Development and Characterization of a Cloned Rat Pulmonary Arterial Smooth Muscle Cell Line That Maintains Differentiated Properties Through Multiple Subcultures

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**Background.** Pulmonary hypertension is associated with abnormal pulmonary arterial contractility and growth. The mechanisms for these abnormalities are largely unknown. To study these processes, we sought to develop a system in vitro. Even though cultured aortic and pulmonary artery smooth muscle cells (SMCs) have been of considerable value in studying smooth muscle biology, one drawback of this system has been that these cells often lose differentiated properties in an unpredictable manner when they are passaged in culture. In addition, there appear to be significant differences in physiological and pathological responses between the systemic and pulmonary circulations, many of which could be directly related to the smooth muscle. We therefore established a cloned population of rat pulmonary arterial SMCs (PASMCs) that maintain differentiated properties through multiple subcultures.

**Methods and Results.** PASMCs were obtained initially by enzymatic dissociation from pulmonary arteries of adult Sprague-Dawley rats. From these cells, clones were isolated. The cloned cells retained expression of functional surface receptors for angiotensin II, norepinephrine, and α-thrombin and high levels of the smooth muscle isoforms of α-actin, myosin heavy chain, myosin regulatory light chain, and α-tropomyosin mRNAs even after multiple passages. The cells could also be transfected and processed exogenous transcripts in a smooth muscle–specific fashion.

**Conclusions.** These cloned PASMCs retain many differentiated characteristics and should be valuable for future studies of pulmonary vascular smooth muscle cell biology. *(Circulation 1992;86:1977–1986)*

**KEY WORDS:** cell culture • smooth muscle • pulmonary artery • contractile protein

Pulmonary hypertension is an important cause of morbidity and mortality in children and adults. Pulmonary vascular smooth muscle plays a key role in the etiology of pulmonary hypertension. In some patients, there is constriction of smooth muscle leading to luminal narrowing, whereas in others, there is fixed obstruction caused by an abnormal increase in the number and size of smooth muscle cells (SMCs). The mechanisms responsible for these pathological changes are poorly understood. One approach to study these processes is to use cultured pulmonary arterial smooth muscle cells (PASMCs). Considerable progress in understanding vascular smooth muscle biology has been made using cultured SMCs; however, one drawback has been that the cells lose differentiated properties in an unpredictable manner after repeated subcultures. Therefore, many studies have been performed on primary or very early subculture cells. The experimental limitations of this approach are that only a small number of cells can be obtained from a single rat artery, and each primary culture contains a heterogeneous population of cells that may have different characteristics including growth properties, receptor type and number, and expression of smooth muscle–specific genes. To establish an SMC culture system that would be more homogeneous and would provide a large number of cells for continued study, we sought to establish a cloned population of PASMCs.

PASMCs were cultured rather than the more widely studied aortic SMCs because there are several important differences between the systemic and pulmonary circulations: 1) Hypoxia and acidosis cause pulmo-
nary arterial constriction, whereas they cause systemic arterial dilation. 2) Although in utero the pulmonary and systemic circulations are exposed to identical pressures, the former develops far less smooth muscle. 26,27 3) Pathological increases in the amount of smooth muscle and connective tissue can occur in the lung at a much faster rate and to a greater degree than in the systemic circulation. 28,29 Although these distinctive features of the pulmonary vasculature may not be solely or directly related to properties of the smooth muscle per se, they raise the possibility that there are significant differences in phenotype and behavior between SMCs of the two vascular beds. 19

We report the isolation of cloned PASMCs that express, through multiple subcultures, functional surface receptors for angiotensin II (Ang II), norepinephrine (NE), and α-thrombin and the smooth muscle isoforms of α-actin, myosin heavy chain (MHC), myosin regulatory light chain (MRLC), and α-tropomyosin (TM) mRNAs.

Methods

Cell Culture

The methods for PASMC isolation and culture were modified from Gunther et al. 30 The main and proximal branch pulmonary arteries of 300–350-g adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were dissected free and excised. Fat, adventitia, and connective tissue surrounding the arteries were removed by blunt dissection. The cleaned pulmonary arteries were cut into multiple small pieces and incubated for 90 minutes in a 37°C oscillating bath with 0.125 mg/ml elastase (type III, 90 units/mg, Sigma Chemical Co., St. Louis, Mo.), 1.0 mg/ml collagenase (CLS type I, 150 units/mg, Worthington Biochemical Corp., Freehold, N.J.), 0.250 mg/ml soybean trypsin inhibitor (type 1-S, Sigma), and 2.0 mg/ml crystallized bovine serum albumin (BSA) (Pentex, Miles Laboratories Inc., Elkhart, Ind.). The tissue suspension was filtered and centrifuged, and the pellet was resuspended in Medium 199 (GIBCO, Grand Island, N.Y.) supplemented with 20% (vol/vol) fetal calf serum (FCS) (GIBCO), 2 mM l-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (standard growth media). The cells were incubated in a humidified 5% CO₂–95% air atmosphere. The medium was changed every other day. PASMCs were harvested with trypsin and passaged when they reached 80–90% confluence. Mouse NIH 3T3 fibroblasts and bovine aortic endothelial cells were used as controls for immunofluorescent staining.

PASMCs were cloned by initially plating 100 subculture 22 cells in a 96-well plate and grown in 50% conditioned: 50% standard growth medium. Conditioned medium was obtained from a dish with PASMCs that were proliferating rapidly in standard growth medium for at least 2 days. Individual cell colonies were subcultured further and characterized. Cloning was started in subculture 22 cells after it was confirmed by extensive characterization that the PASMCs had retained many differentiated properties.

For storage, the cells were harvested with trypsin, centrifuged, and suspended in 10% dimethyl sulfoxide in standard growth medium and immediately frozen at −70°C. Aliquots were thawed, added to dishes containing standard growth medium, subjected to fresh medium change after 2–3 hours, and used for experiments after three passages.

Fluorescent Staining

PASMCs, fibroblasts, and endothelial cells were stained with labeled, acetylated low density lipoprotein (LDL), which is taken up via the “scavenger pathway” by endothelial cells with much greater affinity than SMCs (Biomedical Technologies Inc., Stoughton, Mass.); 31 antibodies against factor VIII–related antigen, which is specific for endothelial cells (Accurate Chemical, Hicksville, N.Y.); 32 and smooth muscle myosin 33 and α-actin antibodies (Enzo Biochem Inc., New York, N.Y.), 34 which stain SMCs but not epithelial, endothelial, connective tissue, or striated muscle cells. Methodological controls included preimmune sera (negative control) and antiplatelet myosin antibody (positive control). 33 Cells on coverslips were rinsed in phosphate-buffered saline (PBS), fixed with acetone or methanol for 5 minutes at −20°C, and air dried. The coverslips were then incubated for 45 minutes at 37°C with the primary antibody at 1:100 dilution in PBS, washed in PBS, incubated with a 1:100 dilution of secondary antibody (goat anti-rabbit IgG-fluorescein antibody) (Sigma) 33 for 45 minutes, washed, and mounted on glass slides using glycerol/PBS. Immunofluorescent staining was examined under epifluorescent illumination on a Zeiss microscope.

[^H]-Thymidine Incorporation and Autoradiography

Proliferation was stimulated with 20% FCS and inhibited by culturing cells in 0.5% FCS (serum deprivation). Assessment of proliferation was performed by cell counts and [^H]-thymidine incorporation followed by autoradiography. 35 Cells in 35-mm dishes were treated with 0.5 μCi/ml [^H]-thymidine (specific activity, 22 Ci/mmol; New England Nuclear, Boston) continuously for 24–72 hours, fixed with a methanol and acetic acid mixture (3:1), washed with increasing concentrations of ethanol, air dried, and coated with Kodak NTB-2 emulsion. After exposure for 5–7 days at 4°C, they were developed with Kodak D19 developer for 2 minutes and fixed in Kodak fixer for 5 minutes, rinsed with water, and stained with Giemsa. 35 At least 1,000 nuclei were scored per dish.

Surface Receptors

Using the calcium-sensitive dye fura-2, intracellular calcium changes were measured in response to Ang II, α-thrombin, and NE. Cells were loaded with 5 μM fura-2/acetoxymethylester (Molecular Probes Inc., Junction City, Ore.) for 30 minutes, washed, and resuspended to a final concentration of 2×10⁴ cells/ml in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM dextrose, 0.25 mM/ml BSA, 20 mM HEPES, pH 7.4). Fluorescence measurements were made with a Spex Fluorog-2 spectrofluorimeter (excitation 340 and 380 nm, slit 3.3 nm, emission 505 nm, slit 4.5 nm). Maximum fluorescence was obtained by permeabilizing the cells with 30 μM digitonin (which exposed the dye to the calcium concentration of the assay buffer). Minimum fluorescence intensity was obtained by chelating calcium with 300 mM EGTA, 1.0 M Tris, final pH >8.8.
RNA Preparation and Blot Hybridization

Total RNA was extracted from PASMC cultures by the guanidinium isothiocyanate/CsCl procedure. Ten micrograms of total RNA were size-fractionated by electrophoresis on 1% agarose gels in 200 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4, 1 mM EDTA, and 3% formaldehyde. Transfer to nitrocellulose and hybridization to \(^{32}P\)-labeled DNA probes were as described. To assess transfer of RNA, filters were stained with methylene blue. Only uniformly stained filters were used for hybridization at 42°C for 14 hours in 50% formamide, 5×SSC (standard saline citrate) (1× = 0.15 M NaCl, 0.015 M sodium citrate), 2×Denhardt’s solution, 0.1% SDS (sodium dodecyl sulfate), 0.025 M sodium phosphate, and 50 mg/ml calf thymus DNA. After hybridization, the filters were washed with 0.1×SSC at 55°C and exposed to Kodak X-Omat AR film at −70°C. Quantitation of relative amounts of mRNA expression was assessed by two-dimensional densitometry. Each experiment was performed at least twice.

Preparation of cDNA Probes

The following cDNA probes were labeled by nick translation using \(^{32}P\)-dCTP (400 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) to a specific activity of about 1.0×10⁶ cpm/μg DNA: RAMHC (rat aortic smooth muscle MHC), PAC269 (chicken skeletal muscle actin), and a clone encoding a MRLC (RLC2) isolated form rat aortic SMC cDNA library. A cDNA clone encoding smooth muscle α-TM was end-labeled with T4 polynucleotide kinase (Bethesda Research Laboratories Inc., Gaithersburg, Md.) and \(^{32}P\)-γ-ATP (Amersham). A rat α-vascular actin cDNA (pRVaA-19), which also recognizes the γ-isoforms of actin, and a probe derived from the 3’ untranslated region of the rat smooth muscle α-actin mRNA (pRVaA-3’UT-DP), which is specific for the α isoform, were oligolabeled with \(^{32}P\)-dCTP (Amersham).

S1 Nuclease Mapping

RNA-DNA hybridization followed by S1 nuclease mapping analysis was performed as previously described. Twenty micrograms of total cellular RNA was hybridized to 8.0×10⁶ cpm of an α-TM probe (end-labeled as described above) in 25 μl of 70% deionized formamide, 400 mM NaCl, 10 mM PIPES (piperazine-N,N’-bis[2-ethanesulfonic acid]) (pH 6.4), 0.05% SDS, and 1 mM EDTA. The hybridization mixture was incubated at 100°C for 3 minutes, in a dry ice ethanol bath for 30 seconds, at 65°C for 1 hour, and then at 60°C for 16 hours. S1 nuclease (100 units) (New England Nuclear) in 300 μl of 200 mM NaCl, 20 mM sodium acetate (pH 4.5), and 3 mM zinc sulfate was added to each sample and incubated at 25°C for 1 hour. The reaction was terminated with 10 mM EDTA and precipitated with ethanol. Dried pellets were dissolved in 90% formamide and electrophoresed on a 5% polyacrylamide–8 M urea gel. The gel was dried and exposed for autoradiography on Kodak X-Omat AR film.

Transfection

A test construct (pTS23D) containing α-TM exons 1–4 under the transcriptional control of the non–tissue-specific SV 40 promoter/enhancer was transfected into passage 25 PAC1 cells and COS cells (serving as control cells) by the calcium phosphate procedure. Cytoplasmic RNA was harvested 48 hours after glucose shock. RNA (30 μg pTS23D and mock-transfected PAC1, 10 μg COS) was analyzed by S1 protection (as described above) after hybridization with probes specific for the 134 or the 124 splicing pathways. The probes were end-labeled within SV40 sequences to distinguish constructs from endogenous TM transcripts and had a 70-bp, nonhybridizing tail that distinguished true full protection from reannealed probe. Protection patterns using the two probes were visualized by electrophoresis in 5% polyacrylamide denaturing gels followed by autoradiography (12 hours at 20°C for COS, 3–5 days, −80°C with intensifying screens for PAC1).

Results

Cell Culture and Cloning

PASMCs obtained from the main and branch pulmonary arteries of adult Sprague-Dawley rats were enzymatically dissociated and placed in a 35-mm culture dish. After 2 days, the cells began to proliferate, and by 5–6 days, they were nearly confluent. Cells were harvested with trypsin and subcultured in 100-mm dishes at a 1:3 to 1:6 ratio.

To demonstrate that the cultures consisted predominantly of SMCs, immunofluorescent staining was performed with a variety of markers. PASMCs stained uniformly (98% of cells) with anti–smooth muscle myosin and α-actin antibodies (Figure 1, panels A–D). PASMCs did not stain with labeled, acetylated LDL (Figure 1E) or anti–factor VIII antibodies (not shown). Endothelial cell controls took up LDL but did not react with the anti–smooth muscle antibodies (not shown). Fibroblasts did not stain with anti–smooth muscle myosin antibodies (Figure 1F). The same pattern of staining was replicated in cells that had been subpassaged 23 times. Subculture 46 PASMCs also stained with the anti–smooth muscle antibodies but in a less distinct and fibrillar pattern (Figure 1G).

To clone the cells, approximately 100 PASMCs from subculture 22 were grown in a 96-well plate. Four individual cell colonies were obtained and further subcultured. All cloned populations exhibited the characteristic “hill-and-valley” culture morphology and had doubling times similar to uncloned cells. The clones also showed the same immunofluorescent staining pattern with anti–smooth muscle myosin antibodies as the PASMCs of origin even after 53 passages (Figure 1H). One of these clones (PAC1) was chosen for further characterization.

Uncloned PASMCs and PAC1 cells have been maintained in culture for >400 doubling times (100 subpassages). In 20% FCS, both PASMCs and PAC1 cells had a doubling time of approximately 24 hours, and in 0.5% FCS (serum deprivation), there was no significant increase in cell number (Figure 2). In the presence of \(^{3}H\)-thymidine, subconfluent cells showed labeling of 97% and 99% of their nuclei after 24 and 72 hours, respectively. In contrast, addition of \(^{3}H\)-thymidine after 72 hours of serum deprivation resulted in labeling of only 3% and 4% of the nuclei at 24 and 72 hours, respectively. Therefore, both uncloned PASMCs and...
PAC1 cells show similar growth characteristics in culture.

**Surface Receptors**

To determine whether PASMCs and PAC1 cells have surface receptors for Ang II, α-thrombin, and NE, we measured changes in intracellular calcium concentration in response to these agonists by using the dye fura-2. This approach was chosen rather than binding assays because it provides evidence that the receptor is functionally coupled to an intracellular response. Subculture 4 PASMCs (Figure 3A) and subculture 36 PAC1 cells (Figure 3, panels B–D) had a significant increase in intracellular calcium in response to Ang II, α-thrombin, and NE. In contrast, subculture 62 ("late subculture") PASMCs retained their response to α-thrombin and NE but lost their response to Ang II.

**Expression of Smooth Muscle–Specific Contractile Protein mRNA Isoforms**

We examined the expression of contractile protein mRNAs in PASMCs (subcultures 10–15) and PAC1 cells (subcultures 15–20 and 37–43) using a variety of cDNA probes representative of both the thin and thick filaments (see "Methods"). As described below, PASMCs and PAC1 cells express high levels of smooth muscle α-actin, MHC, MRLC, and α-TM mRNAs (Figures 4–7).

**Changes in Contractile Protein Expression as a Function of Level of Confluence**

Previous studies of cultured rat aortic SMCs have shown that the expression of contractile protein isoforms is influenced by level of confluence. We examined the changes in contractile protein mRNA expression as PASMCs and PAC1 cells progressed from subconfluence to confluence.

**Actin.** Total RNA from PASMCs was hybridized with a rat vascular α-actin probe, which cross-reacts with the β/γ–isoforms of actin (Figure 4A) as well as the smooth muscle α-isoform of actin (Figure 4B). There was no significant change in the β/γ–mRNAs (confluence mRNA level, 79±18% of subconfluence; n=3, p>0.05), but there was a 115±83% increase (n=4, p<0.05) in the α–smooth muscle actin mRNA as the cells progressed from subconfluence to confluence (Figure 4). Hybrid-
FIGURE 2. Growth curves for rat pulmonary arterial smooth muscle cells (PASMCs). PASMCs (filled squares) and PAC1 cells (open squares) are cultured in 20% fetal calf serum; PASMCs (filled circles) and PAC1 cells (open circles) are cultured in 0.5% fetal calf serum. Results are mean values of duplicate plates of subculture 4, 5, 6, and 60 PASMCs and subculture 6, 22, 28, and 40 PAC1 cells.

Characterization of total RNA from PAC1 cells with a chicken skeletal actin probe, which recognizes predominantly the β/γ-isoforms, demonstrated a decrease in β/γ–actin mRNAs to 12±4% of subconfluent levels (n=2, p<0.005) (Figure 5A). Hybridization of RNA from PAC1 cells with a probe derived from the 3’ untranslated region of the α-smooth muscle actin isoform, which is specific for the α-isoform, showed an increase of 120±49% (n=4, p<0.01) in smooth muscle α-actin mRNA as cells reached confluence (Figure 5B).

MHCI. RNA from PASMCs and PAC1 cells was hybridized with a cDNA probe encoding a rat aortic smooth muscle MHC isoform. This probe specifically identifies the smooth muscle isoform of MHC. Smooth muscle MHC mRNA increased 1,140±670% in the PASMCs (n=3, p<0.05) and 1,870±390% in the PAC1 cells (n=2, p<0.05) from subconfluence to confluence (Figures 4C and 5C).

MRLC. SMCs express two highly homologous MRLC mRNAs. Neither MRLC is specific for smooth muscle, and both are expressed in smooth muscle and nonmuscle cells. Using a probe (MRLC 2) that recognizes both isoforms, PASMC showed a 120±44% increase (n=3, p<0.025) (Figure 4D), whereas PAC1 cells showed no significant change (confluence mRNA level, 126±98% of subconfluence; n=3, p>0.05) (Figure 5D) in MRLC mRNA expression as cells grew from subconfluence to confluence.

α-TM. Rat α-TM is encoded by a single gene whose transcripts are alternatively spliced to produce smooth muscle, nonmuscle, and sarcomeric isoforms. The

FIGURE 3. Graphs of changes in [Ca²⁺], in response to vasoactive agonists. Representative increase in [Ca²⁺], (detected with fura-2) in response to 10 nM angiotensin II (Ang II) in subculture 4 rat pulmonary arterial smooth muscle cells (panel A). For details of the effects of digitonin and EGTA, see "Methods." Concentration–response curves for Ang II (panel B), α-thrombin (panel C), and norepinephrine (panel D) in PAC1 cells. The y-axis denotes percentage of [Ca²⁺], compared with levels in control cells.
smooth muscle isoform differs from the other isoforms in that it contains exon 2, whereas the others lack exon 2 and include exon 3. Exon 3 is thus a “default” splicing choice, whereas exon 2 is smooth muscle specific. S1 nuclease analysis of α-TM in PASMCs and PAC1 cells was performed with a cDNA probe containing exon 2. The results are shown in Figures 6 and 7. Full protection of the probe (smooth muscle, 355 nucleotides) represents the smooth muscle isoform (predominantly expressed in uterus, Figure 7, lane 8), whereas partial protection (nonmuscle, 116 nucleotides) represents one of the other isoforms (nonmuscle isoform in smooth muscle cells, predominant isoform(s) in rat skeletal muscle: Figure 6, lane 6; Figure 7, lane 9). PASMCs and PAC1 cells expressed predominantly the smooth muscle α-TM mRNA isoform, and this isoform increased 380±60% (n=2, p<0.05) and 440±210% (n=3, p<0.05), respectively, as cells grew from subconfluence to full confluence (Figures 6 and 7). The nonmuscle isoform also increased at confluence and by the same magnitude as the smooth muscle isoform (Figures 6 and 7).

**FIGURE 4.** Expression of contractile protein mRNAs in rat pulmonary arterial smooth muscle cells. Total RNA was obtained from subconfluent cells (first column), cells in transition to confluence (second column), and postconfluent cells (third column). Blot hybridizations were performed using the following probes: smooth muscle vascular actin, which cross-reacts with the β/γ–actin mRNA isoforms (panel A) and the α-actin mRNA isoform (panel B); smooth muscle myosin heavy chain (panel D); and myosin regulatory light chain (panel D). Representative methylene blue stain of the 28s rRNA band in a nitrocellulose filter after RNA transfer is shown in panel E.

**FIGURE 5.** Expression of contractile protein mRNAs in PAC1 cells. Total RNA was obtained from subconfluent cells (first column), cells in transition to confluence (second column), and postconfluent cells (third column). Blot hybridizations were performed using the following probes: skeletal actin, which recognizes only the β/γ–actin mRNA isoforms (panel A); untranslated portion of vascular smooth muscle α-actin, which recognizes only the smooth muscle α-actin mRNA isoform (panel B); smooth muscle myosin heavy chain (panel C); and myosin regulatory light chain (panel D). Representative methylene blue stain of the 28s rRNA band in a nitrocellulose filter after RNA transfer is shown in panel E.

**Transfection**

Subculture 25 PAC1 cells were transfected with a construct that contains the mutually exclusive exons 2 and 3 of α-TM. Transcripts derived from this construct were readily detectable by S1 nuclease protection and, moreover, were spliced in a smooth muscle–specific fashion with predominant inclusion of exon 2 (Figure 8). In contrast, transfected COS cells displayed almost complete inclusion of exon 3, the expected non–smooth muscle pattern (Figure 8). PAC1 is the only cell line among several that we have tested including rat aortic SMCs, BC3H1 cells, and A7r5 cells (not shown) that is able to generate smooth muscle–specific splicing of transfected constructs.

**Discussion**

SMC culture has been shown to be of great value in studying the biochemical and physiological responses of aortic1–9,15,30,33,41–43,49–51 and pulmonary venular SMCs in an environment free from other cellular elements. However, one problem inherent in primary and secondary SMC cultures is that although they contain predominantly SMCs, they comprise heterogeneous populations of these cells.52,53 As these SMCs are serially passaged, they show changes in differentiated properties such as the expression of smooth muscle α-actin,4
FIGURE 6. SI nuclease analysis of α-tropomyosin mRNA expression in rat pulmonary arterial smooth muscle cells (PASMCs). Size markers (lane 1), probe (lane 2), negative control t-RNA (lane 3), subconfluent (SC) PASMCs (lane 4), confluent (C) PASMCs (lane 5), L6E9 rat skeletal muscle cell RNA (lane 6). The smooth muscle cDNA Eco RI-PstI restriction fragment with a polylinker tail (hatched) depicted at the top right was 5′ end-labeled at a nucleotide corresponding to codon 120 (see also Figure 7). Full protection of this probe (smooth muscle, 355 nt) represents the smooth muscle isoform, whereas partial protection (nonmuscle, 116 nt) represents the nonmuscle or skeletal muscle isoforms.

FIGURE 7. SI nuclease analysis of α-tropomyosin mRNA expression in PAC1 cells. Hae III–digested ØX nucleotide size markers (lane 1); probe (lane 2); total RNA from cells on day 2 (lane 3), day 3 (lane 4), day 4 (lane 5), day 5 (full confluence) (lane 6), and day 7 (2 days after confluence) (lane 7) in culture; rat uterus RNA (lane 8); rat skeletal muscle RNA (lane 9); and t-RNA (lane 10). The smooth muscle cDNA Eco RI-PstI restriction fragment with a polylinker tail (hatched) depicted at the bottom was 5′ end-labeled at a nucleotide corresponding to codon 120. Full protection of this probe (SM, 355nt) represents the smooth muscle isoform, whereas partial protection (NM, 116nt) represents the nonmuscle or skeletal muscle isoforms.

MHC,7 proteoglycans,11 functional surface receptors,54 sodium and calcium channels,9 and morphological features.12 In addition, each preparation of SMCs can potentially display quite different properties and some may even reach "senescence"55 and cease to proliferate. Attempts to circumvent this problem have led to the development of several clonal cell lines. Among these are rat embryonic thoracic aorta–derived A7r5, A9, and A10 cell lines,56 a rat cardiac vascular SMC line,57 a simian virus 40, large T-antigen–transformed rat aortic SMC line,55 spontaneously hypertensive and normotensive rat aortic SMC lines,53 and a mouse brain neoplasm–derived BC3H1 cell line.58 However, fusion to form multinucleated myotubes observed in A9 cells56 and recent evidence demonstrating expression of sarcomeric muscle–specific contractile proteins in BC3H1 cells59 suggest that these two cell lines are of skeletal muscle origin.

As a means of providing a more standardized culture system composed of a homogeneous population of pulmonary vascular SMCs, we established several clones of PASMCs. Many vascular SMC culture systems have been derived from enzymatic digestion of intact aortas or other systemic blood vessels; therefore, this method was used to isolate the PASMCs. With conditions similar to those previously used to generate aortic SMC cultures,30 we successfully cultured and cloned adult rat pulmonary arterial SMCs. Unlike some SMC lines, these clones were derived by growth selection by using standard cloning procedures and not via mutagenesis or transformation. The clones exhibited similar
growth characteristics, hill-and-valley morphology in culture, smooth muscle myosin immunofluorescent staining, and smooth muscle MHC mRNA expression as early PASMCs. In addition, PAC1 cells, the cloned PASMCs that were extensively characterized, have maintained through multiple subcultures many differentiated properties of intact pulmonary arterial SMCs. They have functional α-thrombin, Ang II, and NE receptors and express high levels of smooth muscle contractile protein mRNAs at passages as high as 43 (the latest subcultures that we tested), further suggesting that these clones represent differentiated SMCs.

A particularly interesting aspect of muscle cell biology is the relation between the expression of contractile proteins and growth and differentiation. In sarcomeric muscle, growing myocytes synthesize little or no sarcomeric muscle-specific contractile proteins. However, upon cell fusion and/or growth arrest, sarcomeric contractile protein genes are induced, and large amounts of protein are synthesized. Previous studies using cultured rat aortic SMCs have demonstrated that these cells synthesize both nonmuscle and smooth muscle-specific actin and MHC isoforms during both growth and quiescence, although the ratios of the nonmuscle and smooth muscle isoforms vary with growth state. We have used a number of cDNAs encoding smooth and/or nonmuscle contractile proteins to examine the changes in contractile protein mRNA expression as PASMCs and PAC1 cells progressed from log-phase growth to quiescence. The smooth muscle isoforms of α-actin, MHC, and α-TM mRNA increased at confluence in both PASMCs and PAC1 cells. MRLC mRNA increased in the PASMCs but did not significantly change in PAC1 cells as they reached confluence. The β/γ-actin mRNA isoforms did not change or decrease, whereas the nonmuscle isoforms of α-TM increased in both PASMCs and PAC1 cells with confluence. Therefore, as previously observed in other cultured vascular SMCs, the smooth muscle–specific isoforms of contractile protein mRNAs generally appear to increase, whereas the nonmuscle isoforms do not change or decrease as pulmonary arterial smooth muscle cells grow to confluence.

There were two instances in which this pattern was not completely followed. MRLC mRNA levels did not significantly change as PAC1 cells grew to confluence. This may be because, unlike the other contractile proteins, there are no clear smooth muscle–specific or nonmuscle isoforms of MLC; the two isoforms that have been identified exist in a variety of SMCs and nonmuscle cells. The reason MRLC mRNA increased at confluence in the PASMCs but did not change in the PAC1 cells is also unclear. The other feature that did not fit the pattern was the apparently paradoxical increase of the nonmuscle α-TM mRNA isoform with confluence in both PASMCs and PAC1 cells. The increase in the nonmuscle isoform quantitatively paralleled the increase in the smooth muscle isoform. One possibility is that because the smooth muscle and nonmuscle isoforms are products of the same α-TM gene, the progression to confluence stimulates an increase in the rate of transcription but no change in the splicing mechanism, resulting in the same ratio of smooth muscle to nonmuscle isoforms.
The significance of this differential regulation of the growth of contractile protein mRNAs with changes in growth state is unclear. Perhaps when the cells reach confluence and curb their growth they are in a more "differentiated" phenotype, resembling their normally quiescent counterparts in vivo. It still remains to be determined whether these changes in contractile protein mRNA are paralleled at the level of protein expression.

An important feature for the study of molecular mechanisms in culture is the ability of cells to be transfected. The introduction of exogenous genetic material can be used to examine the regulation of processes such as transcription, splicing, and translation or to modify the phenotype and behavior of cells. A test construct containing α-TM exons 2 and 3 was transfected into PAC1 cells. They readily expressed the minigene. In addition, the spliced mRNA product included exon 2, demonstrating that the cells have smooth muscle-specific trans-acting factors. Therefore, PAC1 cells can be transfected, and they have the machinery to process RNA transcripts in a smooth muscle-specific fashion.

The similar growth characteristics, functional surface receptors, and contractile protein mRNA expression of PAC1 cells and PASMCs demonstrate that the cloned PAC1 cells are highly representative of cultured rat pulmonary vascular SMCs. However, some differences between the cloned and uncloned cells have been noted. The uncloned PASMCs expressed many differentiated features through 15 subcultures but had weaker staining with α-smooth muscle myosin antibodies at subculture 46 (Figure 1) and lost their response to Ang II when late subculture (subpassage 62) cells were tested. This suggests that the PASMCs also dedifferentiate but perhaps at a slower rate than other vascular SMCs that have been serially passaged in culture.7,8,12,53 However, to strictly confirm this slower rate of dedifferentiation, cells would have to be characterized at regular and more frequent intervals. An additional disadvantage of the uncloned PASMCs was that two of eight initial isolations from different animals became senescent and ceased to proliferate at subcultures 42 and 89, respectively. There was no evidence of infection, suggesting that the longevity of some of these heterogeneous populations is unpredictable. PAC1 cells have been passaged more than 100 times and at these late subpassages continued to demonstrate similar growth rates, morphology, and smooth muscle myosin immunofluorescent staining as earlier subculture cells.

Summary

This study provides one of the most comprehensive characterizations of cultured PASMCs to date. A cloned line of PASMCs was developed that maintains many differentiated properties such as the expression of functional surface receptors and contractile protein mRNAs through many subcultures. This cell line offers advantages relative to noncloned cells: namely, uniformity of phenotype, greater abundance of cells (especially compared with primary cultures) to conduct experiments, and obviating the need for repeated primary cell isolations and characterizations. Initial experiments with these cells have shown that mechanical stretch causes a rapid and short-lived increase in the masses of inositol triphosphate and inositol tetrakisphosphate followed by an increase in calcium efflux.53 PAC1 cells should be an important in vitro model system for studying the mechanisms that regulate the changes in SMC phenotype observed in tissue culture as well as for studying normal and pathological growth in the pulmonary vasculature.

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