MECHANISMS OF RETINOIC ACID EFFECTS ON AIRWAY SMOOTH MUSCLE CELL PHENOTYPE

BY

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A Thesis submitted to

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In Fulfillment of the Requirements for the Degree of

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Department of Surgery

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Purpose Retinoic acid (RA) is a natural vitamin A derivative known to exert multiple effects on cellular proliferation and differentiation. We tested the hypothesis that RA may induce phenotypic and anti-proliferative changes on airway smooth muscle cells. Further, we studied the underlying molecular mechanisms of these effects.

Method Human bronchial and canine tracheal myocytes were grown in primary culture in the presence or absence of RA (1μM). Expressions of RA receptor subtypes were investigated using western blotting and fluorescent immunocytochemistry. Transcriptional regulation of genes encoding the contractile apparatus-associated proteins, smMHC and SM22, was measured by luciferase reporter gene assay and electrophoretic mobility shift assay (EMSA). Expression of the smooth muscle specific contractile protein, smMHC, was analyzed by western blotting. Myocyte proliferation was measured using a Coulter cell counter.

Results Cultured human and canine airway myocytes homogeneously expressed 5 different RA receptors. Luciferase reporter assay revealed RA inhibited SM22 gene transcription by 73.14% ± 4.18% ($n=12$, $p<0.0001$). EMSA demonstrated that RA markedly reduced the binding to the SM22 gene promoter by SRF, a transcription factor essential for smooth muscle specific gene expression. RA also blocked the accumulation of smMHC protein in cultured canine airway myocytes. Further, RA inhibited the proliferation of human airway smooth muscle cells by 52% ($n=3$).

Conclusions Retinoic acid significantly alters the gene expression, phenotype, and proliferation of airway smooth muscle cells. These findings may have important implications in the treatments of emphysema, asthma, and thoracic oncology.

INTRODUCTION

Retinoic acid (RA) is a natural vitamin A derivative known to exert multiple effects on cellular proliferation and differentiation in the developing and mature lung (Malpel ET AL., 2000; McGowan, 2002; Massaro and Massaro, 2001). Naturally occurring retinoids include all-*trans* retinoic acid (atRA), 3,4-didehydroreinoic acid, and 9-*cis* retinoic acid. atRA is bound to albumin in plasma (Smith et al, 1973; Kurlandsky et al, 1995). Its lipophilic nature allows it to transverse cell membranes. Within the cell it interacts with cellular retinoic acid binding proteins (CRABP). It is then metabolized by the P450 system to oxidized RA, or is isomerized to different forms of retinoids (Miano and Berk, 2000). More importantly, it may bind with intranuclear RA receptors (Chambon, 1996). RA receptors belong to the steroid receptor superfamily. Seven RA receptors are known to exist, including RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, and RORγ. RA receptors form heterodimers (Kastner et al, 1997) that binds to retinoic acid response elements (RAREs) within the regulatory regions of many important genes (Gudas et al, 1994). This binding changes the gene-expression profile of the cell (Miano and Berk, 2000). The consensus sequence of RARE is a direct repeat of AGGTCA separated by 1, 2 or 5 nucleotides (Miano and Berk, 2000).

RA may have important effects on pulmonary mesenchymal cells. Regulation of RA signaling is important during proper lung morphogenesis, including early lung bud formation and subsequent branching morphogenesis (Malpel et al, 2000a). RA significantly ameliorates elastase-induced emphysema in rats through the regeneration and repair of pulmonary alveoli (Massaro and Massaro, 1997; McGowan, 2002). atRA effects almost 100% remission rate in acute promyelocytic leukemia (Tallman et al., 1997). Retinoid

therapy is implicated in the prevention of lung cancer in ex-smokers. Smoking reduces RARβ expression, which is considered as a biomarker of pre-malignancy (Kurie et al., 2003). RA treatment restores the expression of RARβ and is associated with a reduction in squamous metaplasia on bronchoscopic biopsies (Kurie et al., 2003). Furthermore, multiple studies have confirmed the significance of retinoids in vasculogenesis, neointimal formation, and growth and differentiation of smooth muscle cell (SMC) (Miano and Berk, 2000). The effects of RA on airway SMC are not well established. In this study, it is hypothesized that RA may induce phenotypic and anti-proliferative changes on airway SMC. The study attempts to answer the following questions:

- Which RA receptors are expressed in human airway smooth muscle cells (HASMC)?
- Does RA have anti-proliferative properties?
- Does RA affect transcription of key smooth muscle genes?
- Does the amount of smooth muscle protein change with RA treatment?
- Answers to these questions will provide us with insights into the mechanisms of effects of RA on airway SMC phenotype.

METHODS

Preparation of cultured HASMC

A segment of bronchus was removed from patients undergoing thoracic surgery in accordance with a protocol approved by the Human Research Ethics Board, University of Manitoba. The specimen was immersed in ice-cold Krebs-Henseleit buffer overnight at 4°C. Buffer composition was : sodium chloride (112.6 mM), sodium bicarbonate (25 mM), anhydrous sodium phosphate (1.38 mM), potassium chloride (4.7 mM), magnesium sulphate (2.46 mM), and dextrose (5.56 mM). The solution was oxygenated with 95% oxygen with 5% carbon dioxide for 30 minutes prior to use. The pH of the solution was adjusted to 7.4 with hydrochloric acid or sodium hydroxide as indicated prior to use.

The smooth muscle of the bronchus was dissected the next day with the aid of a microscope. Bronchial smooth muscle tissue was cleaned of adherent connective tissue with fine dissecting scissors as previously described (Hamman et al, 2000; Naureckas et al, 2000). The smooth muscle tissue was disinfected with 4 successive washed for 15 minutes each in cold sterile HEPES-buffered HBSS containing 1x antibiotic-antimycotic and 0.5x gentamycin solution. HEPES-HBSS s composition was: potassium chloride (5.33 mM), potassium phosphate (0.44 mM), sodium bicarbonate (4.00 mM), sodium chloride (138.00 mM), anhydrous sodium phosphate (0.30 mM), glucose (5.60 mM), and HEPES (20 mM). Antibiotic-antimycotic solution was from Gibco 100x stock (cat. no. 15240-062) diluted to 1x in solutions. Gentamycin reagent solution was from Gibco 1000x stock (50 mg/ml) (cat. no. 15750-060) diluted to 0.5x (25 mg/ml) in solutions. After the dissection, the buffer was removed, the smooth muscle tissue minced with fine scissors, then resuspended in Digestion

Buffer at 37 °C for 30-40 minutes. Digestion Buffer composition was: HEPES-HBSS, pH 7.4 containing 1x antibiotic-antimycotic, 1x gentamycin, collagenase (600 U/ml), Nagarse protease (2 U/ml), and elastase (10 U/ml). The solution was sterile filtered prior to use.

After digestion was completed remaining tissue pieces were disintegrated by mild trituration with a borosilicate glass Pasteur pipette. After allowing remaining debris to settle, the supernatant was removed and divided into two sterile tubes each containing 30 ml of growth medium (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, and 50 U/ml penicillin, 50 μg/ml streptomycin, and glutamine). Smooth muscle cells were collected by centrifugation at 800x g for 5 minutes. The resulting myocyte pellets were re-suspended in fresh growth medium and the number of live cells was counted using a haemocytometer after mixing 2 drops of suspended cells with an equal volume of trypan blue. Thereafter, cells were plated on 100 mm dishes at 8,000-10,000 cells/cm^2 and grown in a humidified incubator at 37 °C in a 5% CO_2/95% air atmosphere. As cell cultures reached confluence they were passaged by lifting myocytes with 0.05% trypsin/0.5 mM EDTA and reseeded into three new culture plates per confluent dish. Cultured HASMC at passage 3 were used for all studies. To induce a functionally contractile phenotype in approximately 1/6 of cultured myocytes, confluent cultures were maintained in serum-free F-12 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferring, 5 ng/ml selenium, 0.1 mM non-essential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin as we have previously described (Halayko et al, 1999; Liu et al, 2003). Cells were maintained in serum-free medium for up to 14 days and fresh medium was provided every

48-72 hours. Cell morphology was assessed with a Nikon Diaphot II phase-contrast microscope equipped with a 35 mm camera.

Preparation of cultured canine airway smooth muscle cells (CASMC)

We used our previously described methods (Halayko et al, 1996; Halayko et al, 1999). Briefly, the trachea was removed from adult mongrel dogs and was dissected free from peri-tracheal and adventitial tissues. It was immersed in ice-cold Krebs-Henseleit buffer overnight at 4 °C. Dissection of the smooth muscle tissue and isolation of smooth muscle cells proceeded as described above for cultured HASMC. Passage 1 cultured CASMC cultures were used in this study.

Preparation of human bronchial tissue lysates

Bronchus segments removed from patients undergoing thoracic surgery and was stored in -80 °C before use. To prepare whole bronchial tissue lysate, the tissue was placed in a pre-cooled tissue grinder. 5x ice cold RIPA buffer with 2% PMSF, 1% sodium orthovanadate, 10% sodium fluoride, and 0.5% Pepstatin A/Aprotinin was added. RIPA composition was: 40 mM Tris (pH 8.0), 150 mM sodium chloride, 1% NP-40 (IGEPAL CA-630), and 1% sodium deoxycholate. The tissue was homogenized and dissociated on ice with a probe sonicator at high setting for 30 seconds. Debris was removed by pipetting and by filtering through 70 μm nylon mesh. Lysate was then centrifuged at 14000x g for 10 minutes and the supernatant and pellet were stored separately at -80 °C.

RA treatment

atRA (Sigma, R-2625, lot 71K1113) was serially diluted in DMSO to 1mM concentration and stored at -80 °C; 1 μL was added to each milliliter of culture medium to create a final concentration of 1 μM. Cultured human or canine airway myocytes were treated with RA 24 hours after initial plating in cell culture and were compared with untreated controls using DMSO as placebo. Medium containing atRA was replaced every 48 hours thereafter.

Western analysis

Cells for western blotting analysis were washed 3 times in 5 mL of ice-cold HBSS. Cells were lysed using 300 μL of radioimmunoprecipitation assay (RIPA) lysis buffer with the addition of 1 μg each of leupeptin, aprotinin, and pepstatin A. RIPA buffer was composed of 40 mM Tris at pH 8.0, 150 mM NaCl, 1% IGEPAL (NP-40), 1% deoxycholic acid, and 1 mM of phenylmethylsulfonyl fluoride (PMSF) stock solution that was dissolved in ethanol. Cells were lifted from plates by scraping with a cell scraper on ice. Cells and cell debris were transferred to 1.5 mL microcentrifuge tubes and sonicated using a sonicator with 2 pulses for 10 seconds each. Sonicated lysates were centrifuged at 20,000 x g for 15 minutes at 4°C. Supernatant was used for subsequent analyses and was stored at - 80°C.

Protein concentration of the sample was determined by spectrophotometry using colorimetric assay from BioRad. A calibration curve was prepared by diluting a protein standard of known concentration in distilled water; 3 μL of sample was added to 27 μL of distilled water in 96 well plates, and thereafter 25 μL of solution A (with 20 μL solution S added to each 1 mL of solution A), and 200 μL of solution B were added to each well. The contents in the

wells were gently mixed and were incubated in room temperature for 15 minutes. Absorbance was determined using a spectrophotometer at 750 nm wavelength.

Samples were prepared for SDS-PAGE and Western Blotting as we have previously described (Halayko et al, 1996; Halayko et al, 1999). Using the calculated protein concentration from the absorbance readings, 20 μg of each sample was mixed with distilled water and 4x sample buffer (50 μL mercaptoethanol in each 950 μL of sample buffer) to create a total volume of 20 μL. This mixture is boiled for 5 minutes and was returned to ice. To prepare 10% separating gels, we mixed 4.05 mL of distilled water, 3.3 mL of 30% acrylamide, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 100 μL of 10% SDS, 50 μL of 10% APS, and 5 μL of TEMED to create a total volume of 10 mL. Stacking gel was made using 6.1 mL of distilled water, 1.3 mL of 30% acrylamide, 2.5 mL of 0.5 M Tris/HCl (pH 6.8), 100 μL of 10% SDS, 50 μL of 10% APS, and 10 μL of TEMED to create a total volume of 10 mL. Stacking gel was poured after separating gel and running buffer (3.03 g Tris, 14.4 g glycine, 1 g SDS in 1 L of distilled water) was added; 20 μL of each sample and molecular weight markers were added to separate lanes on the gel. The proteins within the sample were size fractionated by running at 125 V for 75 minutes. Proteins were then electroblotted to nitrocellulose membrane using transfer buffer composed of: 3.03 g Tris, 14.4 g Glycine, 0.5 g SDS, 200 mL methanol in 1L of water). Transfer was performed at 100 V for 90 minutes at -4°C with stirring. The membrane was rinsed twice with TBS with 0.1% Tween (TBST), then was blocked for 1 hour using TBST with 5% milk. The blot was then incubated with primary antibody in TBST with 1% milk for 16 hours. Primary antibodies used include rabbit polyclonal antibodies anti-RARα, anti-RARβ, anti-RARγ, anti-RXRα, anti-RXRβ,

anti-RXRγ, goat polyclonal antibody anti-RORα (Santa Cruz Biotechnology), mouse monoclonal anti-smooth muscle myosin heavy chain (MHC; clone hSM-V) (Sigma, St. Louis, MS), rabbit anti-laminin, and mouse anti smooth muscle α-actin (Sigma Immunochemicals). Blots were immunolabeled using HRP-conjugated IgG secondary antibodies for 1 hour in room temperature. Blots were washed 3 times using TBST after each antibody incubation. Blots were developed on film using ECL Plus reagents (Amersham). Densitometry was performed to measure relative band intensity using a Hewlett Packard scanner and Scanplot Software.

Fluorescence immunocytochemistry

Human airway myocytes (passage 3) were seeded onto pre-cleaned, sterile glass coverslips in culture dishes. Cells were grown to 50% confluence. They are washed 3 times in cold sterile cytoskeleton (CB) buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, 5 mM glucose; pH 6.1). Fixing was performed using sterile 3% paraformaldehyde (pH 6.1) for 15 minutes in 4 °C. Cells were then permeablized in 3% paraformaldehyde / 0.3% Triton X-100 for 5 minutes in 4 °C. Cells were washed 3 times with cold sterile CB buffer. Coverslips were blocked with 1% BSA and 2% normal serum from host animal of secondary antibody in cyto-TBS (20 mM Tris base, 154 mM NaCl, 2 mM EGTA, 2 mM MgCl2; pH 7.2) and incubated for 1 day in primary antibody in cyto-TBST (cyto-TBS with 0.1% tween 20) at 4 °C. Primary antibodies used include rabbit polyclonal antibodies anti-RARα diluted 1:100, anti-RARβ diluted 1:25, anti-RARγ diluted 1:25, anti-RXRα diluted 1:100, anti-RXRβ diluted 1:25, anti-RXRγ diluted 1:25, and goat polyclonal antibody anti-RORα diluted 1:100 (Santa Cruz Biotechnology). Coverslips were washed 4 times for 15 minutes each

using cyto-TBFT. Secondary immunolabeling was done using FITC-conjugated donkey anti-rabbit IgG diluted 1:50 for RAR and RXR; Cy5 donkey anti-goat IgG diluted 1:50 was used for RORα detection. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were incubated for 2-3 hours at room temperature, and was then washed away using cyto-TBST for 4 times. Nuclei were labeled with Hoechst 33342 (10 μg/mL). Coverslips were again washed using distilled water for 3 times and was air-dried in darkness. Coverslips were mounted using anti-fade medium and were stored at -20 °C. Fluorescent immunostaining was visualized using a Nikon microscope equipped with epifluorescence optics and a Photometrics SenSys 12-bit digital video camera. Digitalized images were taken with Spectrum imaging software (IP Laboratories, Vienna, VA).

Myocyte proliferation studies

Human airway myocytes at passage 2 grown in DMEM containing 10% FBS were used. Cells treated with RA were compared with untreated controls. The number of cellular nuclei was counted using a coulter cell counter.

Plasmids

Luciferase reporter plasmids used and transfection protocols performed were as we have previously described (Liu et al, 2003; Camoretti-Mercado et al, 2000). Three luciferase reporters were used, (1) pSM22luc, in which transcription of the luciferase cDNA in pGL2basic is directed by bp -445 to +41 of the mouse SM22 gene, (2) pSRFluc, in which luciferase expression is directed by an artificial promoter containing 5 copies of the SRF binding site (CC(A/T)GG, or "CArG box") upstream of a minimal TATA box (Stratagene),

and (3) psmMHCluc in which the human smMHC promoter drives luciferase expression; this construct was prepared from a human chromosome 16p13 BAC clone CIT987SK-972D3 (GenBank TM HSU91323; provided by Dr. Ung-Jin Kim) that contained the 5'-end of the human smMHC gene, as evidenced by sequence homology with rat, mouse, and rabbit smMHC genes. BAC DNA was digested with Kpn I and Spe I, and the 3.3-kilobase fragment containing the smMHC promoter and all of exon 1 ligated into Kpn I/Spe I-digested pGL3 basic. In addition to luciferase reporters harboring SRF-dependent promoters, we also used constructs with the viral MSV-LTR promoter controlling luciferase or β-galactosidase expression (pMSVluc and pMSVβgal respectively). For some studies to determine transfection efficiency we also used pSM22βgal, which contained bp -445 to +41 of the mouse SM22 gene directing lacZ expression. All plasmids were purified on CsCl gradients prior to transfection.

Transfections

Transient transfection of plasmid DNA was accomplished with cationic lipids, lipofectAMINEas we have previously described (Liu et al, ,2003; Camoretti-Mercado et al, 2000). Subconfluent canine myocytes in 6-well dishes were transfected in Optimem (Life Technologies, Inc.) with 4 μL of lipofectAMINE, 0.4 μg of luciferase reporter, and 0.4 μg of pMSVβgal (used to normalize transfection efficiency) per well. Myocytes were refed 5 hours later with serum-free Dulbecco's Modified Eagle's Medium (DMEM) and harvested 24 hours after transfection. Luciferase activity was measured for each sample at 410 nm wavelength and normalized to its β-galactosidase activity.

Extraction of nuclear proteins

Serum deprived canine tracheal myocytes were grown to confluence and lysates were prepared as we have described (Liu et al, 2003; Camoretti-Mercado et al, 2003). Cells were washed twice with HBSS, scraped off the plates after adding 1 mL of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mm DTT) and were sat on ice for 10 minutes. Nuclei were pelleted in buffer A after centrifugation. The pellet was gently resuspended in 200 μL of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and mixed for 30 minutes. After centrifugation, the supernatant containing nuclear proteins were dialyzed for 1 hour against 3 changes of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and subsequently clarified by centrifugation at 16,000 rpm for 10 minutes. Protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin, 5 μg/ml each, Sigma) were added, and nuclear extracts were stored at -80 °C.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as we have previously described (Liu et al, 2003; Camoretti-Mercado et al., 2000). Double-stranded DNA fragments harboring the sequences of interest were prepared by annealing complementary synthetic oligonucleotides and were end-labeled with T4 polynucleotide kinase and γ 32P-ATP. CArG-box-containing probes including those encompassing the 5' (5'-GCTGCCCATAAAAGGTTTTTG-3') or 3' (5'-CTTTCCCCAAATATGGAGCCTG-3') CArG boxes (underlined) of the mouse SM22 promoter. 20,000 dpm (1-5 fmol) labeled oligonucleotide were pre-incubated for 15 minutes

with 1.5 μL of binding buffer (50 mM Tris-HCl, pH 7.5, 20% Ficoll, 375 mM KCl, 5 mM EDTA, 5 mM DTT) and 1 μg of poly (dI-dC). Cold probe (without labeled radionucleotide) was used as negative control. For supershift experiments, 3 μg of SRF antibody (gift of Dr. R. Prywes) were added to the incubation mixture. Binding reactions (4 μg of nuclear extract protein) were performed at room temperature in total volume of 15 μL for 30 minutes. DNA-protein complex formation was analyzed by electrophoresis on 4% non-denaturing polyacrylamide gels in TBE buffer (40 mM Tris borate, 1 mM EDTA, pH 8.0).

RESULTS AND FIGURES

Five different retinoic acid receptors were expressed in human airway smooth muscle cells. The expression of RA receptor proteins was determined using Western blot analysis (Figure 1). Cultured human myocytes (passage 3) were harvested at 50% confluence and at 100% confluence. A separate population of human myocytes was serum deprived after reaching confluence and was harvested 12 days after confluence. Whole bronchial tissue lysate was also used to assess and compare the receptors expressed by human airway myocytes in vivo and in vitro. Western blot analysis demonstrated the presence of RAR α, RXR α, RXR β, RXR γ, and ROR α in human airway myocytes, whereas RAR β and RAR γ were absent. Of note, only RAR α and RXR α abundance increased as cells reached confluence and were then serum deprived to induce differentiation into contractile phenotype.

Retinoic acid receptors have a nuclear or peri-nuclear distribution within cultured HASMC. Using fluorescent immunocytochemistry, human airway myocytes (passage 3) were grown to 50% confluence and were immunolabeled using retinoic acid receptors primary antibodies and FITC- or Cy3- conjugated secondary antibodies. Nuclei were labeled with Hoechst 33342. RAR α and RXR α demonstrate strong staining within the nucleus (Figure 2). RXRβ and RXRγ show weak nuclear staining. ROR α shows staining around the nucleus. RARβ and RARγ show no staining, confirming the Western blot results (immunocytochemistry result of RARγ not shown).

Retinoic acid reduces the proliferation of HASMC. Human myocyte proliferation was measured as the number of cellular nuclei per milliliter in solution using a coulter cell

counter. Cell counting was performed at 2-day intervals starting at day 0. Cells treated with RA (1 μM) show much reduced proliferation when compared with untreated controls (Figure 3).

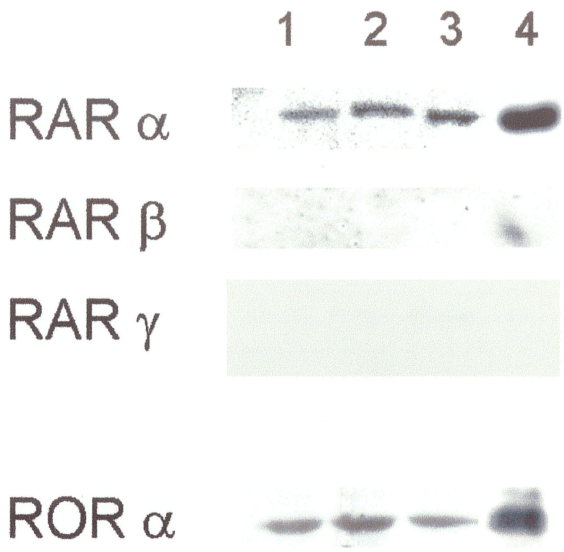
The effects of RA on key smooth muscle gene promotor activities. Figure 4 shows the activities of mouse SM22, SRF, and MHC promotors in subconfluent canine myocytes using luciferase assay. SM22, SRF, and MHC promotor activities are significantly down regulated in RA treated myocytes compared with untreated controls.

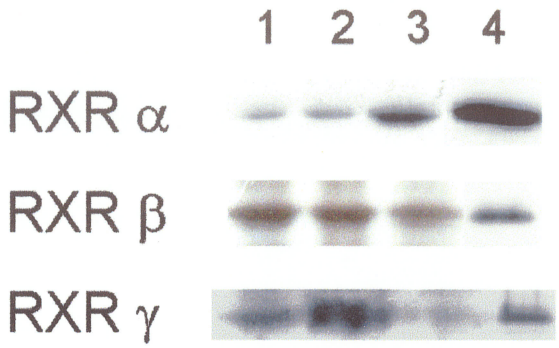
RA markedly reduces the binding of SRF to SM22 gene promoter. SRF is of central importance in activating smooth muscle gene transcription. EMSA (Figure 5) shows that nuclear extracts from cultured canine myocytes contain SRF, which binds strongly to oligonucleotide probe from the SM22 promoter (Camoretti-Mercado et al, 2000). Consistent with our observation that atRA decreased SRF-dependent transcription activity of smMHC, SM22 and an SRF-specific artificial promoter; binding activity is markedly diminished in RA treated myocytes.

The effects of RA on smooth muscle protein accumulation. The accumulation of three key smooth muscle proteins MHC, laminin, and α-actin are analyzed using Western blot (Figure 6). Each of these proteins is a marker for the contractile phenotype (Halayko and Solway, 2001). RA treated canine myoctes were compared with untreated controls at 50% confluence, 100% confluence, and 6 days after confluence and serum deprivation. MHC expression is low before contractile phenotype differentiation induced by serum deprivation.

After serum deprivation and differentiation into contractile phenotype, MHC is heavily expressed in untreated controls. This expression is inhibited by treatment with RA, as was accumulation of laminin and α-actin. These results are consistent with our observations that atRA inhibits transcription of genes encoding smooth muscle contractile apparatus proteins (i.e. smMHC and SM22), and with our observation that SRF binding to CArG box elements in the SM22 promoter is inhibited by atRA.

Figure 1. *Five different retinoic acid receptors were expressed in human airway smooth muscle cells.* Cultured human myocytes (passage 3) were harvested at 50% confluence (1) and at 100% confluence (2). Another sample of human myocytes was serum deprived after reaching confluence and was harvested 12 days after confluence (3). Whole bronchial tissue lysate (4) was included to demonstrate which receptors were present in human airway myocytes that are not cultured. Western blot analysis demonstrates the presence of RAR α, RXR α, RXR β, RXR γ, and ROR α in human airway myocytes. RAR β and RAR γ are absent.





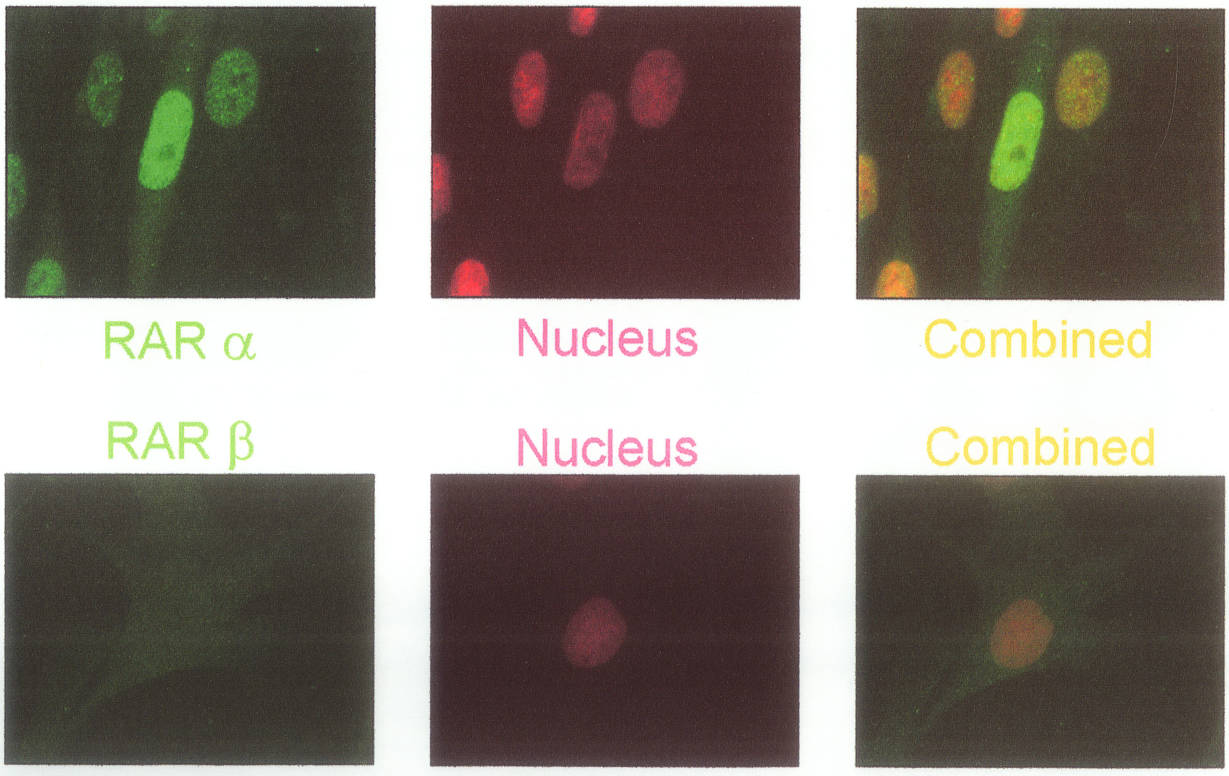
Lane 1: 50% subconfluence

Lane 2: 100% confluence

Lane 3: Day 12 after confluence
Serum deprivation

Lane 4: Whole bronchial tissue lysate

Figure 2. *Retinoic acid receptors distribution within cultured human airway myocytes.* Using fluorescent immunocytochemistry, human airway myocytes were immunolabeled using retinoic acid receptors primary antibodies and FITC- or Cy3- conjugated secondary antibodies. Nuclei were labeled with Hoechst 33342. RAR α and RXR α demonstrate strong nuclear staining, whereas RXR β and RXR γ show weak nuclear staining. ROR α shows peri-nuclear staining. RAR β and RAR γ staining were absent (result of RAR γ not shown), confirming the Western blot results.



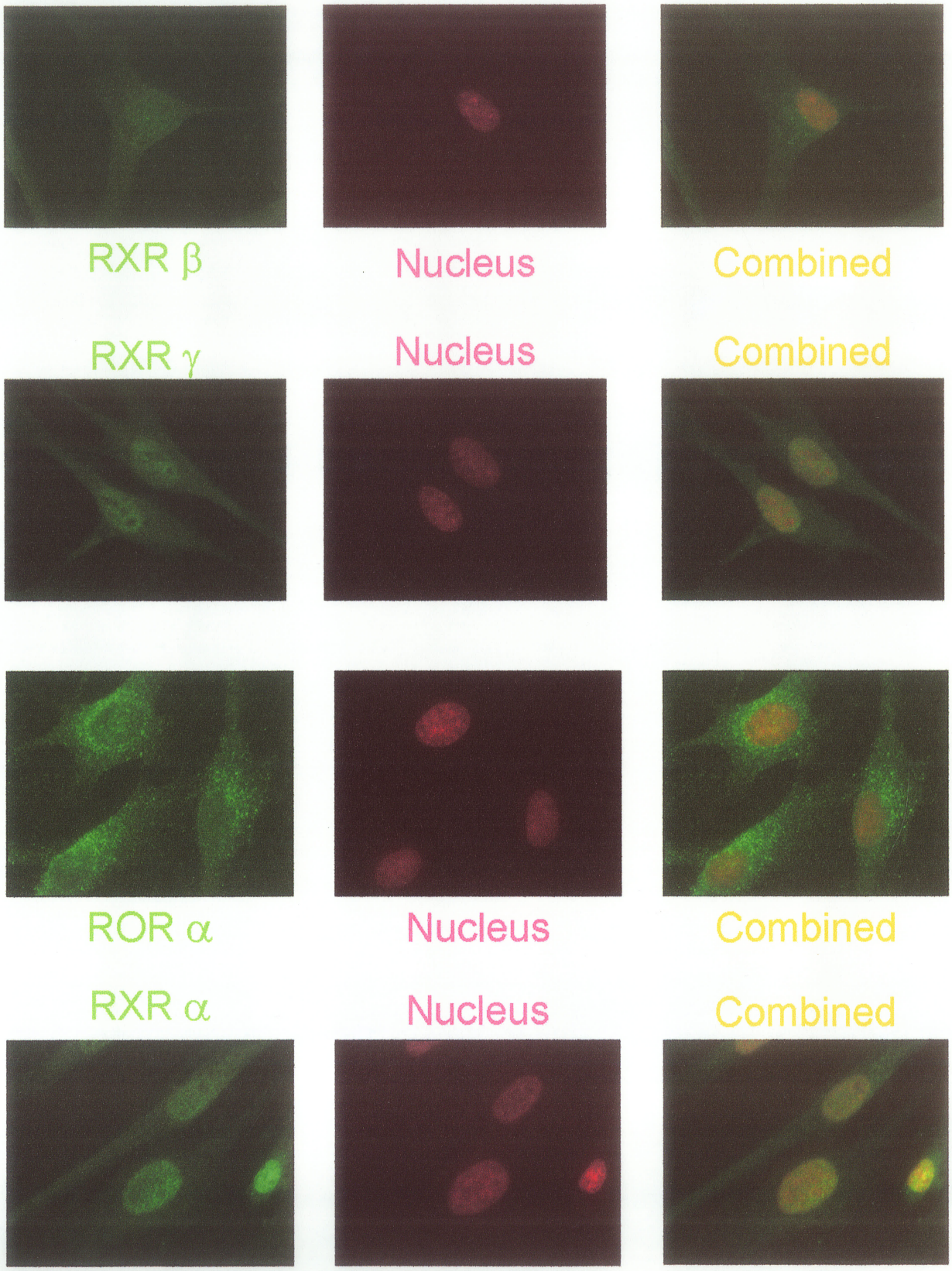

RXR β
Nucleus
Combined
RXR γ
Nucleus
Combined
ROR α
Nucleus
Combined
RXR α
Nucleus
Combined

Figure 3. *The effect of retinoic acid on human airway myocytes proliferation.* Human myocyte proliferation was measured as the number of cellular nuclei per milliliter in solution using a coulter cell counter. Cell counting was performed at 2-day intervals. Cells treated with retinoic acid (1 μM) show 52% reduction in proliferation comparing with untreated cells (n=3)

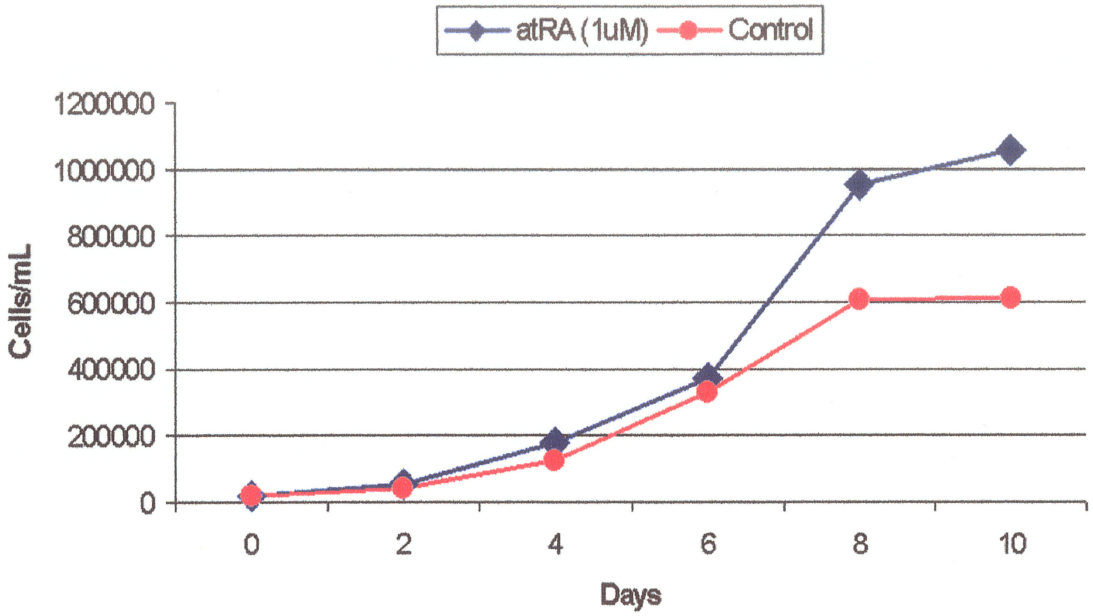

atRA (1uM)
Control
1200000
1000000
800000
600000
400000
200000
0
Cells/mL
0
2
4
6
8
10
Days

Figure 4. *Retinoic acid inhibits key smooth muscle gene promoter activities*. The activities of mouse SM22, SRF, and MHC promoters are expressed as normalize luciferase activity. In retinoic acid treated myocytes, SM22, SRF, and MHC promoter activities are significantly reduced by up to 73.14% ± 4.18% ($n=12$, $p<0.0001$).

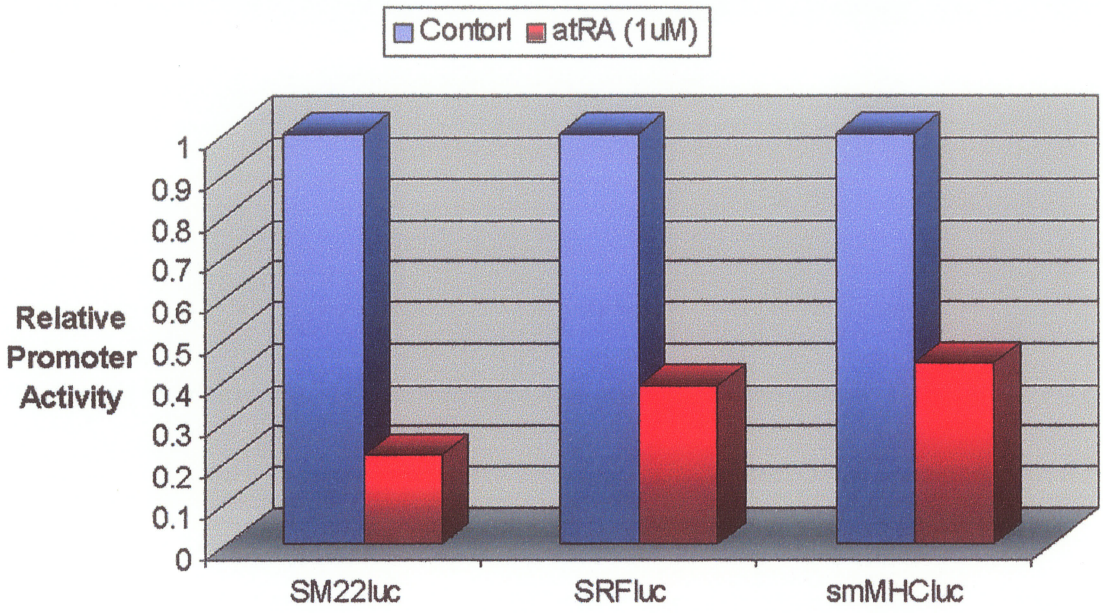

Contorl
atRA (1uM)
Relative Promoter Activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
SM22luc
SRFluc
smMHCluc

Figure 5. *SRF-DNA binding is markedly reduced in retinoid acid treated airway myocytes.* EMSA shows that nuclear extracts from cultured canine myocytes contain SRF, which binds strongly to oligonucleotide probe containing the 5' or 3' CArG boxes from the murine SM22 promoter (15). Retinoic acid treated myocytes exhibit markedly diminished SRF binding activity. Specificity of the SRF-containing DNA complex is demonstrated by selective supershift with anti-SRF antibody and by specific competition with CArG-containing unlabeled (cold) competitor oligonucleotides.

Electrophoretic Mobility Shift Assay (EMSA)

Lane 1: - RA

Lane 2: +RA

Lane 3: SRF antibody added

Lane 4: Cold probe added

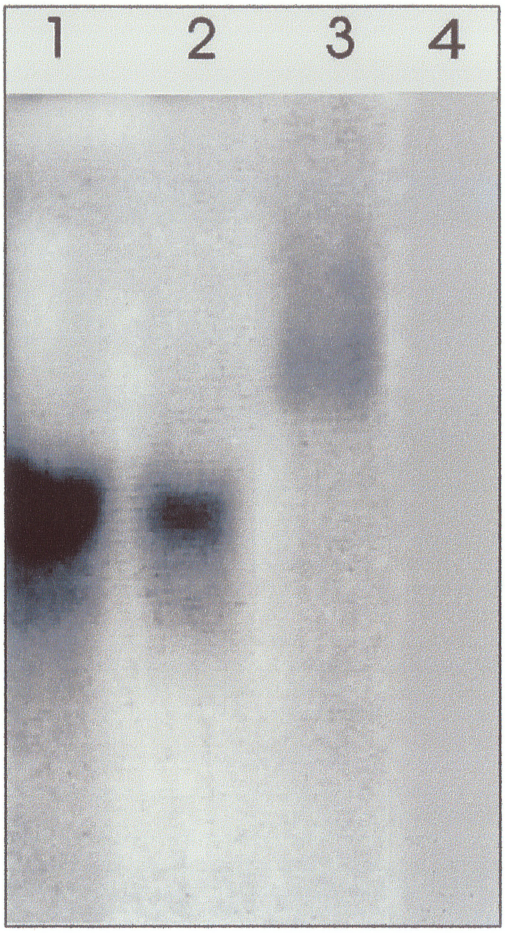
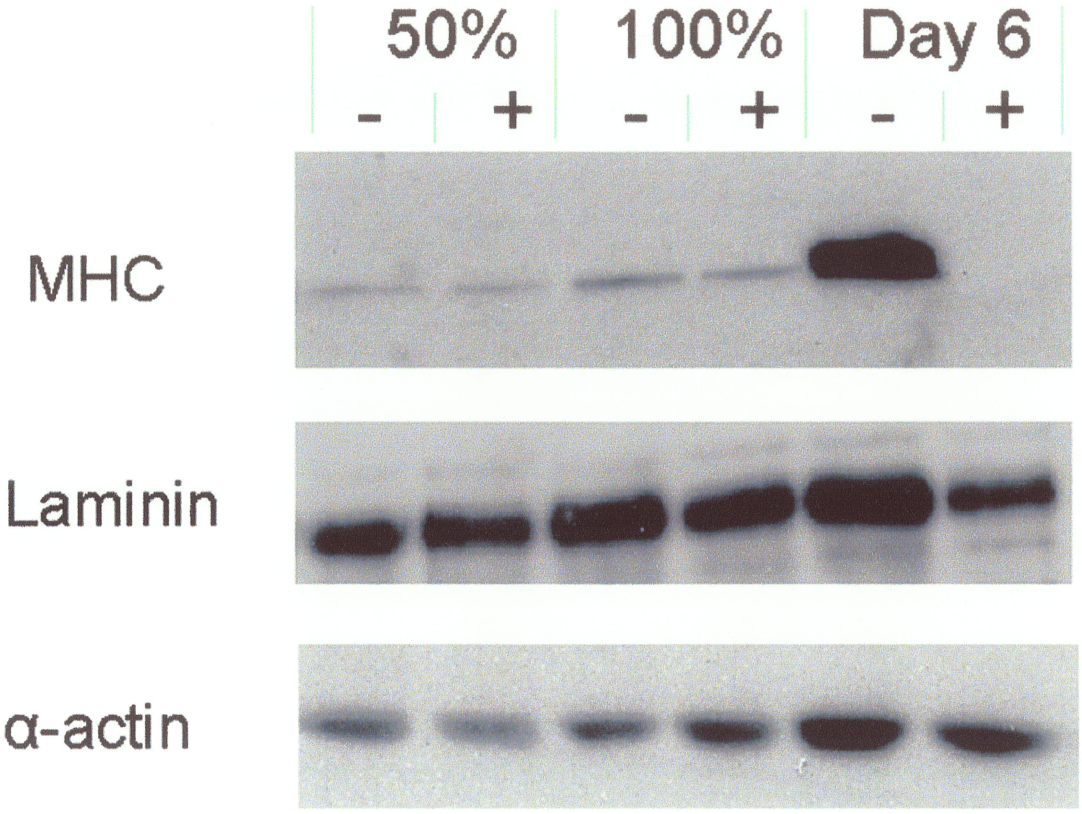


Figure 6. *Retinoic acid significantly inhibits the accumulation of smooth muscle protein.* The accumulation of three key smooth muscle proteins MHC, laminin, and α-actin are demonstrated using Western blot analysis. Myocytes that were treated with retinoic acid were compared with untreated controls at 50% confluence, 100% confluence, and 6 days after confluence and serum deprivation. MHC expression is low before serum deprivation and contractile phenotype differentiation. 6 days after reaching confluence and serum deprivation, MHC is heavily expressed in untreated controls. However, this expression is inhibited by retinoic acid. The accumulation of laminin and α-actin are also reduced in treated myocytes.


50%
100%
Day 6
- + - + - +
MHC
Laminin
α-actin

DISCUSSION

The biology of retinoids on vascular smooth muscle phenotype has been extensively studied (Miano and Berk, 2000). Its effects on airway SMC have not been established before. In this study it is discovered that five of the seven known RA receptors are expressed in HASMC. RAR β and RAR γ are not expressed. RAR α, RXR α, RXR β, and RXR γ have a nuclear distribution whereas ROR α has a peri-nuclear distribution. RA receptor expression may be different in airway and vascular myocytes. Wakino et al (2001) reported that RAR α, RAR β, RAR γ, RXR α, and RXR β are present in human coronary SMC but RXR γ is absent.

Retinoic acid is found to significantly inhibit proliferation of cultured human airway myocytes. The exact mechanism of this anti-proliferative effect is not investigated in this study. Studies in vascular SMC may offer clues to the underlying molecular pathways. Wakino et al (2000) demonstrated that retinoids inhibit mitogen-induced DNA synthesis in human coronary artery smooth muscle cells (CASMC) when those cells are stimulated with platelet-derived growth factor and insulin. Retinoids block the progression of human CASMC into S phase through inhibition of Rb phosphorylation and elevation of CDKI $p27^{kip1}$ levels (Wakina et al, 2000). The same study revealed that atRA inhibited the mitogenic induction of cyclin D1. Chen et al (2001) found that retinoid induced a number ofgenes in vascular smooth muscle, some of which are involved in growth suppression, apoptosis, and differentiation; SSeCKS is a retinoid-responsive gene that is associated with reduced cyclin D1 expression. Miano et al (1996) shows that atRA inhibits growth of cultured rat aortic SMC and attenuates serum stimulated activated protein-1 (AP-1) activity.

Ou et al (2000) demonstrates that atRA induced tissue transglutaminase (tTG) expression and increased tTG-dependent apoptosis in cultured rat aortic SMC. These studies together suggest that modulation of cell cycle regulators, RB hypophosphorylation, and apoptosis likely play significant roles in growth inhibition of SMC.

RA (1 μM) added on day 1 to cultured HASMC significantly inhibits key smooth muscle gene promotor activities, including SM22, SRF and MHC. This is associated with a markedly reduced binding of SRF to the SM22 gene promotor. RA treatment also blocks the accumulation of smooth muscle specific proteins MHC, laminin, and α-actin. Together these findings suggest that RA treatment results in reduced differentiation of cultured HASMC with less active key smooth muscle gene machinery and reduced expression of key smooth muscle protein markers. These results are in contrast to the literature in the vascular smooth muscle field (Miano and Berk, 2000). In subconfluent rat aortic vascular smooth muscle cells, Haller et al demonstrates that RA treatment (1 μM) on days 5 and 6 increases expression of α-actin and PKC-α and promotes a more contractile phenotype (Haller et al, 1995). Similar results are demonstrated in multipotential cell lineages such as P19 mouse embryonal carcinoma cells (Blank et al, 1995; Suzuki et al, 1996) and embryonic stem cells (Drab et al, 1997). Blank et al (1995) demonstrates that treatment of P19 embryonal cells with 1 μM of RA for 48 hours results in induction of smooth muscle α-actin and MHC expression and a cell lineage with smooth muscle characteristics. Similarly, Suzuki et al (1996) shows that RA treatment (1 μM) for 2 days in P19 cells increases α-actin expression and enhances SMC differentiation. In embryonic stem cells, Drab et al (1997) shows that application of RA and db-cAMP between days 7-11 elevates smooth muscle α-actin and

MHC expression and induces differentiation to spontaneously contracting SMC. Kim et al (2001) shows that RA does not affect the DNA binding affinities of SRF in HeLa cells as demonstrated by EMSA; he suggests that RA antagonizes SRF transactivation through competition for transcriptional coactivator proteins such as SRC-1/p300. The differences of RA responses in HASMC from that in vascular SMC and multipotential cell lineages likely represent unique characteristics of different cell types. The difference in RA treatment schedule may also potentially contribute to the discrepancies observed.

In summary, this study shows that five different RA receptors are expressed in HASMC. RA strongly inhibited the proliferation of HASMC. RA treatment results in significant inhibition of key smooth muscle gene promotor activities and DNA binding of SRF. RA also markedly blocks the accumulation of key smooth muscle proteins MHC, laminin, and α-actin. These results suggest that RA significantly alters the proliferation, gene expression, and phenotype of airway SMC. As smooth muscle hypertrophy and airway remodeling are key features in asthma, these findings may have potential implications in its treatment.

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