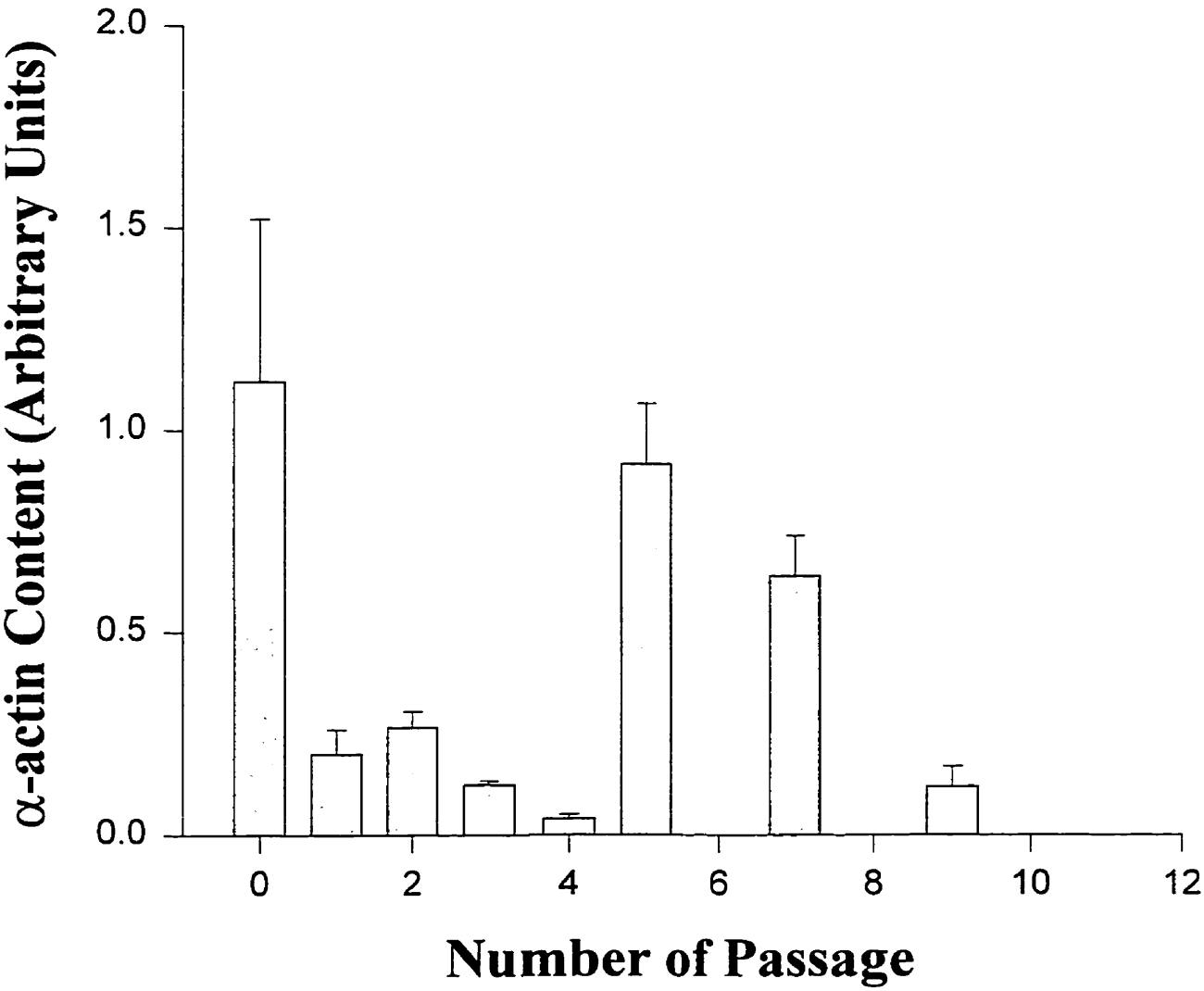
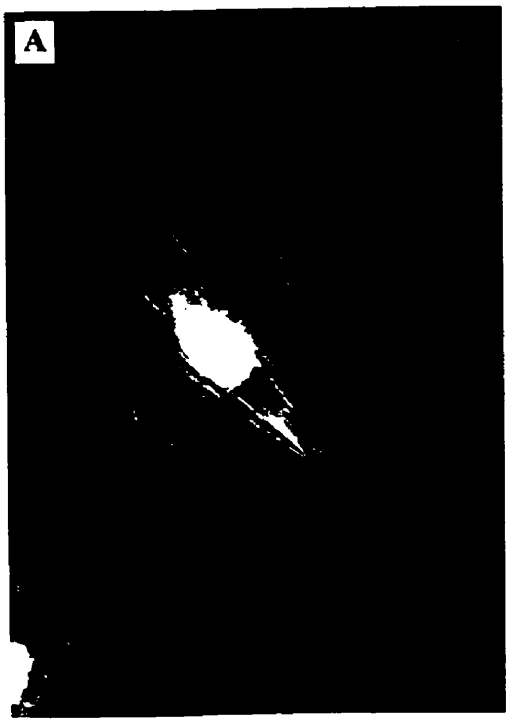


FIGURE 11. (upper panel) Immunocytochemical localization of sm-MLCK isoform in cultured tracheal SMCs. Picture 11A shows the subcellular localization of sm-MLCK. The cells were stained with a primary monoclonal anti-sm-MLCK antibody(1:25) and Cy^3-conjugated secondary antibody(1:200) at day 6. The sm-MLCK was distributed homogeneously throughout the cytoplasm. In the centre of each cell, there was a transparent area indicating absence of sm-MLCK in this area which is presumably nuclear. Figure 11B shows nuclear staining of the same two cells with Hoechst 33342. The shape of the stained nuclei was similar to the transparent area as shown in (A) confirming the nuclei were negatively stained for sm-MLCK.

FIGURE 12.(lower panel) Immunocytochemical localization of nm-MLCK isoform in cultured tracheal SMC. Picture 12A shows the subcellular localization of nm-MLCK. The cell was stained with a polyclonal anti-nm-MLCK antibody (D119, dilution 1:7500) and Cy^3-conjugated secondary antibody at day 6. We can see fibers running mostly in parallel and along the long axis of the cell; an intense nuclear staining is also seen. Picture 12B shows the nuclear staining with Hoechst 33342 of the same cell in picture 12A and we can see the intensely stained area in 10A is the nucleus.


A


B


A

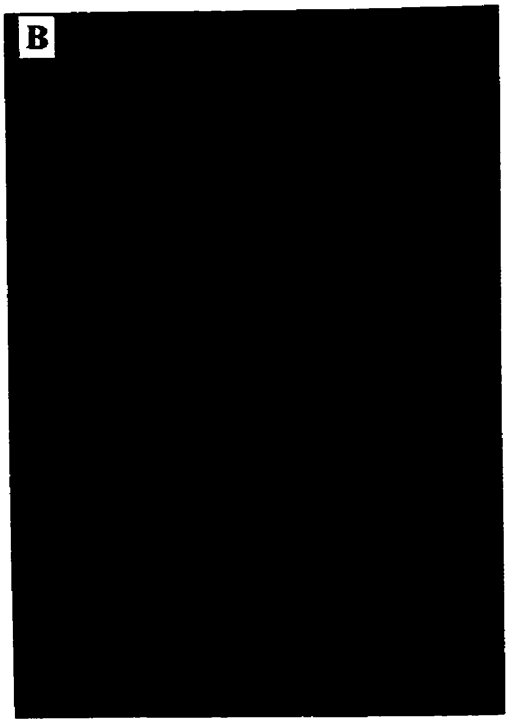
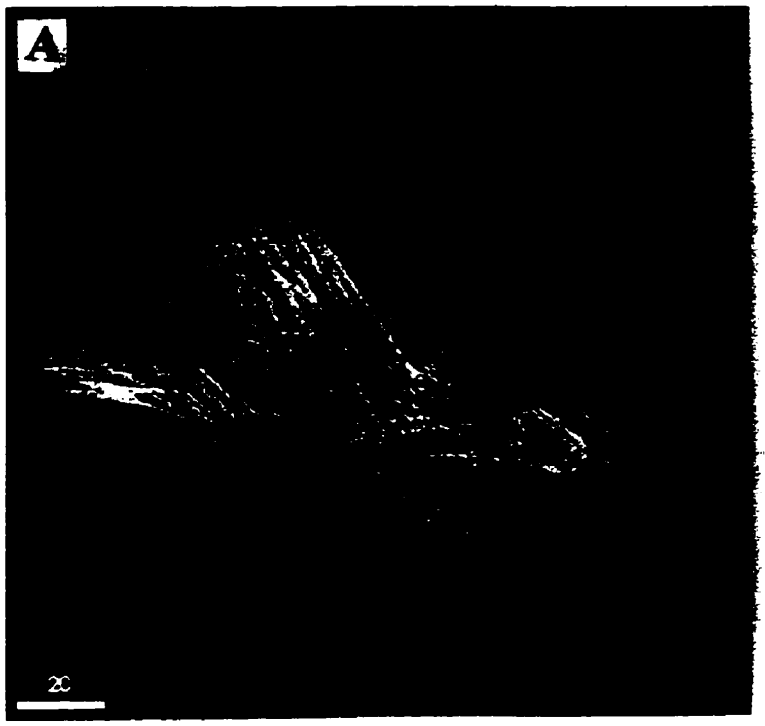

B

FIGURE 13.(upper picture) Distribution of nm-MLCK in canine trachealis smooth muscle cell. This figure shows a series of confocal immunofluorescence images of a single smooth muscle cell labelled with a polyclonal anti-nm-MLCK antibody and a Cy^3-conjugated secondary antibody. Images shown were collected at planes (from top left to bottom right) located 0.8μm, 1.6μm, 2.4μm, 3.2μm, 4.0μm and 4.8μm from the bottom of the cell. Bar=20μm. The intense nuclear staining and stained stress fibers are seen at every plane. The progression of intensity of nuclear staining confirms the immunoreactivity originates from the nucleus.

FIGURE 14.(lower panel) Immunocytochemical staining of mitotic tracheal SMC by nm-MLCK antibody. (A) This figure shows the mitotic cells stained with primary anti-nm-MLCK antibody and Cy^3-conjugated secondary antibody. An intense cytoplasmic staining for nm-MLCK was seen. In (B), the cells were stained with Hoechst 33342 which is specific for DNA staining.

FIGURE 15.(upper panel) (A) This confocal figure shows a single slice of a confocal image of a canine trachealis smooth muscle cell cultured at day 7 stained with primary anti-nm-MLCK antibody and Cy^3-conjugated secondary antibody. An intensely stained nucleus is shown at the upper right corner of the cell. Stress fibers are located mostly in parallel and along long axis of the cell. In (B), the same cell was stained with primary anti-sm-α-actin antibody and a FITC-conjugated secondary antibody. The stress fibers are shown in the cytoplasm, but no nuclear staining is seen. Colocalization of nm-MLCK and sm-α-actin along stress fibres is apparent.

FIGURE 16. (lower panel) (A) This confocal figure shows a single slice of canine trachealis smooth muscle cells at day 7 stained with primary anti-nm-MLCK antibody and Cy^3-conjugated secondary antibody. The nm-MLCK is present both in the nuclei and the cytoplasm along stress fibers. A weakly-stained nucleus is noted in the smaller cell, which indicates the heterogeneity of airway smooth muscle cells. (B) This figure shows the same cells stained with primary anti-β-tubulin antibody and FITC-conjugated secondary antibody. The β-tubulin is distributed in the cytoplasm but not in the nucleus.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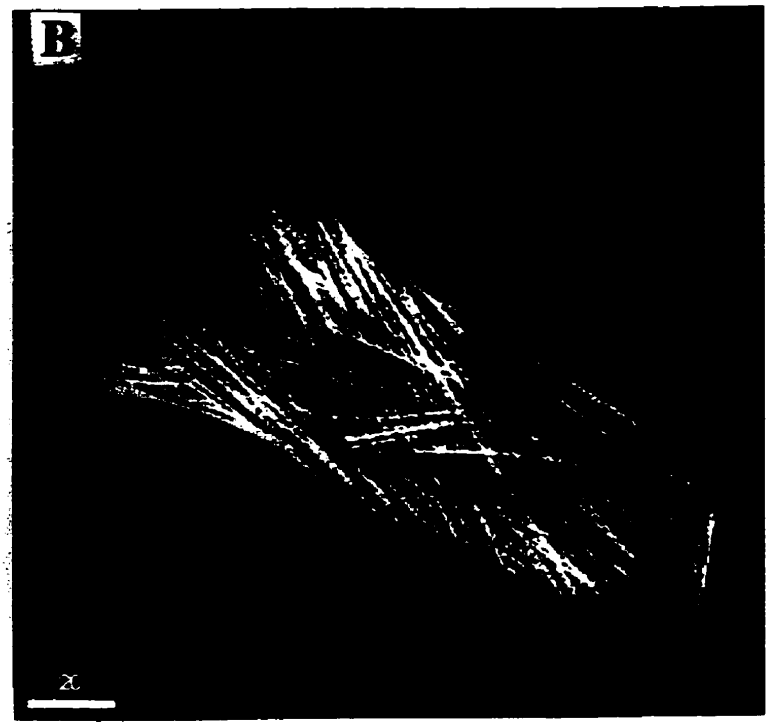
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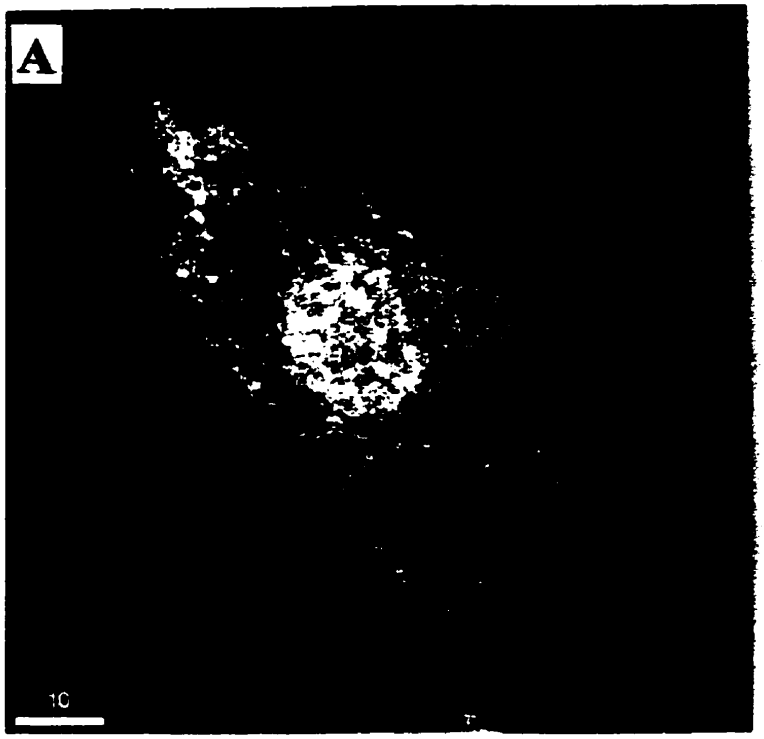
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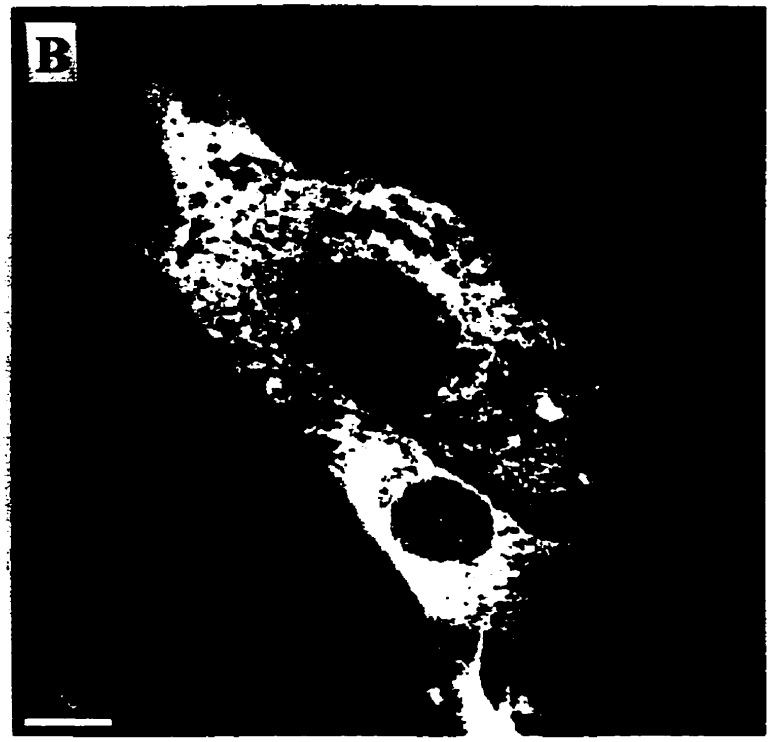
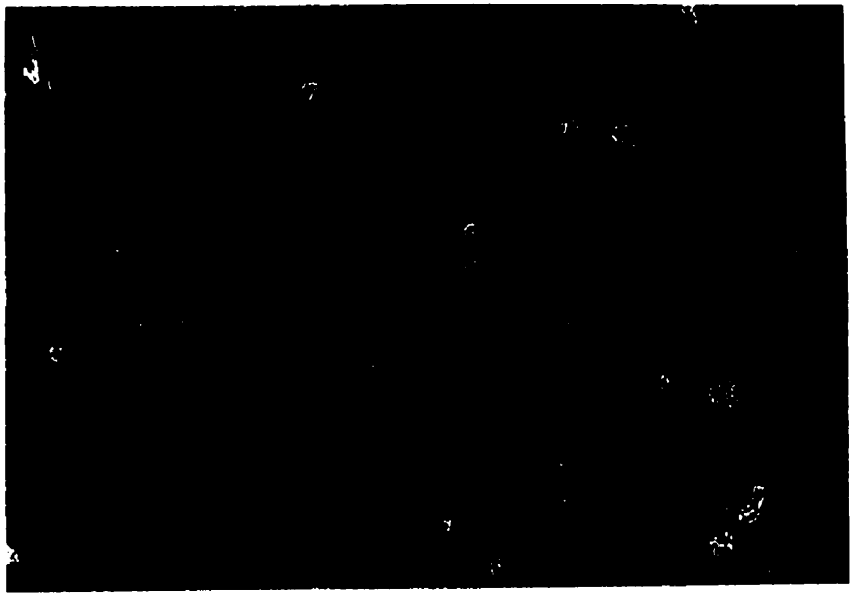

B

FIGURE 17. Induction of two distinct phenotypes of cells in primary culture under prolonged serum deprivation. A (left): phase-contrast microscopy of confluent cultured cells, showing a relatively homogeneous population. B (right): microscopy of 10 days arrested cultured cells, showing two distinct groups of cells: 1) dark flat-circular cells; (There are two kinds of circular cells shown in both picture: the dark circular cells are alive attached cells; the smaller bright circular cells that are floating are dead cells that they should be washed out) 2) spindle-shaped elongated cells (mononuclear) with phase lucent sarcolemma, which appear dark and are aligned side-by-side in most cases.

Emergence of two distinct phenotypes of cells in smooth muscle cell culture under prolonged serum deprivation



Before deprivation

After deprivation

FIGURE 18. Temporal changes in contents of smooth muscle contractile and regulatory proteins in cultured tracheal SMCs during prolonged serum deprivation (there are 40% of elongated cells in each culture dish). A: relative protein content of sm-α-actin and smooth muscle total myosin heavy chain (sm-MHC); B: relative sm-MLCK. Progressive increases in expression of these proteins in cultured SMCs were identified under prolonged serum deprivation.

Temporal Changes in Contents of Contractile and Regulatory Proteins in Growth Arrested Cultures

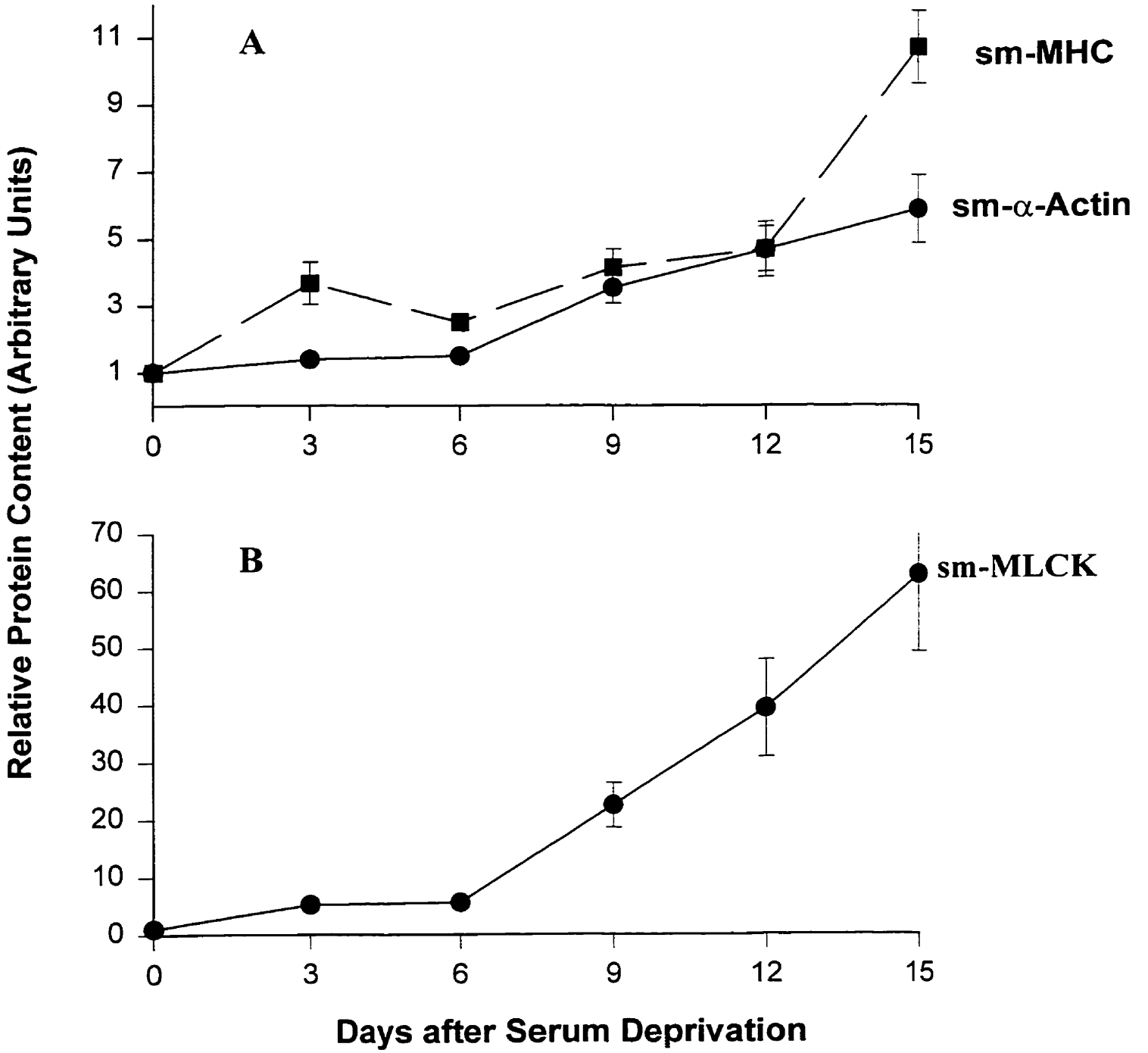


FIGURE 19. Comparison in contents of smooth muscle contractile and regulatory proteins among freshly isolated, cultured confluent (about 7-9 days), and cultured 15-day arrested tracheal SMCs by Wsetern blot analysis on 6% SDS-PAGE minigels. Significant decrease of contractile proteins content in confluent cells, and restoration in arrested cells were found. Surprisingly, the contents of sm-α-actin and sm-MLCK in arrested cells were even significantly higher than those in freshly isolated cells. F: freshly isolated cells; C: cultured confluent cells; A: cultured arrest cells. *$P<0.05$, ***$P<0.001$ compared with freshly isolated cells.

Comparison in Contents of Contractile and Regulatory Proteins Among Freshly Isolated, Cultured Confluent and Growth Arrested Cells

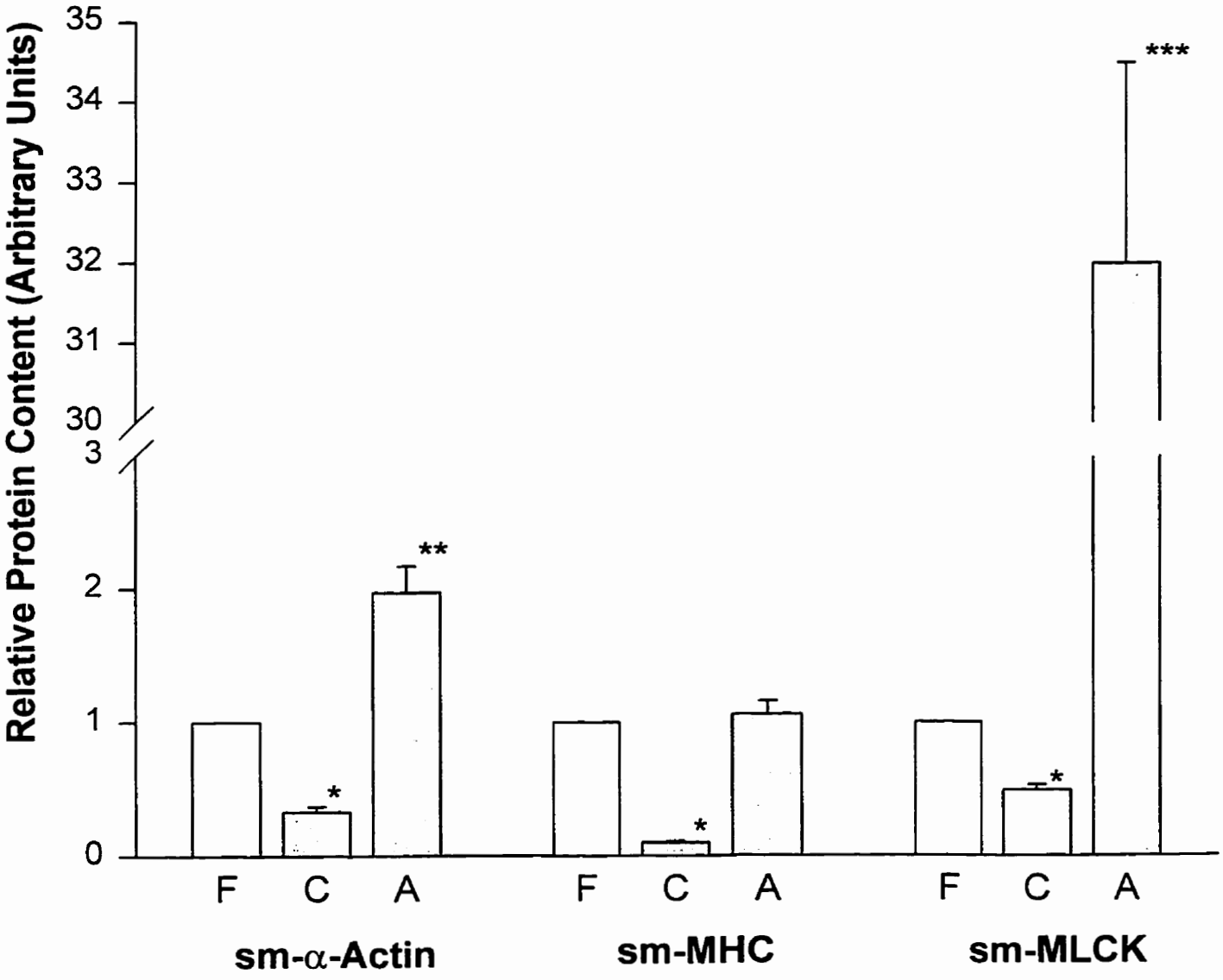


FIGURE 20. Microscopy of cultured, SMCs immunostained for specific smooth muscle contractile proteins after 10 days serum deprivation. A: sm-MHC, B: sm-α-actin, C: sm-MLCK. The corresponding nuclear staining of the same field of cells is presented towards the right of panels A, B, + C. Elongated spindle-shaped contractile cells stained intensively for smooth muscle contractile type and non-contractile type proteins. Flat, circular, non-contractile cells stained relatively weak for nm-MLCK, but almost negatively for sm-MHC and sm-α-actin. Centrally located cigar-shaped nuclei were identified in serum deprivation induced contractile cells, relative round ones in circular non-contractile cells.

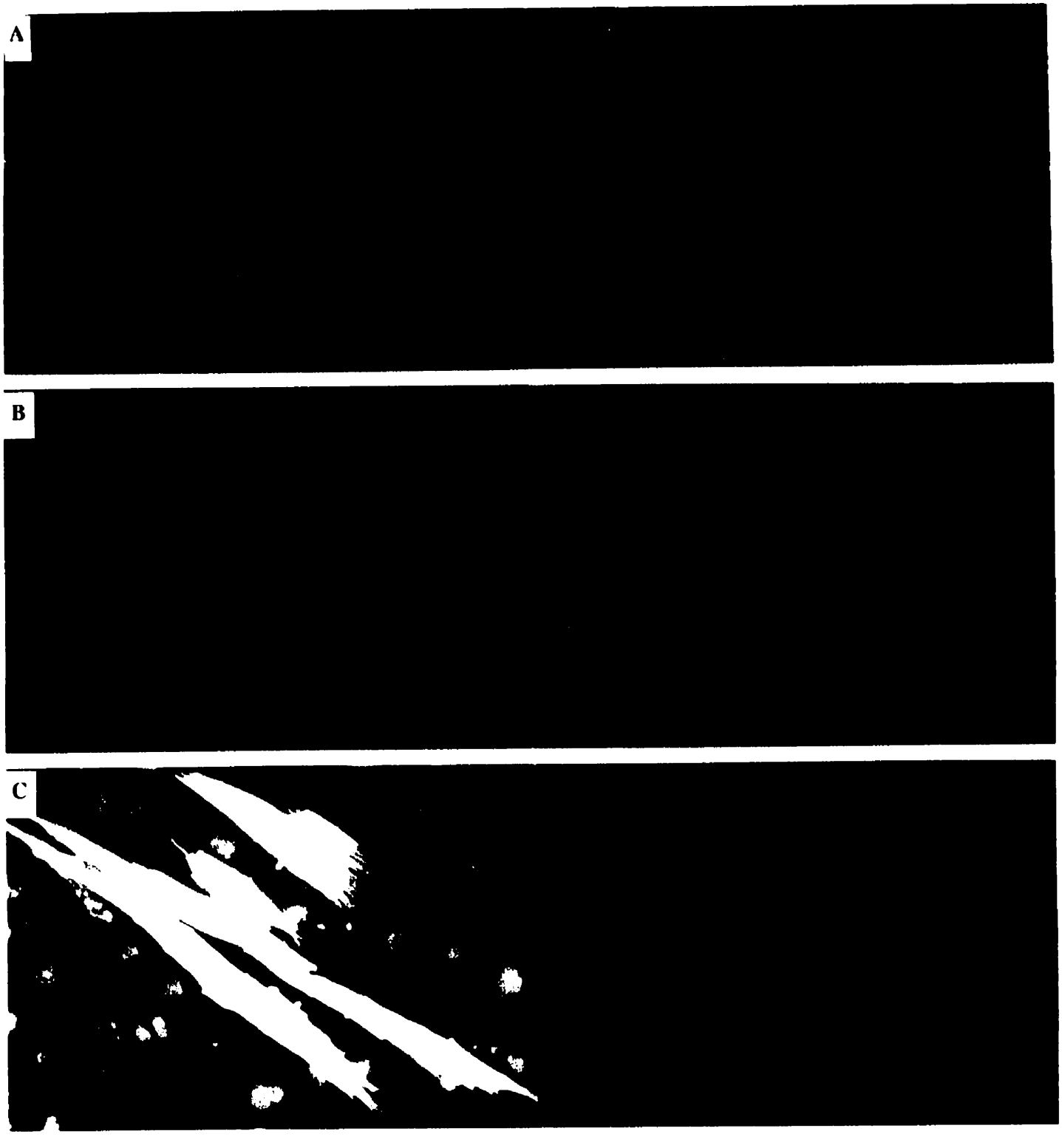

A
B
C

FIGURE 21. Microscopy of cultured tracheal SMCs (after 10 days of serum deprivation) immunostained with specific antibodies for contractile proteins. A: nm-MLCK, B: β-tubulin.

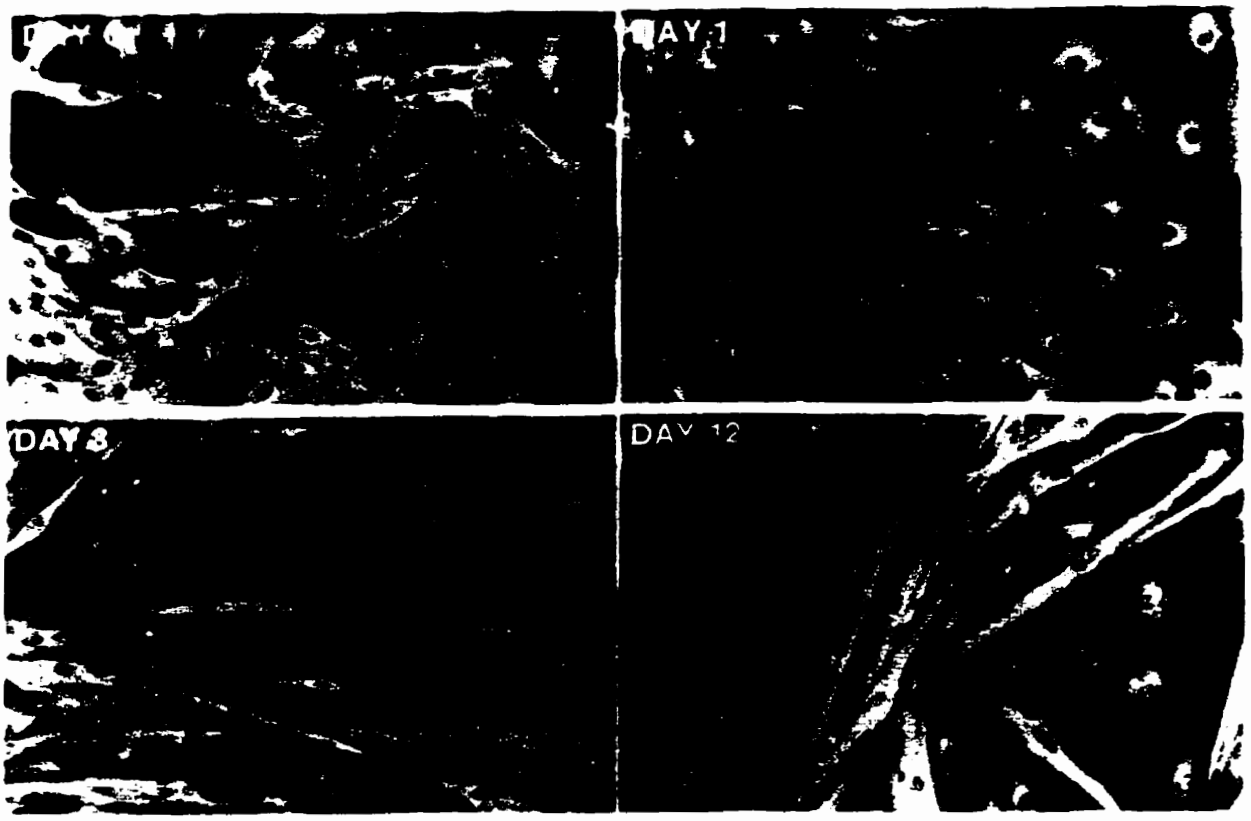
Elongated spindle-shaped cells stained intensely for these non-contractile type proteins. Flat, circular, non-contractile cells stained less intensely for nm-MLCK and β-tubulin.


A
B

FIGURE 22.(upper four pictures) Immunocytochemistry of arrested cultured tracheal SMCs. Cells were stained with sm-MLCK antibody and Cy^3-conjugated anti-mouse antibody. A (upper left): cells before arrest (day 0). B (upper right): cells at day 1 of serum deprivation. C (lower left): cells at day 3 of serum deprivation. D (lower right): cells at day 12 of serum deprivation. Before arrest the cells were stained with sm-MLCK homogeneously. One day after arrest the distribution of sm-MLCK is changed to "spider" like in the cytoplasm and is restricted to perinuclear region. Three days after arrest the elongated cells appeared which were strongly stained for sm-MLCK, while the flat cells were stained less intensely and showed a "spider" like distribution. The cell numbers of spindle-shaped, elongated cells increased at day 12 after serum deprivation and they were arranged like bundles.

FIGURE 23.(lower four pictures) Immunocytochemistry of arrested cultured tracheal SMCs. Cells were stained with anti-desmin antibody and Cy^3-conjugated secondary antibody. A (upper left): cells before arrest (day 0); B (upper right): cells at day 1 of serum deprivation; C (lower left): cells at day 2 after serum deprivation; D (lower right): cells at day 12 after serum deprivation. Before arrest cells were negatively stained for desmin. At day 1 of arrest, the elongated cells were positively-stained for desmin. With the time of growth arrest, the number of these elongated, desmin-positive cells increased, while the flat, circular cells did not stain.

Highly organized cellular re-distribution of sm-MLCK under serum deprivation



Rapid cellular accumulation of desmin in individual cells under serum deprivation

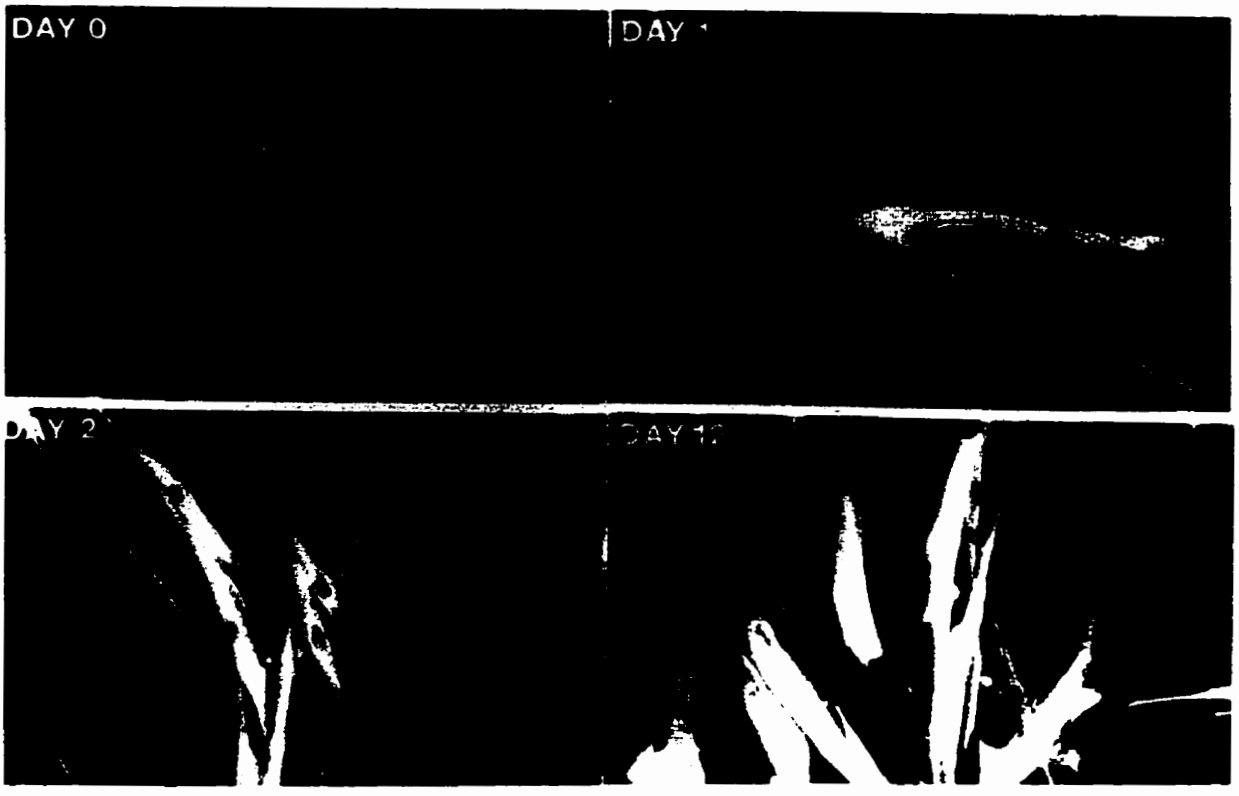


FIGURE 24. Immunocytochemistry of arrested tracheal SMCs. The cells were stained with anti-vimentin primary antibody and Cy^3-conjugated secondary antibody. A (upper left): cells before arrest. B (upper right): cells 1 day after growth arrest. C (lower left): cells 4 days after growth arrest. D (lower right): cells 12 days after growth arrest. Before arrest, the cells were positively stained with vimentin. After arrest both the elongated, spindle-shaped cells and the flat, circular cells were all evenly stained with vimentin. No enhanced vimentin staining was notified in the elongated cells.

No Enhanced Expression of Vimentin in Growth Arrested Contractile Type of Airway Smooth Muscle Cells

5. DISCUSSION

SMCs are multifunctional mesenchymal cells capable of modulating their phenotype in vivo and in vitro. In general, mature SMCs exist in a predominantly "contractile" state, though in response to pathological stimuli they can modulate to a matrix-secreting "synthetic" phenotype. It is now well-established that in primary culture, serum-stimulated SMCs undergo spontaneous, reversible modulation from the contractile to the synthetic phenotype similar to the changes in SMCs seen during injury-repair and proliferative responses in vivo (11, 86). Assessment of the temporal changes occurring in the content of proteins which compose and regulate the contractile apparatus in canine tracheal myocytes indicates that the phenotype of cells modulates rapidly when cultured in the presence of serum. The data obtained from our study describes temporal changes in the expression and content of proteins composing the contractile apparatus, regulating the contractile apparatus and associated with the cytoskeleton of cultured canine tracheal myocytes. From our study, nm-MLCK rapidly increased with culture which indicated a switching of phenotype from contractile to synthetic, along with a significant decrease of sm-MLCK. This change was consistent with the change of sm-α-actin content which has been described as a good phenotype marker for SMCs in cultures. It is suggested that sm-MLCK and nm-MLCK are inversely regulated and these isoforms of MLCK could be used as markers to determine the phenotype of airway SMCs.

Specific antibodies to smooth muscle marker proteins have been used extensively

for immunohistological analysis of the phenotypic diversity of populations of SMCs in different tissues in normal and pathological states (87, 88), but no data have shown the localization of different MLCK isoforms in culture. In our indirect immunofluorescent staining, 90% of the cultured cells were stained for sm-α-actin, a specific contractile marker for SMCs, which indicated that these cultured cells were SMCs. Immunocytological study and confocal microscopy showed a difference in subcellular pattern between sm- and nm-MLCK isoforms. Smooth muscle-MLCK is present only in the cytoplasm, while nm-MLCK is present in both cytoplasm and nucleus. Non muscle-MLCK colocalizes with sm-α-actin-positive stress fibers in the cytoplasm, but does not colocalize with β-tubulin. As is well-known, sm-MLCK is the key enzyme regulating contraction of smooth muscle, and it is colocalized with the contractile proteins present in the cytoplasm. Non-muscle MLCK is expressed at very high levels in synthetic and proliferative cells and as it is present in the nucleus, it might play an important role in the proliferation and differentiation of SMCs. Intracellular localization studies performed in mammalian fibroblast cells have localized MLCK to the spindle apparatus and midbody of mitotic cells, and to stress fibers and the nucleolus of the interphase cells (89). These observations suggest that the phosphorylation of MLC by MLCK in non contractile muscle cells might have a role in cell division and cell motility in culture. This requires further study.

Here a considerable increase in nm-MLCK at day 20 of primary culture (shown in figure 5) is reported. We have no explanation for this occurrence. However nm-MHC

is known to accumulate just prior to entry of the replicating cell into the M phase (90). The increased nm-MLCK would serve to phosphorylate the increased nm-MHC. Further support for this is seen in Figure 14A where cell division is approaching completion and the accumulation of nm-MLCK is remarkable.

One must be cognisant of the fact that there is some controversy as to the exact identity of MLCK isoenzymes. Our measurement, based on molecular weight markers indicates the apparent molecular weights of the two isoenzymes we have detected (nm-MLCK and sm-MLCK) are 206kDa and 138kDa respectively. The latter represents sm-MLCK, while we have identified the former as nm-MLCK because of its molecular weight and its change in phenotypic expression in culture. The controversy stems from the detection of a third isoenzyme by Gallagher et al. (78) which has a molecular weight (M_r) of 208 kDa, is present in embryonic tissues and is termed embryonic MLCK. Fisher and Ikebe feel the two enzymes are the same and have termed the 206 kDa isozyme they have isolated as non-muscle/embryonic MLCK (76). Finally, Garcia et al. have reported the existence of yet another isoform which has a M_r of 214kDa which they have termed endothelial MLCK.

Asthma is considered to be a chronic inflammatory disease of the airway and is highlighted by excessive airway narrowing in response to various stimuli. Thickening of the bronchial wall, which includes subepithelial fibrosis, oedema and smooth muscle hypertrophy, is an accepted feature that contributes to bronchial hyper-responsiveness (44,

45). There is good evidence that requisite factors to promote the phenotypic plasticity of smooth muscle are present in airways of asthmatic individuals. However, the influence of the phenotypic state of airway smooth muscle on modulating abnormal growth and contractility as it might relate to asthma, has not been investigated. Subtle changes in the characteristics, or phenotype, of smooth muscle can greatly affect its physiological role. We have found (59) the shortening velocity and capacity of airway smooth muscle to be increased in ragweed-sensitized dogs. These changes have been shown to be related to increased levels of RLC phosphorylation, increased content and activity of MLCK and reduced acetylcholinesterase activity (91). The presence of nm-MLCK in the cell nucleus may account for the growth changes in airway SMCs in asthmatic patients. So isoforms of MLCK may be used as tools to assess the differentiated state of airway SMCs in the asthmatic airway.

Various cell types can alter their character when the environment changes. These alterations in character are called modulations of the differentiated state and involve reversible interconversions between phenotypes. It has been known that SMCs are capable of expressing a range of phenotypes. At one end of the spectrum of phenotypes is the SMC whose function is almost exclusively that of contraction ("contractile state"). At the opposite end of the spectrum of phenotypic expression is the muscle cell whose function is almost exclusively that of synthesis ("synthetic state"). SMCs express intermediate morphology. It is well-known that the SMC can change its phenotype from contractile to synthetic, and vice-versa, in response to environmental influences (3). The mature contractile SMC was reported to undergo a rapid modulation of its phenotype to the

immature synthetic type during culture in the presence of serum (15,16). Direct evidence for reversal of modulation of cultured SMCs, i.e., re-development of contractile properties, has never been demonstrated, although re-accumulation of sm-MHC was found in post-confluent cultures (16). In this study, prolonged serum deprivation induced the modulation of an immature, synthetic phenotype to a mature, contractile phenotype, which is opposite to the switching of phenotype from a contractile to a synthetic state in the presence of serum and just after plating primary cultures. This was characterized by the significantly increased content of sm-MLCK, sm-α-actin, and sm-MHC in growth arrested cells shown by semi-quantitative Western blot analysis. Indirect immunofluorescence staining also showed intense staining of the arrested cells with sm-α-actin, sm-MHC, sm-MLCK, β-tubulin, and desmin. Increased contractility and shortening velocity was also noticed by others. Also when the serum was given back to the arrested cells the elongated cells changed back to circular ones morphologically and became proliferative again. These changes demonstrated that cultured SMCs (synthetic type) retain the capability to reverse their phenotype back to a contractile state, and undergo differentiation under serum deprivation and vice versa. This suggested that SMCs are not likely to be terminally differentiated in culture and their phenotypic modulation is reversible. This was proved by the switching of the elongated cells to circular cells when the serum was restored to the arrested cells. This cycling could be demonstrated 6 times. In our study, about 35% of the cells become elongated under serum deprivation. Further flow cytometry analysis needs to be carried out to determine the characteristics of these circular and elongated cells with respect to determining what stages of the cell cycle they are in. The expectation

is that the arrested cells are in G_0 phase.

The mechanisms by which the individual airway SMCs elongate during long term serum deprivation are currently unknown. Their shape change is similar to the morphological changes seen when cultured skeletal myoblasts differentiate and fuse into elongated myotubes. But different from skeletal muscle cells our SMCs showed no obvious fusion, nor could we demonstrated the presence of single, multinucleated cells. Electron microscopic study is needed to further confirm this. Parallel and end to end arrangement of these elongated SMCs into bundles was another feature of these growth arrested cells. It is unknown what mechanisms account for this cellular arrangement in culture. Serum deprivation did not result in bundle formation in pre-confluent cultures, suggesting that cell density may be an important factor for the special arrangement of these elongated cells. Cell to cell communication may also contribute to the bundle formation.

Serum deprivation-induced contractile airway SMCs were found to be hyper-reactive and hypersensitive to contractile stimulation. This could contribute to allergic bronchospasm if it were found to be more common in asthma than in normal trachealis. Inflammation and airway wall thickening, including infiltration of leukocytes and macrophages, epithelium denudation, subepithelial fibrosis and medial smooth muscle hypertrophy and hyperplasia are the pathological features of asthmatic airways (45). Inflammatory mediators such as histamine, leukotrienes and prostaglandins which exist

in asthmatic airways, are all potent mitogens (92). Release of these inflammatory mediators during an asthmatic attack may result in proliferation of airway SMCs. After the asthma attack, these mediators could be secreted in smaller amount. So growth arrest, as occurred under serum-deprivation of cultured cells, would then occur *in vivo,* between consecutive asthma attacks. This may result in generation of these elongated, spindle-shaped, hypercontractile and hypersensitive SMCs. These types of cells might contribute to the excessive non-specific hyper-reactivity (elicited by methacholin challenge) of the SMCs, and excessive narrowing of the airway lumen during the post-attack period. The presence of elongated fully contractile cells could also contribute to the specific hyperreactivity seen during the subsequent asthma attacks. This idea was supported by a recent report by Bramley (93) who showed a considerable increase in the shortening capacity of human asthmatic airway smooth muscle compared to non-sensitized muscle. This model may be useful for the study of mechanical properties of asthmatic airway SMCs. It may also be applied to examine the vascular system in which hypertrophy and hyperplasia of SMCs are also present, for example, in the pathogenesis of hypertension.

In smooth muscle, phosphorylation of the RLC of myosin by the Ca^{2+}/calmodulin-dependent MLCK is a well-characterized event in the initiation of contraction. Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complex then binds to MLCK and activates the enzyme. Activation of MLCK results in phosphorylation of the 20kDa RLC subunit on myosin and stimulation of the actin-activated myosin Mg^{2+}ATPase activity. These events lead to force generation or shortening of the muscle. In our study, sm-MLCK content in

cultured cells after 15 days of serum deprivation, increased 62.9-fold compared with non-arrested cells, and was 30 times of that of freshly isolated cells. This finding may contribute to the increased contractility of the spindle-shaped arrested cells. Smooth muscle MLCK is known to be a primary regulator of smooth muscle contraction. Increased sm-MLCK content and activity would lead to an increase of MLC_{20} phosphorylation with a concomitant increase in velocity during smooth muscle activation. Recently, it has been reported that *in vitro* motility of the smooth muscle myosin head was increased with an increased sm-MLCK concentration; this further demonstrated the regulation by sm-MLCK of smooth muscle contractility.

Intermediate filaments constitute an important intracellular structural entity in cells and may serve as a lattice which organizes and anchors the contractile apparatus in mature SMCs (94, 95, 96). Desmin, vimentin and cytokeratin are the structural proteins which comprise intermediate filaments in immature and mature SMCs (94, 97). The expression of these proteins is developmentally-regulated in SMCs (28, 21, 98, 99). Desmin is the muscle-specific intermediate filament protein (100). It is encoded by a single gene (101) which is expressed in all muscle tissues, cardiac, skeletal and smooth. It is generally considered as one of the earliest known myogenic markers of muscle differentiation following the presence of vimentin in the myogenic precursors (102, 103). The accumulation of desmin intermediate filaments is a common feature of the myogenic programs for skeletal, cardiac and smooth muscle (21). Desmin is necessary for the formation of myotubes in tissue culture (104, 105). It is very crucial for skeletal muscle

differentiation and it may be also important for smooth muscle differentiation. Desmin-null mutation affect skeletal and smooth muscle differentiation (106).

In our study immunocytochemical staining was used to show that the spindle-shaped cells stained strongly for anti-desmin antibody. This means that these elongated cells undergo differentiation soon after serum deprivation. During and following the late asthmatic attack cells are likely undergo hypertrophy and hyperplasia. As the attack subsides the cells could enter into a state akin to that of arrested cells in culture. These hyper-reactive and hypersensitive cells could contribute to bronchial obstruction by increasing contractility, in addition to geometrically blocking the lumen by greater size and hyperplasia.

6. CONCLUSIONS

1). Airway SMCs can undergo phenotypic switching from contractile to synthetic state in culture with enhanced expression of non-muscle isoforms and a concomitant reduction in mature smooth muscle isoforms of various proteins. Isoforms of MLCK can be used as phenotypic markers to determine the phenotypic state of SMCs in culture.

2). Isoforms of MLCK are differentially-expressed and localized in airway SMCs of different phenotypic states. While smooth muscle MLCK is distributed only in cytoplasm homogeneously, non-muscle MLCK is present both in cytoplasm along stress fibers and also in nucleus. Disparate regulatory roles may exist for nm- and sm-MLCK isoforms. Smooth muscle MLCK is important for the regulation of contraction. Nm-MLCK may play a role in cytokinesis, migration and mitosis of SMCs in culture although we do not have the evidence for this as yet.

3). Long-term serum deprivation can induce phenotype modulation of airway SMCs from a synthetic to a contractile state with the increased expression of contractile and regulatory proteins.

4). A functional, fully-contractile phenotype is induced in cultured tracheal SMCs as a result of prolonged serum deprivation. This model may be useful for the study of SMC differentiation and contraction.

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IMAGE EVALUATION
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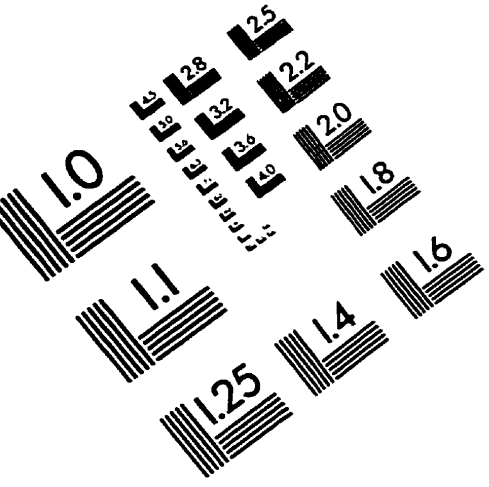

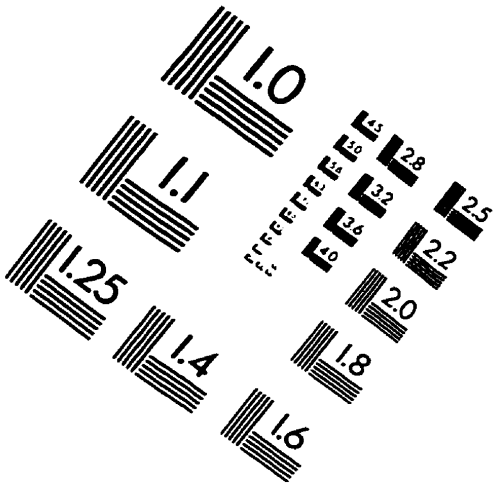

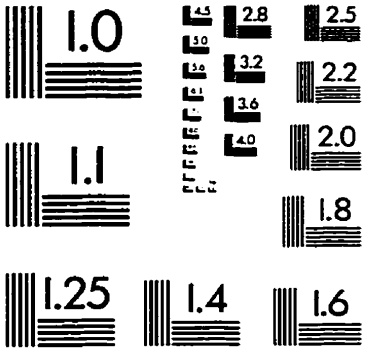

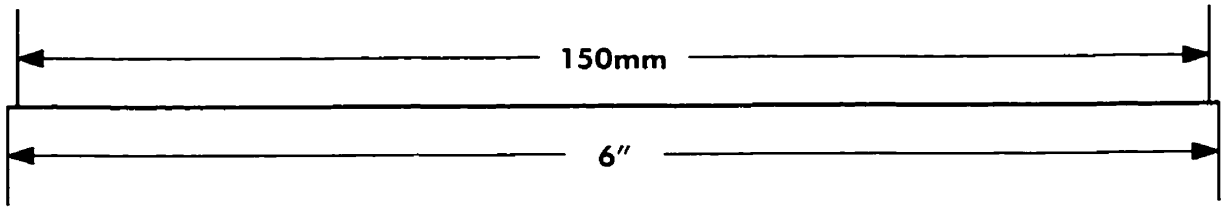

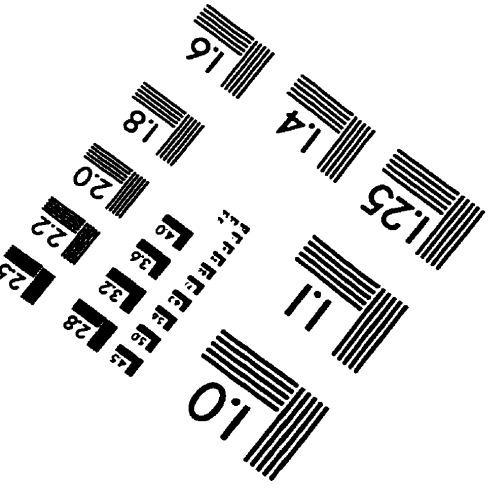

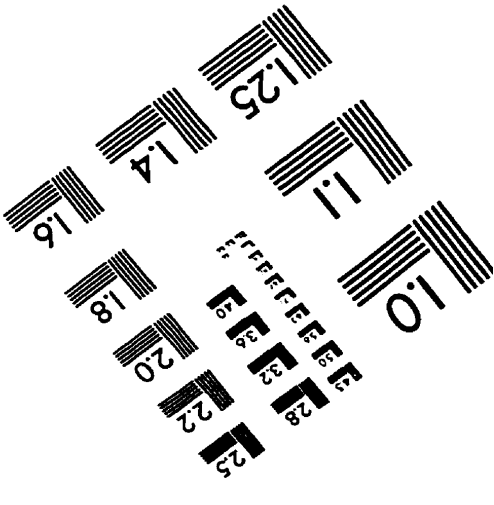

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