Evidence From a Novel Human Cell Clone That Adult Vascular Smooth Muscle Cells Can Convert Reversibly Between Noncontractile and Contractile Phenotypes

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Abstract—Smooth muscle cells (SMCs) perform diverse functions that can be categorized as contractile and synthetic. A traditional model holds that these distinct functions are performed by the same cell, by virtue of its capacity for bidirectional modulation of phenotype. However, this model has been challenged, in part because there is no physiological evidence that an adult synthetic SMC can acquire the ability to contract. We sought evidence for this by cloning adult SMCs from human internal thoracic artery. One clone, HITB5, expressed smooth muscle α-actin, smooth myosin heavy chains, heavy caldesmon, and calponin and showed robust calcium transients in response to histamine and angiotensin II, which confirmed intact transmembrane signaling cascades. On serum withdrawal, these cells adopted an elongated and spindle-shaped morphology, random migration slowed, extracellular matrix protein production fell, and cell proliferation and [3 H]thymidine incorporation fell to near 0. Cell viability was not compromised, however; in fact, apoptosis rate fell significantly. In this state, agonist-induced elevation of cytoplasmic calcium was even more pronounced and was accompanied by SMC contraction. Readdition of 10% serum completely returned HITB5 cells to a noncontractile, proliferative phenotype. Contractile protein expression increased after serum withdrawal, although modestly, which suggested that the switch to contractile function involved reorganization or sensitization of existing contractile structures. To our knowledge, the physiological properties of HITB5 SMCs provide the first direct demonstration that cultured human adult SMCs can convert between a synthetic, noncontracting state and a contracting state. HITB5 cells should be valuable for characterizing the basis of this critical transition. (Circ Res. 1999;85:338-348.)

Key Words: muscle, smooth, vascular differentiation contraction cell movement

The primary function of adult arterial smooth muscle cells (SMCs) is to contract. However, during vascular disease SMCs can be called on to perform noncontractile functions, including replication, migration, and elaboration and degradation of extracellular matrix (ECM) proteins. These noncontractile roles of SMCs are fundamental to the growth of atherosclerotic lesions, as well as lesions that develop after angioplasty/stent therapy and cardiac transplantation.1,2 Noncontractile functions of SMCs are also the means by which SMCs maintain the integrity of the fibrous cap that overlies atheromata, rupture of which can lead to arterial thrombosis and organ infarction.3,4

The basis by which adult SMCs can perform diverse functions has been an active area of inquiry over the past 2 decades. A classic view is that adult vascular SMCs have the capacity to modulate their phenotype, or state of differentiation, in a bidirectional manner. This concept was first hypothesized in 1967 by Wissler5 and was based on the assumptions that (1) SMCs were integral to the pathogenesis of atherosclerosis and (2) SMCs represented a single cell type in the artery wall. Subsequent support for the concept came from the finding that cells enzymatically dispersed from arterial media and placed in culture gradually assumed features of less-differentiated cells with characteristics of SMCs in vascular lesions.6–8 The paradigm thus holds that in response to vascular stress, SMCs specialized to contract can be induced to lose their contractile function and acquire synthetic functions; under different local conditions they can then revert to the more specialized, contractile state. This concept forms an important tenet of the response-to-injury hypothesis of atherosclerosis.9

Despite widespread appreciation of the concept of bidirectional SMC phenotype modulation, there remains no cell culture system whereby SMCs differentiate, reversibly or otherwise, to the extent that they can contract.10 Changes in various markers of differentiation have been identified (eg, myofibrillar content and smooth muscle [SM]–specific proteins),6,11,12 but there is no documentation to our knowledge that vascular SMCs in culture can acquire or reacquire the ability to contract. The absence of such a culture system has...
hindered efforts to dissect the molecular mechanisms that underlie the attainment and/or loss of contractile function of SMCs.

The lack of evidence for complete redifferentiation of cultured SMCs has also been suggested to pose a qualification to the concept of SMC phenotype modulation itself. Although it may be that the appropriate culture conditions for more complete differentiation have yet to be identified, the inability to drive cultured SMCs to a contractile state represents an apparent inconsistency to the SMC-shift paradigm. This is noteworthy in light of growing evidence that SMCs of the artery wall are not, as originally considered, a homogeneous population of cells, and thus there is no a priori need to invoke SMC phenotype shift to explain the emergence of a noncontractile phenotype. Instead, noncontractile or synthetic functions may arise by selective expansion of subpopulations of cells. This concept was supported by a recent observation that cells acutely dispersed from the canine carotid artery, and proven to be mature SMCs, did not have the capacity to dedifferentiate, proliferate, or migrate in culture. Instead, the migratory and proliferative functions seen in culture were performed by cells that, in the vessel wall, did not have markers of a mature SMC lineage.

Ideally, to reliably determine whether a vascular SMC is capable of reversibly modulating to and from a contractile state and to exploit such a finding to model and better define the molecular basis of SMC differentiation, a homogeneous population of SMCs is required. This is because the heterogeneity inherent in primary cultures means that changes in individual cells cannot be easily distinguished from expansion of subpopulations with distinct phenotypes. Stable human SMC clones would overcome this limitation, but none have existed. We report here the generation and characterization of an adult human SMC clone that is capable of reversibly converting between a noncontractile state with migratory, proliferative, and synthetic properties and a well-differentiated state that contracts in response to vasomotor agonists.

**Materials and Methods**

**Cell Cloning**

A primary culture of human SMCs was established by explant outgrowth of a segment of internal thoracic artery retrieved at the time of coronary artery bypass surgery. Endothelial cells were removed by scraping the luminal surface of the vessel with a scalpel blade, and the adventitia was mechanically stripped away. Primary cultures were maintained in medium 199 (M199) supplemented with 10% FBS. SMC identity was confirmed morphologically and by positive immunostaining for SM α-actin (1A4, Dako).

Cloning of human SMCs was performed by a modified cloning ring approach. SMCs in the sixth subculture were dispersed with trypsin-EDTA and replated onto gelatin-coated culture dishes (100 μg/mL) at a density of 2 cells/cm². These cultures were maintained in M199 that was conditioned by primary SMC cultures in log-phase growth and supplemented with 10% FBS. Individual colonies were identified microscopically, released by localized application of trypsin-EDTA, and subsequently expanded. We observed that 3 of the clones underwent striking morphological changes after serum withdrawal and displayed an unusually robust resilience to serum deprivation. Such clones were deemed to be uniquely informative with respect to differentiation of human SMCs. One of the clones, designated HITB5 (human internal thoracic B5) was characterized in this context and is the focus of this report. All experiments with HITB5 cells were performed with subcultures 14 to 19 after cloning. (HITB5 SMCs have been successfully passaged beyond the 40th subculture and have retained the properties described in this report.)

**Cell Migration Assay**

Motility of HITB5 cells was assessed by quantifying migration path and migration speed using digital time-lapse video microscopy. Cells were seeded onto culture dishes precoated with 100 μg/mL type I collagen (Vitrogen, Collagen Corp), and migration was monitored with an inverted microscope (Zeiss Axiovert S100). A charge-coupled device video camera (Sony XC-75) attached to the microscope was used to generate video images that were digitally acquired over an 8-hour recording period (Northern Eclipse, Empix Imaging, Inc.). Ambient temperature was maintained at 37°C by placing the culture dish in a temperature control cell (BC-500W, 20/20 Technology, Inc.). Migration was measured from digital images by tracking the location of cell centroids at hourly intervals. Migration speed was determined as the sum of hourly distances divided by the total time.

**Cell Proliferation and DNA Synthesis Assay**

To evaluate SMC replication, cells were seeded in 24-well plates at a density of 3000 cells/cm² and incubated in M199 with 10% FBS for 48 hours. Cells were then washed with PBS and incubated in serum-free M199 for 7 days, with a medium change on day 3. Serum-supplemented conditions (M199 plus 10% FBS) were then restored, and cells were cultured for 4 more days. On each of the 13 days, cells from quadruplicate wells were dissociated with trypsin and counted with a hemacytometer.

DNA synthesis rates were evaluated by pulse labeling SMCs with [3H]thymidine. HITB5 cells were seeded in 96-well plates at 5×10³ cells/cm² and incubated sequentially in serum-supplemented M199, serum-free medium, and serum-supplemented medium, as described for the proliferation assay. On designated days SMCs were incubated with [3H]thymidine (10 μCi/mL and 71 Ci/mmol, ICN) for 8 hours and then transferred onto glass fiber filters (Wallac) using an automated cell harvester (Tomtec). Incorporated [3H]thymidine was counted on a Trilux 1450 MicroBeta counter (Wallac).

**Protein Synthesis Assay**

Synthesis of proteins, including insoluble ECM proteins, was studied by measuring incorporation of [1H]leucine as previously described. Briefly, SMCs were cultured in M199 supplemented, sequentially, with 10% FBS for 48 hours, with 0% FBS for 3 days, and with 10% FBS for 2 more days. After each change of growth medium, cells were pulsed with 4 μCi/mL L-[4,5-3H]leucine (ICN) for 16 hours. Cells were then subjected to NH₄OH lysis and high-salt extraction. SMCs were washed with cold PBS and disrupted with 0.25 mol/L NH₄OH containing 1 mmol/L PMSF and 1 mmol/L EDTA for 30 minutes. The lysate was withdrawn and the remaining contents in the culture well washed with PBS containing 1 mmol/L EDTA, followed by extraction with 50 mmol/L Tris (pH 7.4)–buffered NaCl (1 mol/L) for 15 minutes. The culture well was then washed once more with PBS-EDTA. All lysis, extraction, and wash solutions were combined to a single “cell fraction,” which was then precipitated in 10% trichloroacetic acid (TCA). The acid-insoluble material was washed twice in 10% TCA and solubilized in 1 mol/L NaOH followed by neutralization with an equal volume of 1 mol/L acetic acid. Radioactivity was then determined by scintillation counting (Cytoscint, ICN). The material remaining on the culture surface after NH₄OH and high-salt extraction is referred to as the ECM fraction. This was solubilized in NaOH (1 mol/L) and precipitated at 4°C by adding 0.1 volume of 100% TCA. The TCA-insoluble fraction was washed and scintillation counted as above. The protein concentration of the “cell fraction” was determined with Bio-Rad DC protein assay reagents.
Apoptosis Assay

Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated dUTP-fluorescein nick end labeling (TUNEL) (Promega). SMCs were washed with PBS, fixed for 25 minutes with 4% formaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS. End labeling of DNA fragments with 0.5 U/mL terminal deoxynucleotidyl transferase and 5 mmol/L fluorescein-12-dUTP was performed for 1 hour at 37°C. Slides were then washed twice in 2× SSC followed by 3 washes in PBS. Cells were counterstained with 5 mg/mL propidium iodide in PBS and examined by fluorescence microscopy (Olympus BX100). For each growth condition, 200 cells were counted and the percentage of apoptotic cells (those with green fluorescence localized to the nucleus) was determined.

Measurement of \([Ca^{2+}]_i\)

Agonist-induced calcium transients were measured in single HITB5 cells loaded with fura-2–acetoxyxymethyl ester (fura-2–AM).

Trypsinized SMCs were loaded with 0.5 mmol/L fura-2–AM and transferred to a custom-made perfusion chamber mounted on an inverted Nikon diaphot microscope. Cells were illuminated with alternating 345- and 380-nm light and the 510-nm emission was detected (DeltaScan System, Photon Technology International). Cytosolic free calcium concentration \([Ca^{2+}]_i\) was calibrated according to the methods of Grynkiewicz et al. Agonists were applied to cells by pressure ejection from a micropipette. Data were corrected for background fluorescence.

SMC Contraction

Contraction of sparsely seeded SMCs was evaluated at room temperature using digital time-lapse video microscopy, as was used for the migration assay. Cells cultured in M199 with 10% FBS or in serum-free M199 for 3 days were imaged before and after addition of either histamine or angiotensin II (Sigma Chemical Co). Two approaches to quantifying contraction were used. With the first approach, cells were maintained adherent to the culture dish and changes in planar area were determined using image analysis software (Empix). Acute contraction was evident within 1 minute of adding agonist, and we recorded the maximum contraction for each cell, expressed as the percentage reduction in cell area. In the second approach, cell length was assessed. SMCs were gently loosened from the substrate with trypsin-EDTA and then released with the addition of M199 with 10% FBS. This procedure maintained the elongated morphology of the SMCs. Cells were then allowed to settle on the culture substrate, and agonist was added. Cell shortening was evident 20 seconds after agonist was added to the medium and contraction was expressed as the maximal percentage reduction in cell length.

Western Blot Analysis

SMCs were washed with cold PBS and disrupted in lysis buffer (in mmol/L, Tris [pH 7.4] 10, phenylmethylsulfonyl fluoride 0.1, and EDTA 0.1, as well as 1% SDS and 10 μg/mL leupeptin). For evaluation of SM myosin heavy chains (SM-MHC), the lysis solution contained 40 mmol/L Na4 P2O7, 1 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin. Equal amounts of proteins were separated on 12% (for SM-MHC) SDS-polyacrylamide gels (gels were electrophotographically transferred to polyvinylidene difluoride membranes (Millipore), which were then blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20. Blots were then incubated for 2 hours at room temperature with designated primary antibodies. Primary antibodies used were the following: anti–SM α-actin monoclonal antibody (1A4); anti–SM-MHC monoclonal antibody that recognizes both SM1 and SM2 isoforms (G4), anti-calponin monoclonal antibody (hCP, Sigma), rabbit anti–caldesmon polyclonal antibody that recognizes both heavy and light caldesmon (kindly provided by Dr M.P. Walsh, University of Calgary, Alberta, Canada), and anti-vinculin monoclonal antibody (hVIN-1, Sigma) that recognizes both metavinculin and vinculin. Horseradish peroxidase–conjugated anti-mouse and anti-rabbit IgG (ab’2) (Boehringer Mannheim) were used as secondary antibodies. Blots were developed with enhanced chemiluminescence detection reagents (Boehringer Mannheim), exposed on Kodak X-Omat blue XB-1 film, and quantified by laser scanning densitometry (GS-700 Imaging Densitometer, Bio-Rad).

Statistics

Results are expressed as mean±SEM. Differences between groups were evaluated by ANOVA with the Scheffé post hoc test.

Results

Serum Withdrawal Induces Reversible Morphological Changes in HITB5 SMCs

When cultured in M199 containing 10% FBS, HITB5 cells appeared as elongated cells that were well spread over the culture substrate (Figure 1a and 1d). The appearance was similar to that of primary cultures of human SMCs, although HITB5 cells were somewhat larger and demonstrably more homogeneous. After serum withdrawal, HITB5 cells underwent a striking change to a highly elongated and spindle-shaped cell that was more refractive to phase-contrast light (Figure 1b). These changes were evident after 12 hours and complete by 72 hours. After restoration of serum, the cells reverted to their spread morphology, which was indistinguishable from that of the cells before serum withdrawal (Figure 1c).

In addition to the morphological changes of individual cells, the population of cells substantially reorganized after serum withdrawal. Twelve hours after serum withdrawal, the elongating SMCs organized to form dense, multilayered cell bundles that appeared as well-aligned ridges reminiscent of the organization of SMCs in blood vessels (Figure 1e). This morphology could be maintained for 2 weeks. Two days after serum restoration, however, the cell bundles disassembled and the cells lost the organized pattern (Figure 1f).

Serum Withdrawal Reversibly Suppresses Motility of HITB5 SMCs

Cell locomotion is a key feature that distinguishes SMCs contributing to vascular disease from the quiescent SMCs of the normal artery wall. Figure 2 illustrates migration paths and migration speeds on type I collagen, for HITB5 SMCs under serum-supplemented conditions (10% FBS), 3 days after serum withdrawal, and 2 days after restoration of 10% FBS. To limit the extent to which serum-deprived SMCs accumulated in multilayered bundles, which would preclude quantitative analysis of single-cell migration, cells were studied at low densities (2×103 cells/cm2). As illustrated, cells in serum-supplemented conditions demonstrated considerable motility; however, the migration path was substantially constrained after serum removal, with a 3.1-fold reduction in migration speed (P<0.001). These changes were fully reversible on readdition of serum. Migration on plastic or fibronectin (10 μg/mL) showed similar profiles (data not shown).

Serum Withdrawal Reversibly Inhibits Cell Proliferation and DNA Synthesis in HITB5 SMCs

Low growth rate is another fundamental characteristic of differentiated, contractile SMCs in the vessel wall. To deter-
mine whether cell proliferation declined in concert with cell elongation and the fall in random motility, we assessed cell number change by hemocytometry. As shown in Figure 3A, growth arrest was induced 2 days after serum withdrawal. The cells remained quiescent in serum-free medium, with no morphological evidence for cell death and no change in cell number for 6 days. SMC proliferation was promptly restimulated on readdition of 10% FBS.

To determine whether the changes in cell number were related to parallel changes in DNA synthesis rate, [3H]thymidine incorporation was measured. As shown in Figure 3B, [3H]thymidine incorporation precipitously dropped to background levels after serum withdrawal and was restored after reintroduction of serum to culture medium 2 days later.

Serum Withdrawal Reversibly Inhibits ECM Protein Synthesis by HITB5 SMCs

Increased protein synthesis, including synthesis of ECM proteins, is a hallmark of SMCs with a synthetic phenotype in vascular lesions. We assessed leucine incorporation into both cellular and ECM fractions by incubating cultures with [1H]leucine and isolating the cell fraction and the insoluble ECM deposited by the cells. As shown in Figure 4, serum withdrawal reduced leucine incorporation into the cellular fraction to 0.08±0.01 of basal levels and reduced leucine incorporation into the ECM fraction to 0.10±0.01 of basal levels. Leucine incorporation into the cellular fraction was fully restored to basal levels 3 days after serum restoration. Leucine incorporation into the ECM fraction also reversed 3 days after serum replenishment, although it did not completely return to basal levels.

Serum Withdrawal Is Associated With Decreased Apoptosis

Despite prolonged periods of “starvation” conditions, HITB5 cells remained viable. In fact, we observed that cells in the serum-starved state had less cytoplasmic granularity and a smoother plasma membrane surface than did cells in serum-supplemented medium, which suggests a healthier cell. There was also less cellular debris in the medium. We reasoned, therefore, that the resilience in serum-starved conditions might, in part, be due to a decreased rate of apoptosis in the serum-starved cells. We tested this by performing TUNEL with dUTP-fluorescein. As shown in Figure 5, 3 days after serum withdrawal the percentage of apoptotic cells was reduced by half (4.3±0.6 versus 9.8±1.2%, \(P<0.001\)). In addition, nuclear fragmentation, assessed from propidium iodide staining, was reduced in serum-deprived cultures. It was also apparent that apoptosis was inversely related to the attainment of the highly elongated, spindle-shaped morphol-
ogy; in microscopic fields in which cells were most elongated, virtually no apoptosis was seen.

HITB5 SMCs Possess Intact Receptors for Vasoactive Agonists That Regulate Ca\(^{2+}\) Signaling

We next sought to determine whether HITB5 cells had functional cell surface receptors for contractile hormones. Agonist-induced Ca\(^{2+}\) mobilization was assessed because this had the potential to establish both the presence of the receptor and functional coupling to a signaling event central to SMC contraction. Changes in [Ca\(^{2+}\)]\(_i\) were quantified in individual SMCs loaded with fura-2 and stimulated with histamine (20 \(\mu\)mol/L) or angiotensin II (1 \(\mu\)mol/L). As shown in Figure 6A, both vasoactive substances induced robust Ca\(^{2+}\) transients in HITB5 cells. This was evident for HITB5 SMCs growing in M199 with 10% FBS, as well as SMCs studied 3 days after serum withdrawal. As shown in Figure 6A and 6B, the peak [Ca\(^{2+}\)], stimulated by histamine and angiotensin II was significantly higher after serum deprivation, which suggests some upregulation of the receptor signaling cascade in the contractile phenotype.

To confirm that the responses were receptor mediated, cells were treated with the H\(_1\)-receptor antagonist pyrilamine (1 \(\mu\)mol/L) or the AT\(_1\)-receptor antagonist losartan (10 \(\mu\)mol/L). Agonist-induced Ca\(^{2+}\) transients were studied serially in the same cell before addition of the antagonist, immediately after its application, and after washout. Both antagonists were found to abrogate the rise of Ca\(^{2+}\) stimulated by the respective agonist (5 of 5 cells treated with pyrilamine and 3 of 3 cells treated with losartan).

Serum-Deprived HITB5 SMCs Contract in Response to Vasoactive Hormones

The ability to contract in response to a physiological stimulus is the only unequivocal criterion for a “contractile” SMC phenotype. It is noteworthy, therefore, that this has never been reported for subcultured SMCs. Having established intact transmembrane signaling cascades for vasoconstrictor peptides, we addressed this question in HITB5 cells. Cells either in M199 with 10% FBS or after 3 days of serum deprivation were stimulated with histamine or angiotensin II. Between 7 and 15 cells were studied for each experimental condition. Contraction was first assessed in adherent cells by quantifying cell area. Administration of histamine (1 \(\mu\)mol/L) to cells that had been subjected to serum withdrawal caused a reduction in 2-dimensional cell area of 27±3%, whereas the area of vehicle-treated cells was reduced by 2±3% (\(P<0.001\)). Preincubation of cells with 1 \(\mu\)mol/L pyrilamine inhibited histamine-induced contraction (Figure 7A and 7B). Stimulation with angiotensin II (1 \(\mu\)mol/L) to serum-deprived HITB5 SMCs also elicited a significant reduction in cell area (21±2%), and this was blocked by losartan (1 \(\mu\)mol/L) (Figure 7B). In contrast, SMCs that were not serum deprived showed no morphological change on agonist stimulation, and this was independent of the culture substrate (cell area reduction after angiotensin II...
stimulation of 0.4 ± 1.2% for cells on plastic, 1.4 ± 1.6% for cells on fibronectin, and 0.1 ± 1.3% for cells on type I collagen). Similarly, SMCs that were serum deprived and then subjected to serum restimulation for 48 hours no longer contracted. We also evaluated contraction in serum-deprived HITB5 cells that had been gently dispersed with trypsin-EDTA. These cells remained elongated and retained this morphology for up to 2 hours. This was associated with delayed readhesion to the substrate, which meant the cell was free to shorten. As shown in Figure 7D, stimulation with histamine shortened the cell by 25 ± 2%, and this was inhibited by pretreatment with pyrilamine. Angiotensin II reduced cell length by 23 ± 3%, an effect blocked by losartan (Figure 7C and 7D), whereas vehicle alone caused an only 3 ± 1% reduction in resting cell length. Depolarizing the cell membrane with iso-osmotic Ringer’s solution containing 100 mmol/L KCl also resulted in cell contraction (data not shown), which suggests the presence of voltage-gated calcium channels.

Contractile Apparatus Proteins Are Expressed in HITB5 SMCs and Modestly Increase as the Contractile State Is Attained

To investigate the relationship between attainment of a contractile phenotype and expression of biochemical markers of SMC differentiation, Western blot analysis was performed on cell lysates harvested before and 16 hours and 1, 2, 3, 5, and 7 days after serum withdrawal. As shown in Figure 8, the contractile apparatus proteins SM-α-actin, SM-MHC isoforms SM1 and SM2, calponin, and heavy caldesmon were present in proliferating HITB5 SMCs before withdrawal of serum. The muscle-specific isoform of vinculin, metavinculin, was also detected. After serum withdrawal, there was a gradual increase in expression of SM-α-actin, SM-MHC isoforms SM1 and SM2, calponin, and metavinculin. There were also substantial increases in the ratios of the SM to nonmuscle isoforms of caldesmon (heavy caldesmon/light caldesmon) and vinculin (metavinculin/vinculin) (Figure 8B). Additionally, the SM2 isoform of SM-MHC, which is expressed after the SM1 isoform during development, appeared to increase in expression to a greater extent than that of the SM1 isoform. Interestingly, 3 days after serum withdrawal, at which time a contraction-competent state had developed, the increases in contractile-apparatus protein expression were relatively modest. As quantified by scanning densitometry, there was a 1.5 ± 0.1-, 1.5 ± 0.1-, and 1.9 ± 0.3-fold increase in abundance of SM-α-actin, SM-MHC (SM1 plus SM2), and calponin, respectively.

Discussion

The physiological properties of HITB5 cells, to our knowledge, provide the first direct demonstration that a cultured human adult...
SMC can bidirectionally convert from a contraction-incompetent state to a contraction-competent state. Conclusive demonstration of this phenomenon was facilitated by 3 unique aspects of the study. First, the human SMCs were clonal; observed changes in cell characteristics can thus be attributed to phenotype changes in individual cells rather than selective expansion of a subpopulation of cells. Second, we were able to establish that the transition between phenotypes was bidirectional, implicating an inherent cell property distinct from the commonly observed unidirectional dedifferentiation response to culture conditions. Third, SMC contraction per se was demonstrated and used to define the contractile phenotype, rather than surrogate features such as ultrastructure or marker proteins, which are not irrevocably linked to contraction.

A number of clonal SMC lines have been developed over the past several years in an effort to generate SMC populations that are longer lived and phenotypically more predictable than primary SMC cultures. This includes cell lines derived from rat embryonic thoracic aorta, a large T-transformed rat aortic SMC line, and a rat pulmonary artery SMC line. The latter cell line retains certain differentiated properties, including expression of SM-specific proteins and functional receptors for vasoactive hormones. However, none of these SMC lines have been shown to display substantial phenotype plasticity, and there is no evidence that they can be induced to contract. In contrast, HITB5 SMCs, a human clone derived from adult internal thoracic artery, underwent a striking functional change on withdrawal of serum from the culture medium. This was characterized by a decrease in random motility, cessation of cell proliferation and DNA synthesis, decreased ECM protein production, spatial alignment and organization of cells suggesting an

Figure 5. A. Fluorescence micrographs showing the effect of serum withdrawal on apoptosis of HITB5 SMCs. Cells plated on chamber slides were cultured in M199 with 10% FBS (a and b) and for 3 days in M199 without FBS (c and d). After fixation with 3% formaldehyde, apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. Cells were then counterstained with propidium iodide to identify all cells (a and c). Nuclei of apoptotic cells appear green (b and d). B. Percentage of apoptotic cells. For each condition, 200 cells were counted, and the experiment was done in quadruplicate. P<0.01 vs SMCs in the presence of 10% FBS.
attempt at organogenesis, and attainment of contractile competence. Each of these changes was fully reversible on readdition of serum, with the exception of ECM protein synthesis, which was partially reversible. ECM protein synthesis did not fully return to baseline levels, possibly because the ECM that had accumulated over the course of the experiment provided feedback inhibition, as has been described in fibroblasts.34

It is important to note that even in the presence of serum, HITB5 SMCs expressed several contractile apparatus pro-
teins, including SM α-actin, SM-MHC, calponin, and heavy caldesmon. It is well established that during vascular development, SMCs sequentially express specific contractile proteins. It is well established that during vascular development, SMCs sequentially express specific contractile proteins. Importantly, these developmental markers appear only after commitment to a SMC lineage and, in the case of calponin and SM-MHC, only after a multilayered artery wall has developed. Therefore, HITB5 cells growing in serum are not primordial cells but are SMCs that are relatively advanced along the differentiation program. This relative maturity was also indicated by the expression of metavinculin and the Ca\(^{2+}\) responses to vasoactive agonists, including the fact that the AT\(_1\) antagonist, losartan, inhibited angiotensin II–mediated calcium influx in serum-supplemented, proliferating HITB5 SMCs. The AT\(_1\) subtype has been found in adult rat aortic SMCs, whereas the AT\(_2\) receptor subtype is predominant in fetal aorta.37

At the same time, HITB5 SMCs in serum were unable to contract and functioned in a nonspecialized and undifferentiated manner. Thus, when in serum, HITB5 cells displayed an intermediate phenotype, in that the cells were differentiated to the extent of being recognizable as SMCs, but not to the extent of having specialized, contractile function. Recent in vivo studies suggest that this intermediate phenotype may be very relevant to the pathophysiology of vascular disease. Neointimal cells of the injured rat carotid artery have recently been reported to express various ECM genes typical of synthetic SMCs at the same time as expressing the SM-specific genes calponin and SM22α.38 Similarly, electron microscopic studies of the balloon-injured rat carotid artery have demonstrated coexistence of a large endoplasmic reticulum, implying synthetic function, and myofilaments in the same cell.39 Furthermore, human atherosclerotic plaques have been shown to have an abundance of SMCs with a well-developed endoplasmic reticulum and Golgi apparatus, in addition to having myofilament bundles and expressing SM myosin and α-actin.40 These data suggest that mesenchymal cells of the vessel wall need not be of a primitive or fetal phenotype to perform synthetic functions; these functions can be performed by SMCs of an intermediate phenotype. The fact that HITB5 SMCs can convert between an intermediate SMC phenotype and a contractile SMC phenotype thus suggests that they may be a valuable model for this aspect of vascular disease.

In addition to the functional changes observed after serum withdrawal, there were biochemical changes consistent with...
SMC maturation/differentiation. This included increases in the ratios of heavy caldesmon to light caldesmon, metavinculin to vinculin, and SM2 to SM1. Expression of SM α-actin and calponin also increased. Although these changes biochemically confirm the process of differentiation in HITB5 SMCs, we speculate that increases in contractile apparatus proteins may not be a dominant mechanism by which the contractile state was attained. This is because the magnitude of change was relatively modest and because, with the exception of SM2 expression, most of the increase in expression occurred several days after the attainment of contractile competence. Development of functional receptors for vasoactive proteins or formation of an intact calcium-release mechanism were also unlikely to significantly mediate the transition to a contractile state, as these properties were already present in noncontractile SMCs. The magnitude of agonist-mediated calcium transients did increase after serum withdrawal; however, we have not yet determined which step(s) in the receptor-Ca\textsuperscript{2+} signaling cascade is subject to upregulation. Other mechanisms mediating the final stage of differentiation to a contracting cell likely remain to be elucidated and could include functional reorganization of existing contractile structures or increased calcium sensitivity of myofilaments. The small GTPase Rho and its target Rho-associated kinase have recently been found to participate in the latter and are candidates in this regard.\textsuperscript{41,42} It is also possible that observed shift in phenotype is dependent on specific changes in the interaction between the cell and ECM. Integrin-ligand interactions are known to mediate cell differentiation, including myogenesis.\textsuperscript{43}

Serum withdrawal precipitated a decline in apoptosis of HITB5 SMCs. This finding contrasts with the observation of increased apoptosis in cultured SMCs after serum withdrawal.\textsuperscript{44} However, an inverse association between death and differentiation has been well documented. In the developing human ductus arteriosus, for example, apoptosis was significantly more prevalent in the less-differentiated SMCs of the inner intima compared with well-differentiated SMCs of the media and outer intima.\textsuperscript{45} We speculate that the resistance to apoptosis in HITB5 SMCs that was induced by serum withdrawal was permissive to, and possibly a prerequisite for, the differentiation program.

The current study also adds to our understanding of SMC multifunctionality by establishing that conversion between contractile and noncontractile states is indeed a property of at least some human SMCs. This notion has recently been challenged. Although several studies have carefully charted changes of enzymatically dispersed arterial cells placed in culture,\textsuperscript{6,46,47} contractile SMCs isolated from the canine carotid artery were recently found not to dedifferentiate in culture. Instead, expansion of less-specialized cells accounted for the in vitro accumulation of cells with characteristics of synthetic SMCs.\textsuperscript{17} It is thus possible that selective emergence of noncontractile cells in culture might erroneously be interpreted as modulation of SMC phenotype. It must also be recognized that the SMC phenotype conversion hypothesis was initially based on the assumption that SMCs constitute a single cell type in the arterial media.\textsuperscript{5} This assumption is probably not valid, given the documented heterogeneity of cells in the normal adult artery.\textsuperscript{14–17} The diverse embryonic origins of arterial SMCs,\textsuperscript{48–50} and the diverse properties of cells that have been cloned from a given artery,\textsuperscript{51,52} the evidence for regions of monoclonal SMCs in atherosclerotic plaques and, occasionally, in arterial media likewise highlights the concept of selective cell expansion.\textsuperscript{53} The current findings do not preclude expansion of less mature cells as a pathophysiological feature of vascular disease, but they do suggest that the potential contribution of SMC phenotype conversion to vascular disease should not be dismissed, and efforts to understand the mechanism are of importance.

In summary, the physiological properties of HITB5 SMCs provide the first direct demonstration that cultured human adult SMCs can reversibly convert between a noncontracting state and a contracting state. This novel cell line may be valuable for clarifying our understanding of SMC phenotype switching and restructuring of the vessel wall.

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