Smooth muscle actin and myosin expression in cultured airway smooth muscle cells

JEAN Z. WONG,1 JANET WOODCOCK-MITCHELL,1 JOHN MITCHELL,1 PATRICIA RIPPETOE,1 SHERYL WHITE,1 MARLENE ABSHER,1 LINDA BALDOR,1 JOHN EVANS,1 KIRK M. MCHugh,2 AND ROBERT B. LOW1

1Departments of Molecular Physiology and Biophysics and of Medicine, University of Vermont, Burlington, Vermont 05405; and 2Department of Anatomy and Developmental Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

In vitro cultured vascular SMC express α-SM actin and SM myosin in a fashion that is regulated by cell growth state (3, 5, 9, 26). The pattern of modulation of the contractile protein phenotype in culture displays certain similarities to that which occurs in vivo during vascular remodeling (9, 26). Cell culture models have proven useful for the study of the mechanisms involved in regulation of contractile protein expression in vascular cells.

We report here analyses of the contractile protein phenotype of cultured airway, visceral-type SMC as a follow-up to studies reporting the basic growth characteristics and response to hyperoxia (1). We demonstrate by both mRNA and protein analyses that airway SMC in culture continue to express several of the SM-specific contractile proteins seen in vivo, including α- and γ-actin MHC and SM1. Furthermore, we find that, as in vascular cells, the level of expression of these markers is different, depending on growth state.

METHODS

In vivo expression of contractile and cytoskeletal proteins. Tracheas were removed from euthanized adult male Fischer 344 rats (NCl, Frederick, MD) and fixed in 100% ethanol before standard paraffin embedding procedures (Medical Center Hospital of Vermont, Laboratory of Histology). Paraffin-embedded sections were dewaxed and immunohistochemically stained using immunoperoxidase techniques described previously (23, 34).

The primary antibodies used in this study were 1) mouse monoclonal anti-α-SM actin (anti-α-SM1; used at 1:800; Sigma, St. Louis, MO; see Ref. 32); 2) monoclonal antibody H9F35 against muscleα- and γ-actins (1:100; ENZO; see Ref. 33); 3) rabbit anti-SM1 and -SM2 both at 1:200 (5, 35) for immunohistochemistry and 1 2,000 for enhanced chemiluminescence (ECL); 4) mouse anti-nonmuscle myosin-B (NMB; used at 1:50 for immunohistochemistry and at 1:500 for ECL); 5) rabbit anti-desmin (at 1:100; see Ref. 34); and 6) guinea pig antivimentin (at 1:100; see Ref. 18).

Cell isolation and culture. All culture media and reagents were obtained from Gibco (Grand Island, NY). Tracheas were excised from adult animals, adherent connective tissue and epithelium were removed by firmly scraping the luminal surface, and the remaining tissue was incubated for a period of 0.1% collagenase II ( Worthington) and 0.1% soybean trypsin inhibitor (Sigma) in Ca2+, Mg2+-free Hanks’ balanced salt solution (HBSS) at 37°C for 20 min (1). Connective tissue was further removed by scraping after the enzyme digestion, and the SM strips were minced into 0.5-mm2 fragments. The tissue fragments were incubated undisturbed in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and

SMOOTH MUSCLE (SM) plays a vital role in the normal function of most organs of the human body. Abnormalities in SM function are associated with a variety of diseases, such as hypertension and asthma (2, 27, 31). Extensive studies of vascular SM have demonstrated the marked organizational and contractile protein phenotypic changes that occur in this tissue in cardiovascular disease (2, 31). Much of what has been published regarding airway SM has been focused on alterations in pharmacological responsiveness of the tissue seen in vivo and as regulatory processes that occur in vitro (2, 27). The recent studies of Halayko et al. (11) have begun to address this issue by assessing the contractile and cytoskeletal protein phenotype of airway smooth muscle cells (SMC) in culture.

The expression of SM actin and myosin isoforms serves as a molecular marker of SM differentiation. The proportions of the two SM actin isoforms, α-SM and γ-SM actin, vary in different SM tissues and during remodeling processes after injury (9, 20, 26). Similarly, the SM myosin heavy chain (MHC) isoforms, SM MHC-1 (SM1) and SM MHC-2 (SM2), are differentially expressed in various SM-containing organs and during tissue remodeling (26, 29, 35).
antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells began to migrate out of the tissue after 3–4 days. Just before the cultures reached confluence (usually 1 wk), the initial explants were removed, and the cells were dissociated with 0.25% trypsin and subcultured in DMEM with 10% FBS at 4 × 10^3 cells/cm^2. Subsequent subculturing was done at weekly intervals. Routinely, the cells underwent three to four population doublings per week. All experiments were done on cultures that were below passage 7 and <21-28 cumulative population doublings. Four basic experimental culture conditions were used to study contractile protein expression in relation to proliferative state of the cells (Fig. 1). They were 1) high density, high (10%) serum (HH); 2) high density, low (1%) serum (HL); 3) low density, high serum (LH); and 4) low density, low serum (LL). We used 1% serum for our low-serum condition to be consistent with our prior experiments (1) and because this amount of serum maintains the cultures in good condition while it prevents proliferation (1). Direct cell counts demonstrated that the cells were at the plateau of growth curve for the high-density cells, whereas the counts for low-density cells were 50–70% of plateau values. Thus the HH, HL, and LL conditions were considered to be states of growth arrest, whereas LH yielded a proliferating culture.

Immunofluorescence staining of cells. SMC were plated on glass coverslips in 35-mm culture plates under the four culture conditions described above. Cells were then fixed in 100% methanol and immunostained (22). The primary antibodies and dilutions were the same as in immunoperoxidase staining of the tissue sections. Fluorescein isothiocyanate antibodies and dihydrofluorescein diacetate were used to visualize the antibody-reactive cells.

Protein electrophoresis and Western blotting. For one-dimensional protein separations, cultured cells were extracted into SDS-cacodylate buffer and electrophoresed in 8.5% polyacrylamide gels (16). Gels consisting of 5% acrylamide and 0.065% bisacrylamide were used to facilitate separation of MHC.

For two-dimensional separations, cultured cells were extracted into urea buffer, and the extracts were loaded onto isoelectric focusing tube gels, which were run at 350 volts for 16 h (25). Isoelectric gels were loaded directly onto a second-dimension SDS-polyacrylamide gel consisting of 8.5% acrylamide. Second-dimensional gels were run at 150 volts for 3 h.

Gels were blotted electrothermically onto nitrocellulose paper (Schleicher and Schuell, Keene, NH) as described previously (6). Protein bands were visualized with fast green, and the blot was subsequently processed for immunoperoxidase sandwich staining using the antibodies cited above for the studies of α- and γ-actin. ECL (Amersham, Arlington Heights, IL) was used for detection of immunoreactive proteins on blots for SM myosin and NMB heavy chains.

RNA analysis. Total RNA was isolated by the acid guanidine-ismiotsiyanate procedure of Chomczynski and Sacchi (7). Electrophoresis of formamide/formaldehyde denatured RNA for Northern analysis was in 0.85% agarose gels containing formaldehyde (19). RNA was then transferred from gel to nitrocellulose paper (Schleicher and Schuell). The cDNA probes used were 1) RAMHC-15 from rat aorta for SM MHC (4); 2) pAC16 from rat skeletal muscle for βγ-cellular actin and α-SM actin (sequence homology to these actins ~80%; see Ref. 10); 3) pRE-γA-11 from rat stomach for γ-SM actin (21); and 4) FSMHC34 from fetal rabbit aorta for nonmuscle MHC (14). After hybridization with [32P]cDNA probe, filters were exposed to Kodak X-Omat AR X-ray film.

Results

Cytoskeletal and contractile protein expression in vivo. The results of immunocytochemical analysis of rat trachea are shown in Fig. 2. The sections contained epithelium, bundles of SM, connective tissue, and hyaline cartilage (Fig. 2A). Airway and vascular SM elements stained positively for α-SM actin (Fig. 2B), SM1 (Fig. 2C), and SM2 (Fig. 2D), as well as for the intermediate filament protein desmin (Fig. 2F). Vimentin (Fig. 2E) and NMB (Fig. 2G) were not detected in either tracheal or vascular SM in these adult tissue sections.

Tracheal SMC in culture. Cells growing at low density were characterized by a flattened, elongated, spindle-shaped appearance, with one or more extended processes originating from the cell body (Fig. 3, A and B). Stress fibers were a prominent feature of the cytoplasm. When cells were at high density (Fig. 3, C and D), they tended to form a monolayer with a hill-and-valley morphology, appeared smaller, and had fewer processes visible at the light microscope level. These morphological features are typical for subcultured SMC (9, 26).

The rat tracheal SMC were distinct from fibroblasts in having SM markers (see Immunocytochemical evaluation, Expression of SM actin, and Expression of SM and nonmuscle myosin in culture). They also were distinct from epithelial cells because they were not stained by anti-keratin antibodies (not shown) and did not have the polygonal shape that would be expected.

Immunocytochemical evaluation. Figure 4 shows results of immunohistochemical staining of cultured cells. These studies were done at near confluence (~90%)

Fig. 1. Experimental culture conditions. HH, high density, high serum; HL, high density, low serum; LH, low density, high serum; LL, low density, low serum.
Fig. 2. Adult rat trachea immunostained with contractile and cytoskeletal protein specific antibodies. A: hematoxylin and eosin stain; B: anti-α-smooth muscle (SM) actin; C: anti-SM myosin heavy chain (MHC)-1 (SM1); D: anti-SM MHC-2 (SM2), length bar 100 µm; E: anti-vimentin; F: anti-desmin; and G: anti-nonmuscle myosin (NMB). Original magnification ×50.

Fig. 3. Giemsa staining of cultured rat tracheal smooth muscle cells (SMC). A: cells at low density, original magnification ×200; B: cells at low density, original magnification ×50; C: cells at high density, original magnification ×200, length bar 10 µm; D: cells at high density, original magnification ×50, length bar 100 µm.

Fig. 4. Immunofluorescence staining of cultured rat tracheal SMC with contractile and cytoskeletal protein antibodies. Studies of cells were near confluency (~90%) such that individual cells could be observed. A: anti-α-SM actin specific antibody; B: anti-SM1 specific antibody; C: anti-SM2 specific antibody; D: anti-NMB specific antibody; E: anti-vimentin specific antibody; and F: anti-desmin specific antibody. Original magnification ×250.

such that individual cells could be observed. α-SM actin was incorporated into the stress fiber-like structures, which were observed in the Giemsa-stained preparations (Fig. 4A). Distinct α-SM actin immunoreactivity was detected in all of the cells examined regardless of the growth state of the culture. SM1 immunoreactivity was detected in high-density cultures maintained in

L788 AIRWAY SMOOTH MUSCLE CELL PHENOTYPE
both high and low serum, as well as in low-density cultures maintained in low serum (Fig. 4B). A stress fiber-like pattern of staining was observed, although the staining was more punctate than was characteristic of α-SM actin staining (Fig. 4A). SM1 reactivity was not observed, however, in cells cultured at low density in high serum (not shown). We were unable to detect SM2 antibody reactivity under any of the culture conditions (Fig. 4C), including primary culture.

NMB antibody reactivity was detected in all cells under all culture conditions. The staining showed a stress fiber pattern very similar to the pattern seen with α-SM actin (Fig. 4D).

Figure 4E shows that the intermediate filament protein vimentin was expressed in the cultured SMC. Cells were desmin negative, however, under all culture conditions (Fig. 4F).

Expression of SM actin. The levels of α-SM actin protein were the same in the growth-arrested cultures (HH, HL, and LL), although there was less α-SM actin in low-density culture in which growth was stimulated by high serum (Fig. 5). Additional Western blots of two-dimensional SDS-PAGE (Fig. 6) demonstrated the presence of both α- and γ-SM actin in the growth-arrested cells.

Northern blot analysis demonstrated expression of α-SM actin cells (Fig. 7). The ratio of α-SM actin to (β + γ)-actin mRNA was approximately the same in confluent high-density cultures with high or low serum and in nongrowing low-density culture in low serum [α/(β + γ); ~0.6]. Somewhat less α-SM actin mRNA was expressed, however, in low-density cells grown in high serum, commensurate with the aforementioned decrease in α-actin protein. Additionally, there was an increase in the amount of (β + γ)-mRNA. These combined results led to a fall in the α-to-(β + γ) ratio (~0.35). γ-SM actin mRNA expression also was evident (Fig. 7). Its mRNA was somewhat reduced under HL conditions and was reduced further in low-density cultures.

Expression of SM and nonmuscle myosin in culture. Immunostaining of the SDS-PAGE blots showed that nearly equal amounts of NMB protein were present under conditions during which there was no cell growth (HH, HL, and LL). Slightly more NMB protein was present under growth conditions (LH; Fig. 8). SM1 was present in nearly equal amounts in cells in HL and LL but was reduced in LH conditions. SM2 reactivity was not observed under any culture condition.

More SM MHC mRNA was expressed in high-density cultures than at low density in the presence of serum (Fig. 7). Serum deprivation restored SM MHC mRNA expression in low-density culture. This pattern of regulation was similar to that for α-SM actin expression. Indeed, within a given experiment, the signal ratio of SM MHC to α-SM actin mRNAs was not affected by the different culture conditions.

RNase protection analysis demonstrated that the bulk of the SM myosin mRNA in these cells was of the SM1 type. The fully protected 380-nt fragment of SM2...
represented only \( \sim 10\% \) of the total protection signal (Fig. 9).

Northern analysis also demonstrated that nonmuscle myosin mRNA was expressed in cultured tracheal SMC in highest amounts in cells at HL (Fig. 7). NMB mRNA was not upregulated in growing conditions (LH).

**DISCUSSION**

The major findings of the present work are that 1) \( \alpha \)-SM actin, \( \gamma \)-SM actin, and SM myosin continue to be expressed in cultured airway SMC; 2) the expression of these contractile proteins is regulated by culture growth state; and 3) the expression of SM myosin and \( \alpha \)-SM actin changes in the same direction as a result of different culture conditions. Results with regard to \( \alpha \)-SM actin and SM myosin expression are similar to what has been reported for vascular SMC (3) and lung mesenchymal cells (5). In contrast, we find that these airway cells control the expression of the fetal myosin isoform (NMB) in a fashion distinct from vascular cells (14, 15, 26). In particular, NMB is not significantly upregulated in conditions of cell growth.

An important new finding that we report is that \( \gamma \)-SM actin is expressed in cultured tracheal SMC. Furthermore, although \( \alpha \)-SM actin expression has been detected in some non-SMC (9, 23, 26) and tissues (9, 26), the expression of the \( \gamma \)-SM isoform appears to be more...
limited (20). Thus we believe the presence of both γ-SM actin and SM1 protein, an isoform unique to SM, in these tracheal-derived cells unambiguously identifies them as being of true SM origin and may indicate a difference between vascular and visceral SM (20).

The pattern of expression that we observed for α-SM actin was similar to that reported in vascular SMC (9, 26) in that the proportion of SM-specific actin mRNA to total actin mRNA rises in our growth-inhibited cultures. This appears to be due to changes in both α-SM actin and (β + γ)-actin mRNA levels. The latter result agrees with those of prior studies of vascular SMC (3, 6, 8, 26). These results suggest the development of a more differentiated state under conditions of growth inhibition. Insofar as there may have been a disproportionate fall in α-SM actin mRNA vs. protein levels, there may be a degree of regulation at the posttranscriptional level (26).

Our conclusions are similar for SM myosin. For example, the low level of SM myosin mRNA seen in growing (LH) cells is elevated by growth arrest caused either by contact inhibition (high density) or nutrient deprivation (low serum). SM1 protein expression changes in a similar fashion. Similarly, this protein can be organized into discrete cellular structures in the high-density cells (Fig. 4).

Halayko et al. (11) also have described the phenotype of airway SMC in culture. Protein and Northern analyses demonstrated that the content and mRNAs for a number of SM proteins, including SM MHC, α-SM actin, desmin, h-caldesmon, and β-tropomyosin, fell rapidly when cells became proliferative. Conversely, the content of several non-SM myosin proteins increased. The expression of SM MHC and α-SM actin increased after cells reached confluency. Our results extend those of Halayko et al. (11) in comparing effects of cell density in high and low serum and by examining the regulation of γ-SM actin, NMB mRNA and protein, and the SM1 and SM2 isoforms of SM myosin.

Panettieri et al. (28) have shown that histamine stimulates proliferation of airway SMC and also increases radiolabeled methionine incorporation into SM MHC. Total methionine incorporation also was increased, indicating a possible precursor pool effect. These results confirm that increased proliferation is not always associated with reduced expression of SM contractile proteins (9, 26).

NMB has been reported to be the major myosin isoform in embryonic and perinatal vascular SM, but its expression is downregulated during subsequent vascular development (15). This myosin isoform has been shown to be reexpressed, however, in proliferating SMC during vascular remodeling in the mature animal (14). Similarly, NMB expression also has been reported in adult vascular SMC growing in culture (17, 30). We report here that reexpression of NMB is observed in cultured airway SMC; however, NMB mRNA and protein appear not to be significantly upregulated in growing cells (LH).

The results of this in vitro study indicate that the expression of SM specific proteins is regulated in the same direction when culture conditions are changed. Further studies utilizing defined growth factors would provide an approach to identifying the detailed steps in the control mechanism(s) for contractile protein gene expression that contributes to this pattern of expression.

Our results may be relevant to understanding the changes in SM phenotype that accompany lung injury. Hershenson et al. (12) and Jones et al. (13) have shown that, during exposure of rats to high oxygen, there is extensive remodeling of airways and vascular elements, with proliferation, migration, and phenotypic changes occurring in the SMC compartment. Rippetoe et al. (29) noted altered expression of SM α-actin and myosin under equivalent oxygen exposure conditions. Similar observations have been made in the case of ozone exposure (24), bleomycin-induced lung injury (23), and in human disease (18). Decreased SM actin and myosin expression together with the reexpression of NMB in proliferating cultures may represent a return to a less differentiated state similar to that seen during remodeling in vivo.

Our results indicate the potential value of the culture system for study of the regulation of contractile protein expression by visceral (airway) SMC. At the same time, such studies must be approached with some caution given the generally reduced expression of contractile and cytoskeletal proteins that occurs in culture and the phenotypic changes that are observed in terms, for example, the switch from desmin to vimentin (11, 26). A detailed study of the relationship between passage or population doubling number and contractile protein phenotype will be important to establish the temporal pattern of change and the stability of the phenotype that we observed (see Ref. 26). Finally, it is highly likely that factors other than growth state, such as extracellular matrix and mechanical forces, are involved in the maintenance of the full level of differentiation of these airway SMC (26).

We thank Dr. Ryozo Nagai for the NMB cDNA and antiserum and Dr. Muthu Periasamy for the smooth muscle myosin and skeletal actin cDNAs. We also thank Julie Lovellette for excellent secretarial assistance in the preparation of this manuscript.

This work was supported by National Institutes of Health Grants HL-14212 (Pulmonary Specialized Center of Research, to R. B. Low), HL-36412 (to J. Woodcock-Mitchell), HL-41213 (to J. Evans), and HD-27252 and HD-00996 (to K. M. McHugh).

Address for reprint requests: R. B. Low, Dept. of Molecular Physiology and Biophysics, Given Bldg. D-204, Univ. of Vermont, Burlington, VT 05405.

Received 18 August 1994; accepted in final form 5 February 1998.

REFERENCES