Myosin in Cultured Vascular Smooth Muscle Cells: Immunofluorescence and Immunochemial Studies of Alterations in Antigenic Expression

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ABSTRACT Vascular smooth muscle cells (VSMC) in the rat mesenteric artery show specific immunofluorescent staining with antisera against purified human uterine myosin (ASMM) but not human platelet myosin (APM). However, in primary cultures produced by enzymatic dissociation of this vessel, VSMC stain specifically with both ASMM and APM within 5 h after plating and throughout growth to confluence (4–10 d). In confluent cultures, APM staining remains bright while ASMM staining is reduced in intensity in most cells. In contrast, cellular myosin content, determined by quantitative SDS PAGE, is comparable in confluent and growing cultures. Immunoprecipitation of high salt extracts of cultured VSMC with ASMM and APM yields myosins with the same mobilities on SDS PAGE. When serial, exhaustive precipitations are performed with one antiserum, followed by reprecipitation with the other, myosin in subconfluent and confluent VSMC cultures is exhaustively precipitated by either antiserum, thus indicating complete immunological cross-reactivity. These results might be explained by synthesis of a new myosin isoform reactive with both ASMM and APM. However, the development of APM staining in cultured VSMC did not require protein synthesis. Therefore, it is more likely that the changes in immunofluorescent staining observed in vitro reflect conformational alterations, perhaps related to cytoskeletal rearrangements. These changes in myosin antigenic expression may be relevant to the problem of VSMC phenotypic modulation both in vitro and in vivo.

Our laboratory has been engaged in developing a functional culture model for the vascular smooth muscle cells (VSMC)[1] of muscular, resistance-type arteries. Primary rat mesenteric artery VSMC cultures, obtained by enzymatic dissociation (13), retain many of the biochemical characteristics of VSMC in the intact vessel. These differentiated functional properties include the presence of specific angiotensin II (AII) receptors (13) and dose-dependent AII-induced phosphorylation of the 20,000-dalton myosin light chain (1). Physiological doses of AII can cause cellular contraction (reversible, agonist-specific cell shape change) in a variable but significant proportion of these cells (13). Thus, it appears that important biochemical steps in receptor activation-contraction coupling continue to be expressed in vitro. These cultured VSMC are, however, "phenotypically modulated" according to some criteria (see reference 5). For example, they lack ultrastructurally demonstrable myosin-containing (12–18-nm diam) thick filaments, and exhibit increased proliferative and biosynthetic activities, prominent cytoplasmic stress fibers and cellular hypertrophy (13).

As an approach to understanding the interrelationship of retained biochemical characteristics and those alterations imposed by culture conditions, we have been studying the

[1] Abbreviations used in this paper: ASMM, antismooth muscle myosin; APM, antiplatelet myosin; F-GAR, fluorescein-labeled goat anti-rabbit IgG; ^{125}I-B-H, monoiodinated Bolton-Hunter reagent; NRS, normal rabbit serum; VSMC, vascular smooth muscle cells.

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expression of myosin, an important contractile and cytoskeletal protein (for a recent review, see reference 12) in VSMC, using immunofluorescent and immunochemical techniques. We have used antisera raised against purified human smooth muscle (uterine myometrial) and nonmuscle (platelet) myosins, which show cell type-selective immunofluorescence in frozen sections of various tissues (17–19). For example, the antiuterine myosin selectively stains visceral and vascular smooth muscle, including the medial layer of rat mesenteric artery. In contrast, the antiplatelet myosin does not stain medial VSMC in the mesenteric artery but does stain intimal endothelial cells in this vessel, as well as a variety of connective tissue and epithelial cells. Using these immunologic reagents, we have characterized both qualitative and quantitative changes in myosin antigenic expression that occur in rat mesenteric artery VSMC during their adaptation to in vitro culture conditions. These in vitro changes may be relevant to the process of phenotypic modulation as it occurs in vivo in certain disease states involving vascular smooth muscle such as atherosclerosis or hypertension (5).

MATERIALS AND METHODS

Antisera: The rabbit antiserum against human platelet myosin (purified according to Pollard et al. [23]) has been described previously (8) and is specific for the rod portion of this nonmuscle myosin type. In Ouchterlony double diffusion, this antiserum cross-reacts with extracts of human uterine myometrium (22); therefore, it was extensively absorbed with purified uterine myosin until immunofluorescent staining of frozen sections of visceral (rat tracheal and duodenal) smooth muscle was lost. All experiments reported here employed the absorbed antiplatelet myosin (APM). We have further characterized the reactivity of APM by immunofluorescence microscopy on frozen sections of a variety of mammalian tissues (17, 19). In general, APM stains platelets, vascular endothelial, epithelial (renal, intestinal, tracheal), and connective tissue cells, but not cardiac, skeletal, or visceral smooth muscle or the vascular smooth muscle in muscular arteries, arterioles, or veins (cf. 7, 12). In contrast, however, APM does specifically label the smooth muscle of elastic arteries in all species tested (rat, calf, guinea pig, human).

Antismooth muscle myosin (ASMM) was raised in rabbits against human uterine myosin purified by slight modifications of the protocol cited above (23). In immunodiffusion, ASMM forms a single precipitin line with extracts of human uterus but not with extracts of human platelets or cardiac or skeletal muscle (22). ASMM is also specific for smooth muscle myosin heavy chain (200 kd) as shown by Western blot tests of rat aortic smooth muscle peptides separated by SDS PAGE under reducing conditions (19). In immunofluorescence microscopy on frozen sections, ASMM stains all visceral and vascular smooth muscle tested, but no other cell type, including epithelial and connective tissue cells and skeletal and cardiac muscle (17, 19).

Cultured Cells: Rat mesenteric artery smooth muscle cells were isolated and cultured as previously reported (13). Briefly, male C-D strain Sprague-Dawley rats (225–250 g) were killed by cervical dislocation and the superior mesenteric arterial arcade was aseptically excised. Fat, adventitia, and veins were removed by blunt dissection and the remaining arterial tree was incubated with elastase, collagenase, soybean trypsin inhibitor, and bovine albumin at 37°C in a gyratory shaker bath. After repeated trituration and sieving, the resulting cell suspension was washed by centrifugation and plated in Dulbecco's modified Eagle's medium with 10% calf serum, 2 mM L-glutamine, 25 mM HEPES buffer, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (M. A. Bioproducts, Walkersville, MD). Similar methods were used for production of rat aortic smooth muscle cultures by enzymatic dissociation.

The cells were normally plated at a density of $\sim 5 \times 10^3$ viable cells/cm^2 in plastic culture dishes (Falcon Plastics, Oxnard, CA) or on glass coverslips that had been precoated with human fibronectin (Collaborative Research, Inc., Lexington, MA; 5 μg/cm^2, air-dried from aqueous solution). Fibronectin-coated coverslips were used for increased cell adhesion; this treatment had no apparent effect on growth or staining patterns. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air with thrice weekly media changes.

Explant outgrowth smooth muscle cell cultures were established, following the method reported by Ross (24), on fibronectin-coated coverslips and cultured as described above. A strain of bovine aortic endothelial cells, used for comparison purposes, was originally isolated by collagenase digestion and characterized by standard methods (10).

Indirect Immunofluorescent Staining: Smooth muscle cells on coverslips were rinsed briefly in phosphate-buffered saline (PBS: 0.15 M NaCl, 10 mM Na_2HPO_4, 3 mM NaN_3, pH 7.4), treated with acetone at 4°C (3 × 45 s), and air-dried. Coverslips were used immediately for immunofluorescence or stored, desiccated, at 4°C until use. Storage for at least 2 mo had no appreciable effect on staining intensity or pattern nor did other preparative protocols tested (formalin/Triton X 100; formalin/acetone; methanol/acetone; acetone at −20°C).

Acetone-treated cells on coverslips were incubated for 30 min at 37°C with ASMM, APM, or control sera (see below) at a 1:100 dilution in PBS. They were washed for 15 min (three changes) in PBS, incubated with a 1:100 dilution of goat-anti-rabbit IgG labeled with fluorescein (F-GAR; Miles-Yeda, Ltd., Rehovot, Israel), washed again in PBS, rinsed briefly in deionized water, and mounted on glass slides using glycerol/PBS.

Immunofluorescent staining was examined under epifluorescent illumination (100 W Mercury) on a Leitz Orthoplan microscope with Leitz L-2 filter block. A 63 × Zeiss Planapo (NA 1.4; oil) and a 25 × Zeiss Plan-Neofluar (NA 0.8; oil) objective lens were used for both fluorescence and phase contrast microscopy. Time-exposure micrographs were taken for comparison of relative fluorescence intensities using Kodak Tri-X film pushed to an effective ASA 1200 with Acufine developer (Acufine, Inc., Chicago, IL) and processed under standardized conditions.

As controls for specificity of staining, cells were incubated with (*a*) preimmune or normal rabbit serum (NRS, M. A. Bioproducts) followed by F-GAR; (*b*) no first antibody then F-GAR; (*c*) first antibodies but no F-GAR; (*d*) no antibodies; (*e*) ASMM and APM followed by fluorescein-conjugated normal goat serum; (*f*) ASMM that had been preabsorbed with uterine myosin, then F-GAR; (*g*) APM that had been preabsorbed with platelet myosin, then F-GAR; and (*h*) ASMM and APM followed by F-GAR that had been preabsorbed with NRS. None of these control treatments resulted in positive staining of VSMC in vivo or in vitro.

Immunochemistry: Myosin was metabolically labeled by incubating cultures with [^{35}S]methionine (0.5 mCi in 3 ml medium/100-mm diam culture dish; New England Nuclear, Boston, MA; specific activity 1,000 Ci/mmol) in methionine-free medium (RPMI-1640) with 10% dialyzed (against Dulbecco's PBS) fetal calf serum for 12–18 h at 37°C. Cultures were washed in Hank's balanced salt solution (Ca^{++}- and Mg^{++}-free) at 4°C to remove extracellular label. Cells were scrape-harvested in 0.5 ml of extraction buffer (0.5 M NaCl; 0.5 mM EDTA; 0.5 mM dithiothreitol (DTT); 30 mM Tris, pH 7.5; 0.1 mg/ml BSA (Pentex^R, Miles Laboratories, Naperville, IL); and 0.1 μg/ml phenylmethylsulfonyl fluoride (PMSF); as modified from Scordilis et al. [26]), disrupted in a Dounce homogenizer on ice, and cleared of particulates by centrifugation at 100,000 *g* for 1 h at 4°C. In preliminary experiments, this extraction protocol yielded a mean recovery (supernatant) of 70 ± 17% (SD) of the total cellular myosin (as judged by comparison of supernatant and pellet fractions on quantitative SDS PAGE, as below). Recovery was not significantly greater when 10 mM ATP was added to the extraction buffer. Other unlabeled cell extracts, prepared as above (extraction buffer without dithiothreitol, BSA, or Tris and with 50 mM borate buffer), were radioiodinated for 1 h on ice with monoiodinated Bolton-Hunter reagent (^{125}I-B-H; New England Nuclear, Boston, MA; 2,000 Ci/mmol). After the iodination step, DTT, BSA, and Tris were added to the same final concentration as in the original extraction buffer.

Purified uterine and platelet myosins were iodinated using ^{125}I-B-H in a high salt buffer (0.6 M NaCl; 1 mM dithiothreitol; 0.1 mM $MgCl_2$; 10 mM Imidazole, pH 7.0) at a B-H:myosin molar ratio of 2:1 (0.250 mCi ^{125}I-B-H added to 13 μg myosin at 0.5–1.0 mg/ml).

Myosin, labeled with ^{35}S or ^{125}I, was immunoprecipitated from the cell extracts at room temperature (compare reference 1) using ASMM, APM, and heat-killed, formalin-fixed *Staphylococcus aureus* (15). To reduce nonspecifically precipitating material, purified myosins and cellular extracts were first cleared by a preimmune or normal serum precipitation (0.1 ml serum/0.5 ml extract for 90 min followed by incubation with the packed equivalent of 0.2 ml of a 10% *S. aureus* suspension for 45 min, and centrifugation). The supernatants were divided into three equal aliquots and then reprecipitated with specific antisera (ASMM or APM) or NRS (0.05 ml serum/aliquot for 60 min followed by the packed equivalent of 0.1 ml of *S. aureus* for 30 min). To avoid myosin solubility problems at low ionic strengths, 3 M NaCl was added to all sera to increase the molarity to 0.5 M. The pellets were washed repeatedly by centrifugation: twice with NET buffer (140 mM NaCl; 5 mM EDTA; 50 mM Tris, pH 7.3; 0.02% NaN_3; 0.5% NP40) supplemented with 0.5 M NaCl, twice with NET buffer supplemented with 0.25 M NaCl, and twice with NET buffer. The final pellets were boiled directly in 2 × Laemmli sample buffer for SDS PAGE (16).

Samples were electrophoresed on Laemmli SDS PAGE gels (7–15% linear acrylamide gradient) that were subsequently fixed with trichloroacetic acid/sulfosalicylic acid/methanol (30). [^{35}S]labeled gels were treated with the gel fluor solution En^{3}Hance (New England Nuclear). Dried gels were autoradi-

ographed at −80°C (an intensifying screen was used with ^{125}I) on Kodak X-omat AR film which was developed by an X-omat automatic processor.

Quantitation of Myosin Content of VSMC: Myosin content of VSMC was determined by densitometry of Coomassie Blue stained gels. Freshly isolated, washed pellet cells were suspended in 2 × Laemmli sample buffer, sonicated, and boiled for 3 min. Cultured cells were washed twice with Hank's balanced salt solution (Ca^{++}- and Mg^{++}-free) and scrape-harvested. Pellets of these cells were solubilized in 2 × sample buffer as above.

Samples were run reduced on Laemmli SDS PAGE gels (6% acrylamide), fixed, and stained with Coomassie Blue R-250 (1% with trichloroacetic acid, sulfosalicylic acid in 30% methanol; [30]) and destained in 10% 2-propranol/10% acetic acid. After destaining, gels were incubated in 1% glycerol/10% acetic acid for 30–45 min, and dried, under vacuum, between sheets of cellophane (Bio-Rad Laboratories, Richmond, CA). Dried gels were scanned in an LKB Bromma 2202 Ultroscan laser densitometer. Myosin content (heavy chain only) was quantitated by comparison with a standard curve based on densitometry of known amounts of rabbit skeletal myosin (Sigma M1636, Sigma Chemical Co., St. Louis, MO; additionally purified by precipitation by dialysis against distilled water) run in parallel gel lanes. For comparisons of myosin content at different times in culture, cellular DNA and total protein assays were carried out on parallel cultures or pellets of freshly isolated cells. DNA determinations were made by the technique of Cesarone et al. (3) with 33258 Hoechst dye and protein was determined by the method of Lowry et al. (20). The DNA assay was used as an indication of cell number in these studies. In preliminary experiments on both growing and confluent cultures, the correlation coefficients of linear regression between DNA content and hemacytometer cell counts were >0.9.

RESULTS

Immunofluorescent Staining

As we have reported previously (17, 19), vascular smooth muscle cells in the media of the rat mesenteric artery show specific immunofluorescent staining with ASMM but not with APM (Fig. 1). However, when these cells were isolated by enzymatic dissociation and plated in culture, they rapidly acquired reactivity for the "non-muscle-specific" antiserum, APM. By 5 h in primary culture, all cells stained with both ASMM and APM in a punctate, fibrillar pattern (Fig. 2). These brightly staining particles, which resembled those reported by Gröschel-Stewart et al. (11), were often aligned in linear arrays, arranged circumferentially or radially. After 1–2 d in culture, staining with both antimyosins (Fig. 2) was localized in structures corresponding to stress fibers as visualized by phase contrast microscopy (Fig. 3). These stress fibers were found to stain with either antiserum suggesting co-localization of ASMM and APM reactivity within these microfilament bundles. Preimmune sera, normal rabbit serum, and preabsorbed antisera (see Materials and Methods) did not stain any of these cultured VSMC. In addition, as a cellular control for antisera specificity and fixation artifacts, we examined cultured bovine aortic endothelial cells using ASMM and APM. The staining of these cells retained the staining pattern seen in vivo (Fig. 1): staining with APM was bright whereas ASMM did not stain (Fig. 4*D*).

Cells in subconfluent primary cultures stained brightly and specifically with both ASMM and APM at all times examined, as illustrated in Fig. 2. However, at confluence, most of the cells showed reduced staining with ASMM (although still in a specific, fibrillar pattern), while only a small proportion retained the previous bright labeling (Fig. 2), thus giving rise to a "mosaic" appearance. In contrast, the reaction with APM continued to be bright, specific, and fibrillar in all cells (Fig. 2). The same temporal sequence was also seen in primary cultures of VSMC obtained by enzymatic dissociation of rat aorta. In addition, primary cultures of bovine and canine coronary artery VSMC, isolated by the same method, showed a similar staining pattern (Larson, D., P. Miao, and P. Libby, unpublished observations).

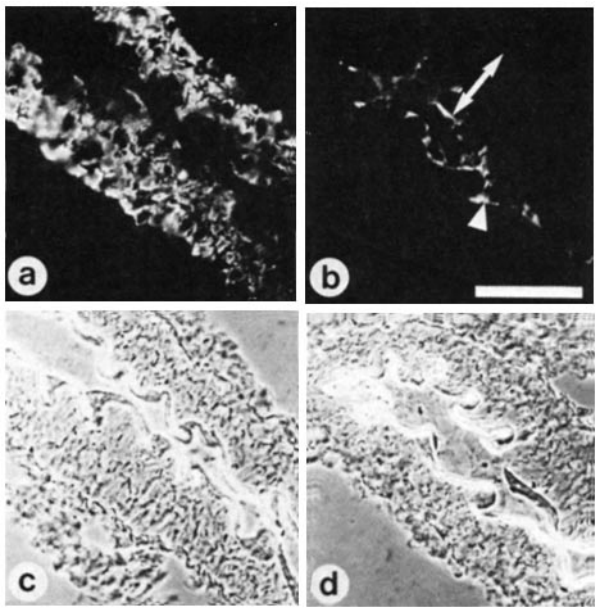

FIGURE 1 Immunofluorescence micrographs of myosin staining in frozen sections (3 μm) of rat mesenteric artery. (*a*) ASMM staining in medial smooth muscle cells; (*b*) APM staining of intimal endothelial cells (arrowhead) but not medial smooth muscle cells (double arrow indicates width of medial layer); (*c*) phase-contrast micrograph of *a*; (*d*) phase-contrast micrograph of *b*. Bar, 50 μm; × 280.

In mesenteric artery VSMC, the shift to the mosaic staining pattern at confluence was independent of plating density and hence time in vitro. These cells begin to show mitotic activity at ~60 h in culture. When we examined cultures that became confluent after one population doubling (at 4 d), the staining pattern was indistinguishable from that in cultures reaching confluence after approximately six population doublings (10 d).

After a given primary culture of rat mesenteric artery or aortic VSMC reached confluence, the staining pattern described above was retained throughout postconfluent growth and persisted in subsequent subcultures. In addition, when cultures were prepared by the explant-outgrowth technique, bovine aortic (Fig. 4*A*), rat aortic (Fig. 4*B*), and rat mesenteric artery (Fig. 4*C*) VSMC displayed a similar staining pattern, viz., uniformly intense staining with APM in all cells, specific but reduced staining with ASMM in most cells, and bright staining with ASMM only in occasional cells. In fact, the initial halo of cells migrating from explanted fragments of rat aortic media showed the same staining pattern.

Immunochemistry

The change in myosin antigenic expression (as detected by immunofluorescent staining) from the in vivo to in vitro states in rat mesenteric artery VSMC, and the variable antigenic expression of myosin in cultured VSMC, suggested that an alteration was occurring in this cytoskeletal component as the cells became adapted to culture conditions. To further define these culture-related changes, we used immunochemical methods to better characterize the myosins expressed by cultured VSMC.

When high-salt extracts of subconfluent or confluent, metabolically-labeled, rat mesenteric artery VSMC cultures were immunoprecipitated with ASMM or APM, each antiserum

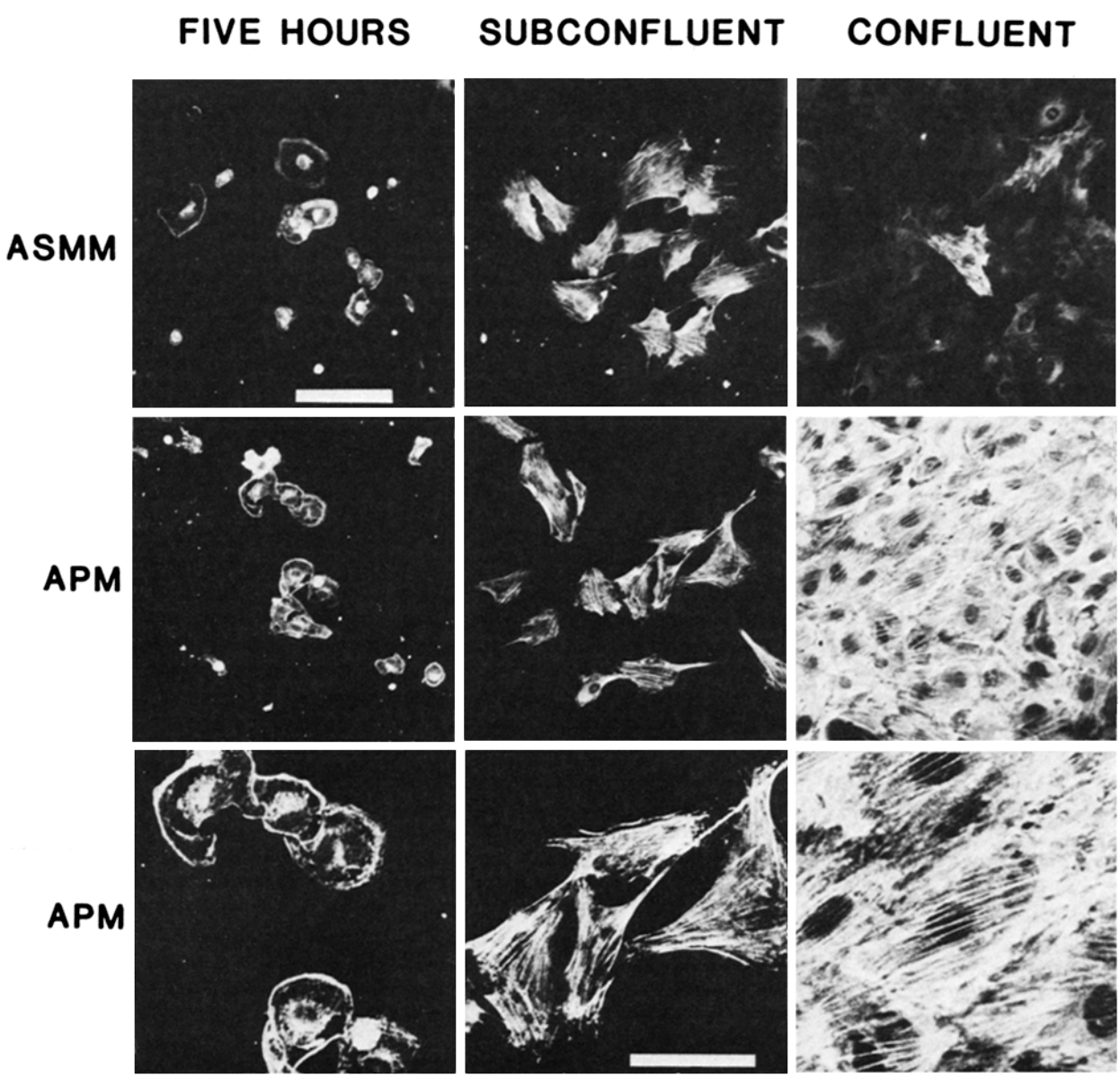

FIGURE 2 Immunofluorescence micrographs of myosin staining in cultured, enzymatic dissociation, rat mesenteric artery smooth muscle cells using ASMM (*top*) APM (*middle* and *bottom*) at 5 h in culture, in subconfluent culture, and in confluent culture. Bar, 100 μm; × 140. The bottom row contains higher magnification micrographs of APM staining. Bar, 50 μm; × 380. These photographs reflect the relative fluorescence intensities observed microscopically.

precipitated myosin selectively (Fig. 5). The myosin recognized by both antisera ran as one heavy chain (M_r = 200 kd) and two light chains (M_r = 17 and 20 kd) of similar mobilities on SDS PAGE. Treatment, in parallel, of aliquots of the culture extracts with control NRS did not result in precipitation of detectable myosin. A similar result was obtained with a nonimmunological precipitation of myosin from these high-salt extracts using purified rabbit skeletal muscle or chicken gizzard F-actin (prepared according to the method of Spudich and Watt [28]). F-actin precipitated myosin of the same mobility on SDS PAGE in the absence but not in the presence of 10 mM ATP (data not shown). The above experiments relied on protein synthesis for radiolabeling and thus would not detect any myosin that was carried over from before the labeling period. Therefore, we extracted unlabeled rat mesenteric artery VSMC cultures, radioiodinated all of the proteins in these extracts using ^{125}I-Bolton-Hunter reagent, and then immunoprecipitated with ASMM and APM. This approach gave essentially the same results as obtained with [^{35}S]methionine-labeled cells, one myosin heavy chain, and two light chains of the mobilities indicated above on SDS PAGE (data not shown).

To determine whether the antisera used in this study could distinguish between different myosins in solution, we initially tested them against radioiodinated purified uterine and platelet myosins (the original immunogens) in an exhaustive immunoprecipitation protocol (Fig. 6*A*). Each myosin preparation was repeatedly precipitated with one antiserum until no further myosin was removed from solution (Fig. 6*A*; lanes *1–4*). The resultant supernatant was then reacted with the other antiserum (lane *5*). Note that in this protocol, the critical tests for antigenic cross-reactivity are the cross-over precipitations (lane *5*). Using this technique, we determined that each antiserum could exhaustively precipitate its original immunogen.

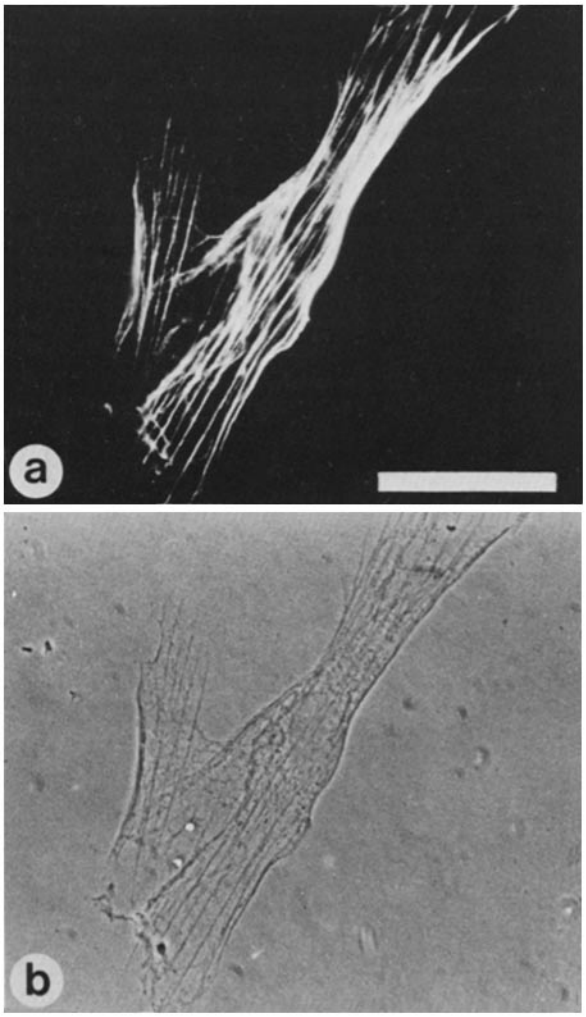

FIGURE 3 Immunofluorescence (a) and parallel phase-contrast (b) micrographs of cultured rat mesenteric artery smooth muscle cells. Note the co-linearity of stained (ASMM + F-GAR) and phase-dense stress fibers. Bar, 50 μm; × 480.

Thus, APM exhaustively precipitated platelet myosin (ASMM, in lane *5*, did not precipitate myosin after four serial precipitations with APM) but not uterine myosin. Similarly, ASMM exhaustively precipitated uterine myosin but not platelet myosin. Presumably, the reactivity of APM with uterine (myometrial) myosin (lane *1*) is due to the presence of nonmuscle cell myosin in this heterogeneous tissue preparation (2, 22).

To test the immunoreactivity of myosin in the tissue used for the VSMC cultures, rat mesenteric artery segments (dissected free of adventitia) were homogenized in extraction buffer, and the extracted proteins radioiodinated and subjected to serial immunoprecipitation. Although the cellular heterogeneity of this tissue (endothelium, fibroblasts, residual blood cells, in addition to VSMC) makes definitive interpretation of the results difficult, the great majority of cells in the sample were smooth muscle (vessel segments chosen were of the same size as those shown in Fig. 1). Interestingly, however, >50% of the extracted myosin was precipitated by APM, thus suggesting the presence of APM determinants in VSMC in situ (data not shown).

In contrast to the results obtained with the original immunogens and with myosin extracted from the vessel wall, when similar tests were performed with high-salt extracts of [^{35}S]-methionine-labeled cultured rat mesenteric artery VSMC (Fig. 6*B*), both ASMM and APM exhaustively precipitated the metabolically-labeled myosin (lanes *1–4*), so that no additional myosin was precipitated by subsequent incubations with the other antiserum (lanes *5*). This result was obtained with myosin extracted from both subconfluent and confluent VSMC cultures (Fig. 6*B*).

Myosin Content of Cultured VSMC

To determine whether the myosin content of VSMC was altered during the course of primary culture, we measured myosin in these cells by quantitative SDS PAGE and gel scanning densitometry. To provide an estimate of the amount

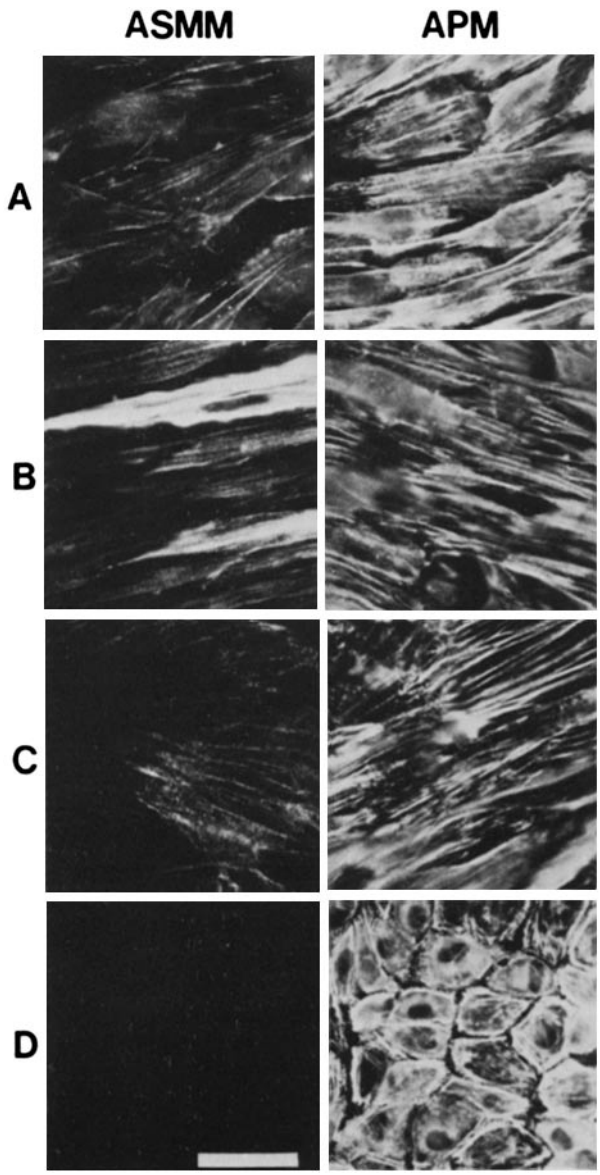

FIGURE 4 Immunofluorescence micrographs of myosin staining. (*A–C*) Confluent primary, explant-outgrowth, vascular smooth muscle cultures: (*A*) bovine aortic; (*B*) rat aortic; (*C*) rat mesenteric artery. Note uniform staining with APM and mosaic pattern with ASMM. (*D*) Bovine aortic endothelial cell culture. Note staining with APM but not with ASMM. *Bar*, 50 μm; × 260.

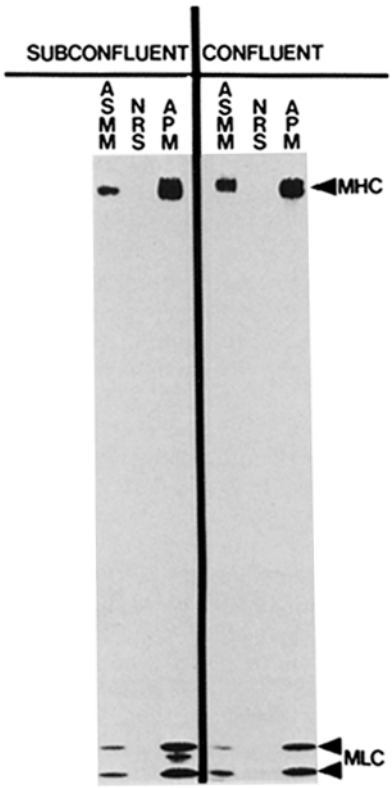

FIGURE 5 Gel autoradiography of immunoprecipitates from high-salt extracts of subconfluent and confluent rat mesenteric artery smooth muscle cultures. Metabolically labeled myosin was precipitated using ASMM or APM and run reduced on SDS PAGE as described in the text. (Markers from parallel Coomassie Blue-stained lanes: *MHC*, myosin heavy chain, M_r = 200,000 mol wt; *MLC*, myosin light chains, M_r = 20,000 and 17,000.) Lanes labeled *NRS* are parallel precipitations with normal rabbit serum. The band migrating between the 17,000- and 20,000-mol-wt MLC (subconfluent, APM) was presumed to be a partial proteolytic product of the 20,000-mol-wt MLC since it varied in quantity from experiment to experiment and with the concentration of phenylmethylsulfonyl fluoride used. A similar band was also seen with ASMM precipitation and from confluent cultures in other experiments.

of myosin present in VSMC in vivo, we used freshly isolated cells harvested by the enzymatic dissociation protocol. Cultured cells were prepared for gel samples at subconfluent (in log-phase growth) and confluent densities, in parallel with the cultures used for the immunofluorescence and immunochemical studies described above. Multiple samples of cells, dissolved in sample buffer, were run on SDS PAGE gels for each culture time point. For each gel, a standard curve was constructed using known amounts of rabbit skeletal myosin. Scanning densitometry of the myosin heavy chain peaks in these standards yielded correlation coefficients of linear regression of 0.986–0.997. Interpolation on these standard curves then provided values for the myosin content of cell samples run in parallel lanes. These data, normalized for DNA to permit comparison at different time points, are presented in Table I. Cellular myosin content in some subconfluent cultures tended to be decreased from the baseline value calculated for freshly isolated cells, although the overall difference in the mean values is not statistically significant ($P > 0.5$). In contrast, total protein DNA ratios were increased in both subconfluent and confluent cultures compared with freshly isolated cells, consistent with cellular hypertrophy (13).

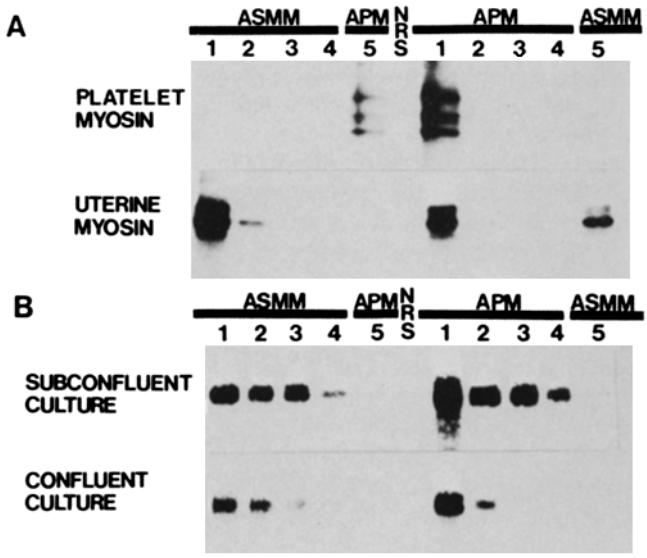

FIGURE 6 Gel autoradiography of serial immunoprecipitations of radiolabeled myosins. (*A*) Precipitations of radioiodinated purified platelet and uterine myosins; (*B*) Precipitations of metabolically labeled ([^{35}S]methionine) myosin from high-salt extracts of subconfluent and confluent rat mesenteric artery smooth muscle cultures. Each experimental sample was divided into three equal portions. One was subjected to a control precipitation with normal rabbit serum (*NRS*). The remaining two were serially precipitated with ASMM or APM as noted (lanes *1–4*). The resultant supernatants in each case were then reacted with the contrasting antimyosin as noted (lanes *5*). Only those parts of the autoradiograms containing the 200,000-mol-wt myosin heavy chain are shown. Note that partial degradation products of platelet myosin heavy chain are also immunoprecipitated.

TABLE I

Total Protein and Myosin Content of Freshly Isolated and Cultured Rat Mesenteric Artery VSMC

Cell preparations* (*n*)	Total protein‡/DNA§ (± SEM)	Myosin‖/DNA§ (± SEM)
Freshly isolated (6)	5.5 ± 0.3	0.30 ± 0.06**
Subconfluent culture (5)	25.2 ± 6.0¶‡‡	0.10 ± 0.05**§§
Confluent culture (5)	20.6 ± 2.6¶‡‡	0.13 ± 0.05**§§

* Freshly isolated cells were collected at the end of the enzymatic dissociation protocol. Cells were plated at a standard density (≃5 × 10^3 viable cells/cm²) to achieve confluence at 6–8 d (5.9 ± 1.6 (SEM) × 10^4 cells/cm²). Subconfluent cultures were harvested during log-phase growth after 4 d in culture (2.3 ± 0.6 (SEM) × 10^4 cells/cm²). *n*, number of preparations.

‡ Total protein was determined by the method of Lowry et al. (20) and is expressed in weight/weight ratios with DNA for comparison purposes.

§ DNA was determined by the method of Cesarone et al. (3). DNA content was proportional to cell number (see Materials and Methods) and averaged 10.7 ± 2.4 pg per cell.

‖ Myosin content was determined by SDS PAGE and scanning densitometry and is expressed in weight/weight ratios with DNA for comparison purposes. Standard curves were contructed for each gel from scanning densitometry of myosin heavy chain bands in parallel lanes containing known quantities of rabbit skeletal myosin. Values for the mass of VSMC myosin were determined by interpolation on these curves assuming, as a first order approximation, that the quantities of heavy chains were proportional to total myosin.

¶ Different from freshly isolated cells (Fisher's t distribution for unpaired variates; $P < 0.01$).

**,‡‡,§§ No significant difference (at $P = 0.05$) between values indicated by same symbol.

DISCUSSION

The purpose of this study was to characterize the antigenic expression of myosin in cultured rat mesenteric artery VSMC, cells which retain vasoactive hormone receptors and other differentiated properties, including contractility, in vitro (13). We have demonstrated several changes in myosin antigenic expression during the course of primary culture. First, the selective staining with ASMM expressed by medial VSMC (Fig. 1) in the rat mesenteric artry in situ was rapidly altered in vitro by the acquisition of staining with APM (Fig. 2). This dual staining was retained throughout growth to confluence (Fig. 2). Second, the majority of immunofluorescently demonstrable myosin in cultured cells (Figs. 2 and 3) was associated with punctate arrays and stress fibers, not the thick filaments typically seen in vivo (13, 27). Finally, ASMM staining was greatly reduced in intensity in most cells at confluence, while APM staining (Fig. 2) and total myosin content was unchanged (Table I).

Thus, we detected two major shifts in myosin immunofluorescent staining in culture: a rapid appearance of APM staining following the initiation of culture and a diminution of ASMM staining at confluence. There are several potential explanations for the de novo appearance of APM staining (Fig. 2). One possibility is that the cultured VSMC make new myosin isoforms with different immunoreactivities. This explanation seems unlikely for two reasons. First, preliminary experiments, in which the freshly isolated VSMC were plated and cultured for 6 h in the presence of 0.1 mg/ml cycloheximide (Sigma Chemical Co.; resulting in >95% inhibition of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material), revealed that the acquisition of APM staining was not dependent on protein synthesis (D. M. Larson, unpublished observations). Hence, APM staining was unlikely to be due to de novo expression of a different myosin isoform. Second, the complete cross-reactivity of ASMM and APM in immunoprecipitating myosins extracted from the cultures (Fig. 6*B*) suggested that these antisera recognized the same molecular species in solution. This conclusion is reinforced by the results obtained with confluent cultures. Since confluent VSMC stained heavily with APM (Fig. 2) and lightly with ASMM, discrimination between the stained species should be greatest at this point in culture. However, this difference was not reflected in the immunoprecipitation of soluble, extracted myosin (Fig. 6*B*).[2]

The most plausible explanation for the appearance of APM staining in the cultured cells is that APM-reactive antigenic determinants were present in situ, but masked. Two lines of evidence support this interpretation. First, the cycloheximide experiments indicated that protein synthesis was not required for APM reactivity. Second, the immunoprecipitation studies on myosin extracted from the rat mesenteric artery suggested that >50% of the precipitable myosin could be recognized by APM. Although the vessel preparations contained both smooth muscle and nonmuscle cells (see Fig. 1), the quantity of myosin precipitated by APM strongly suggested the presence of APM determinants in the smooth muscle cells in situ.

This postulated masking of antigenic determinants might reflect differences in macromolecular assemblages of myosin in situ and in vitro (e.g., thick filaments vs. stress fibers). Such differences would presumably be abolished under dissociative conditions in solution, allowing the complete cross-reactivity seen in the serial immunoprecipitations from the cultured cells. Whether the differences in immunoreactivity observed in this study are related to the recently reported salt- and phosphorylation-dependent conformational alterations in solubilized smooth muscle myosin monomers, from both gizzard (e.g., reference 14) and vascular (29) sources, remains to be studied.

The other major alteration in myosin antigenic expression detected during the course of primary culture was the diminution of ASMM staining at confluence. This shift appeared to be a cell contact or density-related phenomenon, since neither the time elapsed in growth to confluence nor cumulative population doublings were significant factors. It is unclear what effects cell contact might have on the expression of cytoskeletal elements in this system. Certainly, there was not a concomitant dramatic reduction in cellular myosin content at confluence, based both on the quantitative gel analyses (Table I) and on the observation that APM staining remained uniform in intensity in all cells (Fig. 2). In addition, confluent cultures of rat mesenteric artery VSMC can phosphorylate the 20 kd myosin light chain (1) and a substantial number of these cells can contract in response to physiological doses of angiotensin II (13). The lack of direct correlation between ASMM-specific staining, phosphorylation, and cellular contraction suggests that myosin in certain altered antigenic states is still capable of functional interactions with actin in this in vitro system. Whether the myosin must somehow be mobilized or shifted to a different conformation for this activity to occur is an interesting possibility. If this hypothesis is correct, then it indicates that the functional defect(s) in the characteristically noncontractile VSMC cultures produced by explant-outgrowth (which share the same myosin staining pattern as confluent enzyme-dissociation VSMC; Figs. 2 and 4*A–C*) may be a more proximal point in the cellular contractile system, such as a lack of specific agonist receptors (compare with reference 13) or receptor-activation-response coupling.

Chamley-Campbell and co-workers (4–6) have used rabbit antisera raised against gizzard smooth muscle myosin to study myosin expression in cultured rabbit, human, and monkey VSMC. In their experience, cultured VSMC characteristically lose smooth muscle specific myosin staining at the onset of cell division and only variably regain it at confluency (depending on initial plating density). These observations formed one of the bases for their definition of "phenotypic modulation." In contrast, we have observed that ASMM staining in rat, bovine, and canine VSMC was retained at all times in culture and not lost during log-phase growth. In addition, our results indicated that there was no quantitative reduction in total cellular myosin in culture, but rather a shift in antigenic reactivity that was independent of plating density. The reasons for the discrepancies between our results and those reported by Chamley-Campbell et al. are unclear, but may be related to the differences in species and antisera employed. Nonetheless, our observations clearly indicate that changes in myosin

[2] Since the extraction protocol used in the immunoprecipitation studies did not quantitatively solubilize the total cellular myosin (as seen in preliminary studies on confluent cultures), it is conceivable that a subpopulation of myosin, with a different antigenic reactivity, was not represented in solution. However, the wide variability in the proportion of extractable myosin (50–82%) obtained under controlled conditions, and the lack of any detectable heterogeneity in the immunoprecipitation results, suggest that there was not, at least, any clearly defined insoluble subpopulation of myosin in these cells.

antigenic expression are not necessarily a simple monitor of the state of "phenotypic modulation" in cultured vascular smooth muscle cells.

Currently, there is much interest in alterations in contractile and cytoskeletal proteins in various pathological processes (25) and, in particular, in the modulation of intermediate filament protein expression in vascular smooth muscle in experimental models of atherosclerosis (9, 21). In the case of myosin expression in vivo, it is interesting that human aortic intimal VSMC, in both diffuse intimal thickening and well developed fibromuscular plaques, can be stained with both ASMM and APM (unpublished observations; [19]). Such cells have been considered to be an in vivo counterpart of phenotypically modulated cultured VSMC (5, 6, 25). Further understanding of the factors regulating the expression of antigenically altered states of myosin may help to better define the problem of "phenotypic modulation" in cultured VSMC, and also provide useful information on the cell biology and pathology of vascular smooth muscle cells in vivo.

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