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TGF-β as Differentiating Factor for Cultured Smooth Muscle Cells

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Running Title: Airway Smooth Muscle Cell Phenotype

Abstract

Background

In cultured smooth muscle cells starved by removal of 10% FBS for 7 days, growth arrest was seen; 30% became elongated and demonstrated super contractility. Study of conditioned medium suggested the differentiating factor was TGFβ.

Methods

SDS-PAGE was carried out on conditioned medium from the arrested cells. Two protein bands were identified as MMP-2 and TGF-β1. To determine second messenger signaling by SMAD2, Western blotting and confocal microscopy were employed.

Results

Conditioned medium from arrested cultures showed presence of MMP-2 and TGF-β1 as revealed by SDS-PAGE, 68kDa and 25kDa bands were seen. Differentiation was confirmed by up-regulation of marker proteins, smooth muscle type myosin heavy chain and myosin light chain kinase. Confirmation was obtained by down-regulating these proteins with decorin treatment which reduces levels of active TGFβ, and an adenoviral dominant-negative vector coding for a mutated type II TGFβ-receptor. Activation of second messenger signaling was demonstrated by presence of phosphorylated SMAD2 and SMAD4 immunocytochemically.

Conclusion

TGF-β is the likely differentiating factor responsible for the development of these super-contractile smooth muscle cells.

Significance

Such cells developing *in vivo* after cessation of an asthmatic attack, could contribute to the non-specific hyperreactivity of airways seen in patients.

Key Words: decorin, double mass spectrometry, matrix metalloproteinase-2, SMAD-2, TGF-β1, TGFβ-RII

Introduction

The most significant advance [1-5] in the pathogenesis of chronic asthma has been recognition that it is a chronic inflammatory disease associated with hypertrophy and hyperplasia of airway smooth muscle cells (ASMC). As the ASMC changes, engendered by hyperplasia, are accompanied by modulation of the cell to a secretory phenotype with reduced contractility, it may seem that asthmatic airway narrowing is more due to geometric alterations than increased contractility. However increased contractility may yet be an important factor.

Arrested Airway Smooth Muscle Cells in Culture

Support for the view stated above has come from studies of cultured canine tracheal smooth muscle cells (TSMC). These cells are normally of synthetic type and feebly contractile. However, if replication under 10% FBS stimulus is arrested at 70% confluence by serum withdrawal, 30% change their phenotype and became supercontractile [6]. They express mature smooth muscle type contractile and regulatory proteins and appear to be differentiating into mature cells.

TGF-βI

Evidence suggests a contribution of factors such as transforming growth factor-beta (TGF-β) to the pathogenesis of asthma [8,9]. TGF-β is secreted as a latent complex [10,11]. Several processes can activate TGF-β, allowing it to bind to its type II serine/threonine kinase receptor (TβR-II) [11]. Ligand binding to TβR-II causes phosphorylation of the intracellular domain of TβR-I. In turn, TβR-I phosphorylates

receptor-activated Smad2 (R-Smad2) which forms a complex with common-mediator Smad4 (co-Smad4); this dimer, directly or indirectly, interacts with various target genes, after translocating from the cytoplasm to the nucleus.

Decorin

As receptor activation requires binding of active TGF-β, activation of latent TGF-β is needed. This is brought about by activity of matrix metalloproteinase 2 (MMP-2) [12-14]. Another component of the ECM, decorin, binds only the activated and not the latent form of TGF-β [4] and subsequently blocks TGF-β function [15,16] suggesting a regulatory role. Previous research has shown co-localization of decorin and TGF-β in human connective tissues [10,17] which may thus serve as a reservoir for TGF-β.

Airway smooth muscle cells release TGF-β which may promote differentiation. Serum from conditioned medium of starved airway smooth muscle cells in culture has been reported to be associated with a new supercontractile phenotype [6]. It has been speculated that under these conditions TGF-β is released, that, by an autocrine effect, induces differentiation.

Over-expression of AdenoVirus-mediated-dn-TGFβ-RII

TGFβ-RII is required for TGF-β signaling [18]. The latter acts by binding to and TGFβ-RII which then activates TGFβ-RI [19]. Use of a mutated TGFβ-RII has been shown to block TGF-β signaling [20-26]. This block is affected by prevention of phosphorylation of downstream effectors such as Smad proteins [22]. This technique has been reported to be effective in a variety of cell types [26,27].

Smad Proteins

In most mammalian systems TβR-I phosphorylates Smad2 (R-Smad2) which forms a complex with common mediator Smad4 (co-Smad4) which translocates to the cell nucleus [24] with the aid of the cytoplasmic protein importin [24]. This results in binding of the complex to appropriate gene promoters. Though Smad complexes do bind to DNA, the low affinity of this reaction necessitates binding of additional factors for efficient transcription [22].

The results reported here suggest a mechanism for differentiation of airway smooth muscle cells. We showed that serum starvation of cultured cells was accompanied by an increase in the expression of TGF-βI and MMP-2. This was accompanied by switching of expression of proliferating smooth muscle cell type proteins (non-muscle types of myosin heavy chain, and myosin light chain kinase) to those of differentiated cells (smooth muscle type myosin heavy chain and kinase). These changes can be partially prevented with the use of decorin blocking antibodies and by transfection with adenovirus dominant negative TGF-βRII constructs. While such differentiation has been reported in vascular [28] and other smooth muscle types, the current report is among the first for airway smooth muscle [6,7], and the only one in which the role of TGF-β has been studied as a differentiating factor. This process of differentiation could occur in any smooth muscle where inflammation and proliferation occur.

Materials and Methods

Cell Culture

Canine tracheal SMC were isolated according Ma et al [6]. The procedures were those recommended by our Animal Ethics Committee. For each experiment, we used a seeding density of 5.0 x 10^4 cells/cm^{-2} plated into 100-mm diameter plastic culture dishes. At 70% confluence, cultures were switched to serum-free medium (F12) containing selenium (1 nM), insulin (1 nM) and transferrin (65.8 nM), (SIT), and maintained in this arrested state for as long as required. Five cell lines were used for each group of animals.

Collection of Conditioned Medium

After incubation of cells for the required period of time, conditioned medium (CM) was collected from cultures, placed in a sterile Eppendorf tube with 1 mg/ml each leupeptin, aprotinin and pepstatin, and stored at -80^{o}C.

Measurement of Single Cell Mechanics

Arrested cells were obtained as described above. The methodology has been carefully described by Ma et al [6]. Individual cells could be separated from the bundles and used for studies of their mechanical properties. After an adequate number of bundles (20-30) were collected from culture dishes they were used for studies of myosin heavy chain and myosin light chain kinase isoforms. Cell length was measured with an inverted microscope. Maximum shortening of cells was elicited by applying electrical pulse stimulation (10 Hz, 40 V, 1 ms width). Images of the cells were recorded by a video camera mounted on the microscope. Cell shortening was then analyzed for maximum shortening capacity (ΔL_{max}) and maximum velocity of shortening (V_o). The shortening

measured was that under zero applied load. We have operatively termed the increased V_o and ΔL_{max} elicited by EFS as "supercontractility", [6].

Western Blotting and Immune Western Blotting and Immune Detection

Seventy percent cultured, confluent, canine tracheal SMC were lysed in 150 µl modified RIPA lysis buffer. Protein content was measured using the bicinchoninic acid method. Protein extracts (25 µg) and broad-range molecular weight markers were separated by 8-12% (w/v) SDS-PAGE and then transferred to nitrocellulose membranes. After blocking, membranes were incubated over-night at 4°C, in solution with a dilution of 1 µg/µl of monoclonal antimouse myosin light chain kinase (Sigma clone K36), anti-α-smooth muscle actin (Sigma clone 1A4), anti-myosin heavy chain (Sigma clone HSM-V), anti-rabbit TRII, anti-rabbit TGF-βI antibody, or polyclonal anti-mouse MMP-2 antibodies in 0.01% TBS-T containing 5% skim-milk powder. Blots were washed and then incubated with streptavidin-HRP conjugate (1:4000) diluted in TBS-T containing 1% skim-milk powder for 1 hour at room temperature. Blots were visualized with enhanced chemiluminescence (ECL), and subjected to densitometry.

Fluorescence Immunocytochemistry

Immunofluorescence analysis was performed as previously described [6]. Freshly isolated cells were plated in six-well dishes on acidified alcohol-treated cover slips. At 70% confluence, cells were arrested for seven days, fixed in 1% paraformaldehyde – PBS (pH 7.6), permeabilized with 0.1% Triton X-100 and then blocked. Primary antibodies (diluted to 1:25) included rabbit anti-smooth muscle MLCK, mouse anti-smooth muscle

type MHC, and anti-smooth muscle α-actin (α-SMA). After treatment with secondary antibodies cover slips were washed and nuclei were stained with Hoechst 33342 dye (20 μg/ml). Slides were photographed using a Nikon Diaphot microscope.

Translocation of Smad2 complex from cytoplasm to nucleus was detected by confocal microscopy employing phosphospecific Smad2 antibodies.

TGF-β ELISA

The Duo-Set® TGF-β ELISA kit was used to determine TGF-β content in neutral culture medium (representing active TGF-β) or culture medium that was acidified and subsequently neutralized (representing total TGF-β) according to the manufacturer's instructions. One hundred micro liters of mouse anti-TGF-β capture antibody were coated onto a 96-well micro-plate overnight at room temperature. After washing (3 x 200 μl) with wash buffer (0.05% Tween-2 in PBs, pH 7.2-7.4), samples were blocked with 200 μl blocking buffer (5% Tween-20, 5% sucrose in PBS with 0.05% NaN3) for a minimum of one hour at room temperature. The plate was washed (3 x 200 μl) with wash buffer, and then incubated with 100 μl biotinylated chicken anti-human TGF-β detection antibody (diluted to 300 ng/ml in reagent diluent) for 24 hr at room temperature. The plate was next washed (3 x 200 μl) with wash buffer and then incubated with 100 μl streptavidin horseradish peroxidase (HRP diluted 1:2000) in each well for 20 min at room temperature and shielded from direct light. The substrate solution consisted of a 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (tetramethylbenzidine). Fifty micro liters of 2 N H_2SO_4 were used to halt the colorimetric reaction. The optical density of the colored complex was read using a Power Wave micro plate reader employing KC4 software on a

Windows-Based PC set a 562 nm, from which the concomitantly measured value of density read at 450 nm was subtracted to yield a corrected optical density. TGF-β concentrations in the samples were calculated by a four-parameter/coefficient logistic regression equation with the KC4 program.

Double Mass Spectrometric Analysis of Phosphopeptides

To examine differential expression of proteins, we collected 1 ml conditioned media from serum-starved, arrested TSM cells (TSMC). The medium was dialyzed in PBS for 24 hr at 4^{o}C and proteins were separated using 10% (w/v) SDS-PAGE, and stained with the Silver Staining Kit (Protein) according to manufacturer's instructions. A ~68 kDa band was seen, excised, and subjected to reduction, carboxyamidomethylation and digestion with trypsin. Tryptic peptides were analyzed by micro-capillary reverse-phase high-performance liquid chromatography and nano-electrospray tandem mass spectrometry at Harvard Microchemistry and Proteomics Analysis Facility (Cambridge, MA). Peptide sequencing was also carried out. To identify potential matches with our sequencing results, homology searches were conducted with the aid of the BLAST algorithm (www.ncbl.nlm.nih.gov/blast).

Decorin Pre-treatment to Partially Block Increased TGF-βI Protein Accumulation in Conditioned Medium from Serum-deprived Cell Cultures

Decorin regulates matrix assembly by binding to collagen [10,17]. It also binds several other proteins important among which is TGF-β [17]. Co-localization of decorin and TGF-β has been reported in bronchial biopsies [29]. The complex of the two may

serve as a reservoir for TGF-β from where it can be liberated by appropriate stimuli. Recombinant human decorin was purchased from a commercial source in 100 μg lots and was diluted in 1 ml of 10nM PBS free of Ca^{2+} and Mg^{2+}. In experiments that required decorin the 100nm dismeter culture dish that contained canine airway myocytes was rinsed with 100nM PBS free of Ca^{2+} and Mg^{2} (2x3 ml) and samples were treated with 5 μg/ml in 3 ml of F-12 medium containing 1% ITS for 24 hours. At the completion of the treatment the culture dish was rinsed with 10nM PBS free of Ca^{2+} and Mg^{2} (2x3 ml) and 10 ml of F-12 medium containing 1% ITS was added and cells were further serum-deprived for the required duration of time.

Transfection with Adenovirus Vector Containing Mutated TGF-β Receptor Type II

Canine TSMC were transfected with an adenoviral vector expressing a mutated human type TβRII that was truncated of its kinase domain (Adv-dn TβRII); it was made by us (FS and PC). This construct abrogates TGF-β ligand-mediated signaling because deletion of the cytoplasmic serine/threonine domain prevents signal propagation by intracellular mediators [20]. Seventy percent confluent cultures were transfected with Adv-dn TβRII [multiplicity of infection (m.o.i.) = 10] or control adenovirus (Adv-TrLacZ, which expressed bacterial-galactosidase), in serum-free medium for 24 hours while being incubated at 37^{o}C in 5% CO_2, and then were serum-starved for six more days.

X-Gal Staining of Fixed Cells

Canine tracheal SMC were transfected with control adenovirus (Adv-TRLacZ) in serum-free medium for 24 hours while being incubated at 37°C in 5% CO_2, and then serum-starved for six more days. Cells were fixed with PBS containing 1.25% glutaraldehyde for five minutes at room temperature. Solution "A" (5mM potassium ferricyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride in PBS) was added in 1:40 dilution to X-gal (5-bromo-4-chloro-3-indolylβ-D-galactoside) to yield "final X-gal solution". Following two washes with Ca^{2+}-free PBS, "final X-gal solution" was added and cells were incubated at 37°C in 5% CO_2 until a blue color developed in 4-6 hours. This staining was stopped when cells were rinsed with PBS containing Ca^{2+}/Mg^{2+} and images were captured using a CCD camera.

Gelatin Substrate Zymography

To evaluate the effect of serum deprivation on expression and activity of MMP-2, conditioned medium was analyzed by in-gelatin zymography. Briefly, samples (50 µl) were run on an 8% (w/v) SDS polyacrylamide gel containing 1 mg/ml porcine gelatin and washed three times to remove SDS and allow enzyme renaturation. Gels were incubated overnight in MMP activation buffer (10mM Tris HCl, pH 7.5, containing 1.25% Triton X-100, 5mM $CaCl_2$, 1 uM $ZnCl_2$) prior to staining with 0.25% (w/v) Coomassie blue in the fixing solution. Cleared zones of gelatinolytic activity were observed upon destaining.

Data Analysis

Data were expressed as means ± SE. A fixed constants, one-way analysis of variance (ANOVA) was used to analyze the differences among mean values at different time points. Duncan's new multiple-range test was used to determine which mean values were responsible for the differences indicated. To compare differences in protein expression, statistical significance was determined by Student's t-test using the SPSS™ program with $p < 0.05$ considered significant.

Results

Morphology of the Supercontractile Phenotype of Starved Canine TSMC in Culture

Morphologically two distinct groups of cells were seen on immunocytology of cultured, 15 day-arrested TSMC as previously reported by us [6]. Figure 1 shows that one group appeared as small, flat, bright cells (short cells); these comprised numerically, about 70% of the cells in culture (see A) and the second group (long cells) showed an elongated, spindle shape that was aligned in parallel bundles (see B). Halayko et al [30] have reported the presence of muscarinic M3 receptors on the sarcolemma of the arrested cells; gap junctions were also seen between the latter types of cells. We found they possessed a shining sarcolemma and were 190 ± 25.5 μm (SE) in mean length on the seventh day of serum deprivation. Numerically they comprised 28.5 ± 4.6% (SE) of all cells but by virtue of their cytoplasmic volume occupied almost 40% of the total area of the dish. Because of their super-contractility an attempt was made to determine whether these cells were transforming into skeletal muscle cells. However, in five experiments there was no expression of sarcomeric myosin heavy chain, troponin C or T, nor MyoD.

Mechanics of the Supercontractile Phenotype of Canine TSMC in Culture; Marker Proteins

Measurements:

Shortening capacity (% of cell optimal length or L_o) versus time showed the arrested cell shortened almost twice as much as the freshly isolated control cell (control). The V_o for the arrested cell was double that of the control.

Western blot analysis using specific antibodies showed that the long cells contained the smooth muscle type of myosin heavy chains, smooth muscle type of myosin light chain kinase, and α smooth muscle type of actin. Non-muscle cell types of the same proteins were considerably down-regulated. Proliferating small cells showed a mitotic pattern of protein expression predominant – non-muscle types of myosin heavy chain and myosin light chain kinase.

Identification of a ~68 kDA Protein Band Present Exclusively in Serum-deprived Conditioned Media

Tryptic peptides were analyzed by micro-capillary, reverse-phase high-performance liquid chromatography nano-electrospray tandem mass spectroscopy at Harvard Microchemistry and Proteomics Analysis Facility (Cambridge, MA). To identify potential matches with our isolated protein, sequencing was carried out and yielded the following unique sequence: WYDVLR. To investigate whether serum starvation differentially expressed any proteins, conditioned medium (CM) from proliferating and seven day serum-deprived canine TSMC was examined. A ~68 kDa protein band was seen (see Figure 2, lane 2), excised and subjected to reduction, carboxyamidomethylation and

digestion with trypsin. Subsequent Western blot analysis with specific antibody confirmed this.

Figure 3, panel A shows Western blots of MMP-2 of conditioned media obtained on days 3, 6, and 9 of serum starvation. Panel B is a zymogram showing proteolytic activity of MMP-2, as a function of time.

Increased TGF-β Protein in CM Obtained from Cell Cultures Subjected to Increased Duration of Serum Starvation

We analyzed CM from canine TSMC for content of TGF-βI protein. The medium was dialyzed, proteins were separated by 8% (w/v) SDS-PAGE, transferred to nitrocellulose and subjected to autoradiography. Immunoblotting of CM showed the TGF-β protein content was high when the cell was proliferating (day zero), became absent at day three of starvation and then reappeared in increasing concentrations on days six, nine and twelve. This indicates that the time course of TGF-β protein expression in serum-starved cells mimicked that of MMP-2. Note that though the protein content had increased by day nine, it was considerably less than that at day zero. A similar increase in TGF-β was demonstrated on day 6 using a Duoset ELISA kit. Decorin pre-treatment attenuated the serum deprivation-induced increase in total and active TGF-β.

Effect of Decorin on TGF-βI Protein Expression

An ELISA detected a significant increase in both active and total TGF-βI protein in CM that peaked at day six of serum starvation of the cultured cells.

Decorin pre-treatment (5 μg/ml) for 24 h prevented the serum deprivation-induced increase on day six.. A two way analysis of variance combined with Duncan's new multiple range tests was carried out to arrive at the above conclusions, ($p<0.05$).

Effect of Addition of Exogenous TGF-β on Cell Length

To cultures of proliferating and arrested cells TFG-β was added in concentrations of 1 ng/ml, 2 ng/ml, 10 ng/ml and 20 ng/ml. These were added to separate cultures on day 1, 3, 4, 7, 10, 11, and 14. Their lengths were measured using an objective lens graticule. A 3-dimesional plot of the data is seen in Figure 4. The mean cell lengths for the serum-fed cells at day 6 when this proliferation culture is 70% confluent, is 85.4 μm ±1.9 (SE), n=60. It is at this time serum starvation is commenced for prospective "arrested" cells which are termed day 0 cells. ™ Their mean lengths are 81.3 μm ±1.8 (SE); n=60. While peak length for proliferating cells is 106 μm ±2.0 (SE) achieved on day 7, that for serum-starved, arrested cells is 155.7 μm ±4.2 (SE). Thereafter the arrested cells become smaller in length.

TβR-I and TβR-II Cross-linking of Decorin Pre-treated Serum-deprived Cells

Canine TSMC, serum-deprived for seven days showed bands corresponding to proteins of appropriate molecular weights for TβR-I (53 kDa) and both the minor (75 kDa) and the major (110 kDa) isoforms of TβR-II (see Figure 5, lane 1). This confirms, in canine TSMC, the presence of receptors which provide the proteins for TGF-β signaling. Figure 5, lane 2 shows that decorin (5 μg/ml pre-treatment for 24 h followed by six additional days of serum deprivation) partially blocked appearance of TFβR-I and βR-II

bands. Competition studies (Figure 5, lane 3) show that 2-fold excess of TGF-β (10 ng/ml) was able to block binding of ^{125} I-TGF-β; this demonstrated binding specificity.

TFβ-RII Protein Accumulation in Serum-deprived Canine TSMC Pre-treated with Decorin

No significant effect ($p>0.05$) of serum-deprivation or decorin pre-treatment on TFβ-RII protein expression was seen. Combined with the cross-linking experiment data, these data suggest that TGF-β blockade by decorin likely occurs by disrupting the TGF-β and TFβR-II and TFβR-I association and that the effect is not compounded by altered levels of TFβR-II.

smMHC and smMLCK Protein Accumulation in Canine TSMC Pretreated with Decorin

Figure 6, panel A shows Western blots of experiments that show that decorin significantly reduced smMLCK content while panel B histographically confirms this. The results of statistical analysis of data from six experiments are shown in the bar graphs (see panel B). With respect to smMLCK, significant differences are seen on day seven, while with respect to smMHC (panels C and D) significant differences are evident on day three and seven. The disparity of timing in the onset of decorin's effect could be explained by the fact that smMHC and smMLCK are regulated by different mechanisms.

Adv-dn TGF-βRII Blocks Accumulation of Markers of TSMC Phenotype

To further substantiate the hypothesis that TGF-β is a differentiating factor for TSMC in culture we expected that its down-regulation would be associated with a change of phenotype from differentiated to synthetic, with a corresponding change in marker

proteins. We therefore transfected canine TSMC with an adenoviral vector expressing a mutated human type II TGF-β receptor (Adv-dn-TGF-βRII). Previous research [31] indicated that this construct, whose serine/threonine catalytic domain is truncated, would interfere with intracellular TGF-βI-βRII-βRI signaling.

We cultured airway smooth muscles with 10% FBS and then serum-starved them for 24 hours. Careful analysis showed that the cells were morphologically homogeneous. The field shown was selected from four randomly chosen ones, taken from each of four different dishes of cells. These data indicate that the long, supercontractile phenotype of cell is not present in any appreciable number (less than 5%) initially and develops later. A time course study is needed to determine when the changes start. A culture of proliferating of myocytes was infected with Adv-TβII-βGal (m.o.i. =10). After 24 h; serum-fed medium was replaced and cells were further serum deprived for 6 additional days (mag x 100). The cells were then incubated with X-gal (1mg/ml) for 4 h at 37^{o}C until the blue color indicative of successful gene transfer of β-galactosidase was seen. Numbers of cells, stained blue in which a nucleus could also be seen, averaged 90% indicating efficient adenovirus infection.

Cultures at 70% confluency were then transfected with Adv-dn-TGF-βRII (m.o.i. = 10) or control adenovirus (Adv-TGF-βR-LacZ which expressed bacterial galactosidase) in serum-free medium for 24 h while incubated at 37°C in 5% CO_2 and then serum-starved for 6 more days. See Figure 6.

Transfection with Adv-dn-TGF-βRII was able to down-regulate the serum starvation-induced accumulation of smMLCK and smMHC compared to the control vector (See Figure 7). In addition, neither construct caused changes in expression of sm-α-actin.

Smad Proteins

Using antibodies against Smad2 proteins, Western blots identified a phosphospecific Smad2 protein.

With these antibodies immunocytochemistry of serum-starved (for seven days) canine TSMC was carried out by confocal microscopy. Figure 8, left hand panel shows Smad2 immunoreactive protein in the cytoplasm and nucleus of an arrested cell. The presence of a signal in the nucleus indicates translocation of Smad2 into the nucleus. Since Smad2 can only enter the nucleus when in a complex form, the intranuclear location of Smad2 suggests Smad4 is present also. Arrested cells treated with decorin shown in the right hand panel indicate accumulation of lesser amounts of Smad2 in the nucleus. See right hand panel.

Discussion

We determined whether TGF-β, expressed in greater abundance in the conditioned medium of cultured cells subjected to serum starvation, could be a differentiation factor for the supercontractile, differentiated cells that develop under these conditions. To achieve this we identified marker proteins for these cells. Smooth muscle type myosin heavy chain and myosin light chain kinase proved to be effective as markers of the differentiated state. Proliferating cells show very low levels of these markers and instead up-regulate levels of non-muscle type myosin heavy chain and non-muscle type of myosin light chain kinase. The expression of these marker proteins should thus help us to identify the phenotype of the various cells.

The highest concentration of TGFβ was found in medium from proliferating cells. On initiating serum starvation levels dropped precipitously by day 3 and proliferation rate became considerably reduced. By day 6 and 9 the levels became increased though never to the same levels as on day 0. It is known that while high levels favour proliferation, lower levels favour either apoptosis or differentiation. To test the hypothesis that TGF-β in the levels seen at day 6, 9 and 12 could be a differentiating factor for these supercontractile cells, down-regulation studies were conducted. Two methods of down-regulation were used; treatment of the arrested cells with decorin that returned the arrested cells to a partially proliferating state, transfection of the cells with Adv-dn-TβR-II which produced the same change [32,33].

To determine whether TGF- β added exogenously to the culture media of proliferating and arrested (serum started) cells would show the same qualitative effects as those of the arrested cells alone appropriate experiments were carried out. See Figure 6. The 3-dimensional plots show the difference in cell lengths between proliferating and arrested cells. The 3-D surfaces show this difference more clearly than any other plot or histogram.

Interestingly, while the proliferating cells start at a smaller length as expected, they show a progressive increase with time right to day 14. The arrested cells show a sharp increase in day 1 and under no exogenously added TGF- β. This is likely due to production of endogenous TGF- β and its export to the culture medium. At a concentration of 1 ng/ml exogenously added TFG- β there is a considerable increase in cell length. Thereafter, at 10 and 20 ng/ml concentrations cell length diminishes to that of the proliferating cells. Our explanation for this behaviour is the contact inhibition that occurs

in the serum-deprived cells whenever the cells are increasing in length and contacting each other [35, 36, 37]. It must be noted that though the number of cells is greater in proliferating cultures, the amount of cytoplasm in the arrested cells is much greater and establishes inter-cellular contact much more easily.

Further proof of the role of TGF-β was provided by showing that specific second messenger signaling could be initiated by TGF-β. Immunocytochemical phosphospecific Smad2 antibodies demonstrated, in the arrested cells, expression of phosphorylated Smad2 in the cytoplasm. Its presence in the nucleus indicate the occurrence of translocation and the likelihood of its involvement in gene expression. To delineate downstream mechanisms further studies of SM22α promoter activation and of myosin heavy chain, and myosin light chain kinase promoter activation will be conducted in the future, along with assessment of the activities of inducer and co-repressor molecules, and activation of specific genes.

The conversion of cells from proliferative to differentiated type is not unique. Pickering's group has reported similar findings in vascular smooth muscle [28].

With respect to gaining insight into the significance of smooth muscle differentiation we can only speculate. Were these cells to develop in the airways of asthmatics they could contribute considerably to bronchoconstriction. The scenario is that during the acute attack of asthma, inflammatory exudates appear. The growth factors that they contain lead to airway myocyte hyperplasia and hypertrophy which in turn lead to airway obstruction. Once the acute attack subsides a state akin to that of our cultured cells grown in serum-deprived media could then develop with resultant development of the supercontractile cell phenotype. Its timing would occur at the time of emergence of non-specific hyper-reactivity of the airways which commonly follow an acute attack of asthma.

Zhou et al [34] have reported similar findings in cultured human bronchial smooth muscle cells.

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Figure Legends

Figure 1

Indirect immunofluorescent staining, using a Cy3-conjugated secondary antibody, of smooth muscle Myosin Heavy Chain(1:100) in canine airway myocytes that were serum starved for 15 days (magnification x 200).

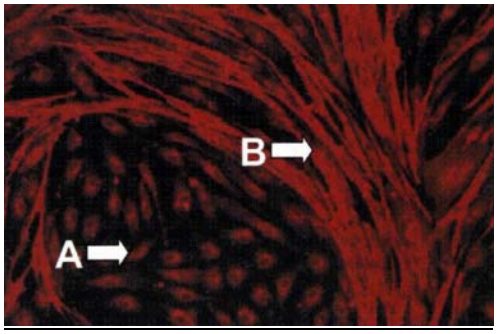

Figure 2

Conditioned medium from confluent, serum-fed cell cultures 48 hours since their last feeding (**lane 1**), and cells deprived of serum for seven days (**lane 2**) were dialyzed; proteins (25 μg/lane) were fractionated via SDS-PAGE, and silver-stained. A more intense ~70 kDa band in conditioned medium was obtained from cells deprived of serum for seven days, than conditioned medium from proliferating canine airway myocytes. The figure is typical of those obtained from two samples which are representative of cell lines.

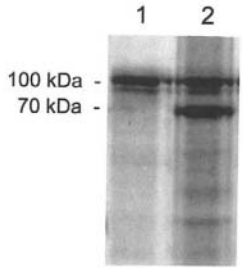

Figure 3

Panel A conditioned medium from airway myocytes serum-deprived for three, six and nine days. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose, probed with anti-matrix-metalloproteinase-2 (MMP-2) antibody (1:1000) via Western blot. This representative blot shows that accumulation of MMP-2, began at day three of serum-deprivation, and increased with duration of serum-deprivation. Panel B shows similar samples as in A subjected to gelatin substrate zymography (performed in triplicate). This representative zymogram shows a ~70 kDa band of metalloenzyme proteolytic activity, corresponding to MMP-2 that showed increased activity beginning at day three of serum-deprivation, and increased with duration of serum-deprivation.

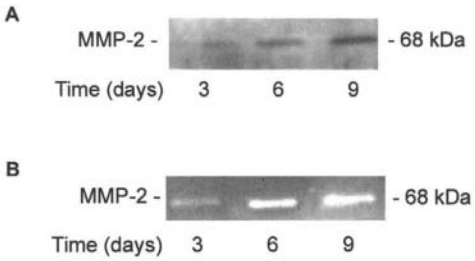

Figure 4

Cell length (μm) is plotted as a function of 'days in culture' and 'TGF-beta concentration (ng)'. The upper surface shows values for the arrested (serum starved) cells. The lower surface represents values obtained from proliferating cells. Not shown on the graph, but important to note is that cell length at day 0 with no TGF-beta added (control) is the same in the arrested and proliferating cells: 81.29167 ± 10.68116 vs. 85.375 ± 14.43003 (mean ± std).

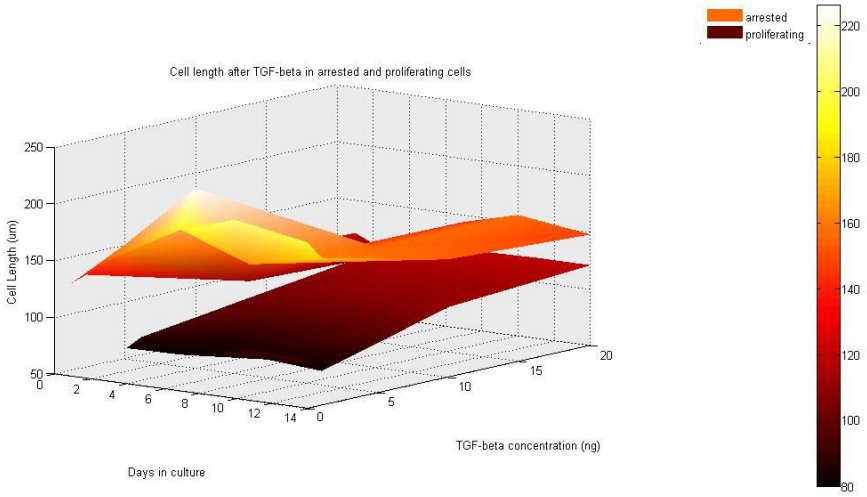

Figure 5

Canine airway myocytes, serum-deprived were cross-linked with 0.27 mM DSS, and then incubated with ^{125}I-TGF-β_1 (5 ng/ml). Cell lysates (25 μg/lane) were fractioned by 10% (w/v) SDS-PAGE followed by autoradiography. Cells that were serum-deprived for seven days **(lane 1)** show bands of appropriate sizes, confirming the presence of TβRI and TβRII in canine tracheal smooth muscle cell. **Lane 2** shows cells pretreated for 24 hours with decorin (5 μg/ml) and serum-starved for six additional days before cross-linking. **Lane 3** shows that 2-fold excess cold TGF-β_1 (10 ng/ml) was able to block binding of ^{125}I-TGF-β_1 to TβRI and TβRII, indicative of binding specificity.

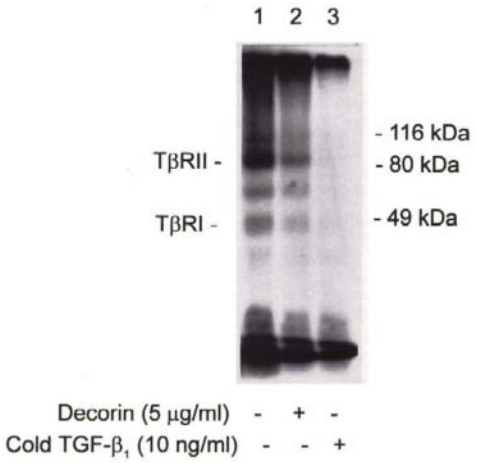
1 2 3
- 116 kDa
TβRII -
- 80 kDa
TβRI -
- 49 kDa
Decorin (5 μg/ml) - + -
Cold TGF-β1 (10 ng/ml) - - +

Figure 6

Canine airway myocytes were serum-deprived for one, three or seven days and/or treated with decorin (5 μg/ml) for the first 24 hours, serum-free medium was replaced and cells were serum-deprived for the same total time as the first group. Proteins (25 μg/lane) were fractionated by SDS-PAGE and probed with anti-smooth muscle myosin light chain kinase (smMLCK) or anti-smooth muscle myosin heavy chain (smMHC) antibodies (both at 1:1000) via Western blot. Experiments used two cell lines with three replications each and gels were performed in triplicate. Representative blots of smMLCK **(panel A)** and smMHC (**panel C)** are shown. These were relatively quantified by the ratio or protein obtained from serum-deprived cells divided by the amount of protein obtained from cells treated with decorin (5 μg/ml) during the first 24 hours of serum-deprivation. Densitometry results below each blot (**Panels B and D**) are expressed as a mean ± SE from three independent experiments. * $P<0.05$ for the quantity of protein from airway myocytes that were serum deprived for seven days (smMLCK) and for three or seven days (smMHC) versus airway myocytes that were serum deprived for one day, respectively (paired Student's t-test).

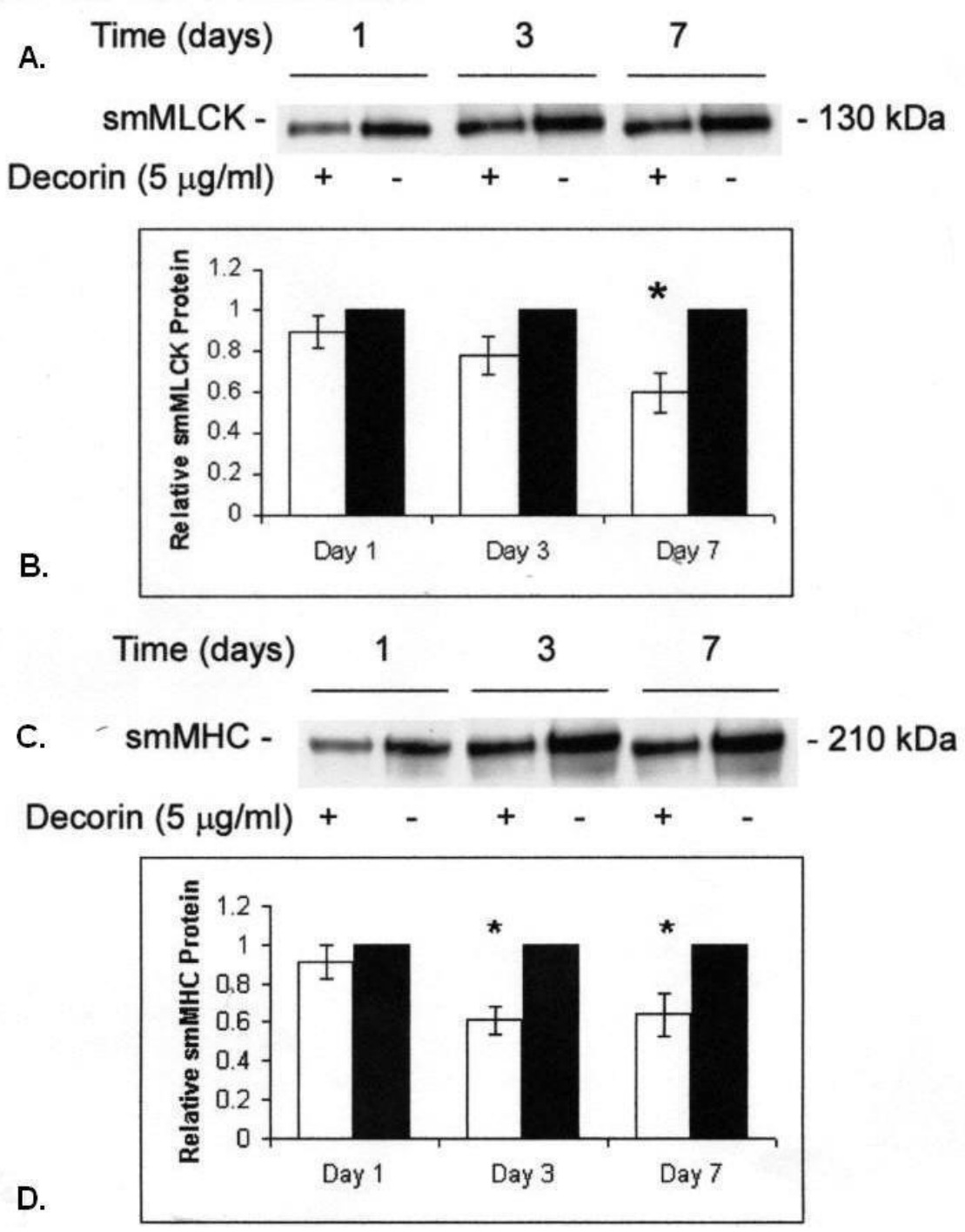
A.
Time (days)
1
3
7
smMLCK -
- 130 kDa
Decorin (5 μg/ml)
+ - + - + -
B.
Relative smMLCK Protein
1.2
1
0.8
0.6
0.4
0.2
0
*
Day 1
Day 3
Day 7
C.
Time (days)
1
3
7
smMHC -
- 210 kDa
Decorin (5 μg/ml)
+ - + - + -
D.
Relative smMHC Protein
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Day 1
Day 3
Day 7

Figure 7

Western blots (using antibodies against smooth muscle myosin heavy chain (smMHC), smooth muscle myosin light chain kinase (smMLCK) and smooth muscle actin (smActin) of cell lysates of canine airway myocytes infected with Adv-dn-TβRII ("+") or Adv-dn-TβRII-βGal ("-") (both at m.o.i. 10) for 24 hours culture medium was replaced and cells were serum deprived for six additional days. Proteins (25 μg/lane) were fractionated by SDA-PAGE, transferred onto nitrocellulose and were probed with antibodies against smMHC, smMLCK, and sm-α-actin (all 1:1000) via Western blot. Experiments used two cell lines with three replications each and gels were performed in triplicate. Smooth muscle cells (SMC) infected with Adv-dn-TβRII decreased accumulation of smMHC and smMLCK versus SMC infected with Adv-dn-TβRII-βGal. However, infection with Adv-dn-TβRII did not alter smooth muscle-α-actin accumulation versus infection with Adv-TβRII-βGal.

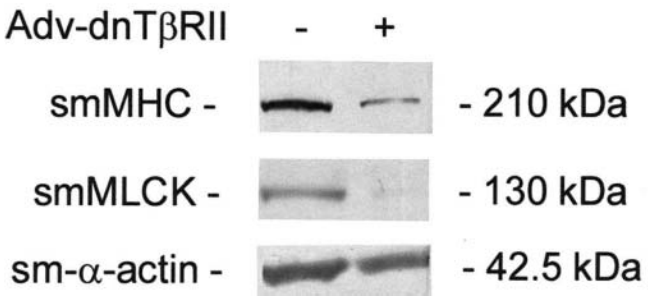

Figure 8

Immunocytochemistry of serum-deprived (seven days) airway smooth muscle cells. Phosphospecific Smad2 antibodies were used. The left hand panel shows phosphospecific Smad2 signal in the cytoplasm and the nucleus. The right hand panel shows similar immunocytochemical analysis except that the cells were pre-treated with Decorin which

bound TGFβ1 in the extra-cellular matrix. A reduced intensity of green fluorescence is evident both in the cytoplasm and the nucleus, indicating reduced amounts of Smad proteins in these locations.