Expression of Smooth Muscle–specific α-Isoactin in Cultured Vascular Smooth Muscle Cells: Relationship between Growth and Cytodifferentiation

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Abstract. The relationship between growth and cyto-differentiation was studied in cultured rat aortic smooth muscle cells (SMCs) using expression of the smooth muscle (SM)-specific isoactins (Vanderkerck-hove, J., and K. Weber, 1979, *Differentiation*, 14:123–133) as a marker for differentiation in these cells. Isoactin expression was evaluated by: (*a*) measurements of fractional isoactin content and synthesis ([^{35}S]methionine incorporation) by densitometric evaluation of two-dimensional isoelectric focusing sodium dodecyl sulfate gels, and (*b*) immunocytological examination using SM-specific isoactin antibodies. Results showed the following: (*a*) Loss of α-SM isoactin was not a prerequisite for initiation of cellular proliferation in primary cultures of rat aortic SMCs. (*b*) α-SM isoactin synthesis and content were low in subconfluent log phase growth cells but increased nearly threefold in density-arrested postconfluent cells. Conversely, β-nonmuscle actin synthesis and content were higher in rapidly dividing subconfluent cultures than in quiescent postconfluent cultures. These changes were observed in primary and subpassaged cultures. (*c*) α-SM actin synthesis was increased by growth arrest of sparse cultures in serum-free medium (SFM; Libby, P., and K. V. O'Brien, 1983, *J. Cell. Physiol.*, 115:217–223) but reached levels equivalent to density-arrested cells only after extended periods in SFM (i.e., >5 d). (*d*) SFM did not further augment α-SM actin synthesis in postconfluent SMC cultures. (*e*) Serum stimulation of cells that had been growth-arrested in SFM resulted in a dramatic decrease in α-SM actin synthesis that preceded the onset of cellular proliferation. These findings demonstrate that cultured vascular SMCs undergo differential expression of isoactins in relation to their growth state and indicate that growth arrest promotes cytodifferentiation in these cells.

S TUDIES by Chamley-Campbell et al. (2, 4) and others (37, 41) demonstrate that vascular smooth muscle cells (SMCs)[1] undergo marked changes in phenotype when grown in cell culture. These changes include diminished contractile filaments and contractile proteins, loss of contractility, alterations in biosynthetic properties, and changes in sensitivity to mitogens. Based on observations that most cells did not proliferate in cell culture until these changes occurred, Chamley-Campbell et al. (2) proposed that cells normally exist in a nonproliferating contractile state and must modulate to a synthetic state before proliferating. Furthermore, they proposed that established SMC cultures remain permanently in a synthetic state. However, recent studies demonstrate that established cultures of SMCs continue to express smooth muscle (SM)-specific contractile proteins (10, 13, 20), retain high affinity receptors to various agonists (5, 18), and undergo

agonist-induced changes in membrane conductance (5, 24). Whereas these studies demonstrate that cultured SMCs continue to express a variety of differentiated characteristics, they did not specifically address how growth and cytodifferentiation were related. An understanding of this relationship may have profound implications for investigators interested in studying various aspects of SMC contractile function in cultured cells, as well as in studies of growth control. It is not known, for example, whether present methods for inducing quiescence in cultured SMCs (e.g., serum-free medium [SFM, 22], plasma-derived serum [33]) are associated with increased expression of cell-specific proteins indicative of entrance into a true G$_0$ state.

A major limitation in previous studies of cytodifferentiation in SMCs was the lack of a sensitive, easily measured, quantitative means of assessing their state of differentiation. The contractile proteins are logical candidates for a group of cell type–specific proteins whose synthesis might be coordinately regulated during growth and differentiation. This is true in skeletal muscle, where cessation of myoblast proliferation and

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subsequent differentiation is characterized by a large increase in myosin synthesis, and a switch in actin synthesis from the nonmuscle isoactins (i.e., β- and γ-nonmuscle [NM]), to the skeletal α-isoactin form that makes up nearly all of the actin in fully differentiated skeletal muscle cells (6). Similar alterations in expression of muscle and nonmuscle actins appear to occur during differentiation of smooth muscle (12, 29, 34). Based on amino acid sequence analysis, Vandekerckhove and Weber (38) demonstrated that SMCs express four different actin polypeptides or isoactins, each representing a different gene product. These include two SM- specific isoactins (α-SM and γ-SM; note that α-SM is distinct from the α-isoactin found in skeletal and cardiac muscle) and two isoactins expressed in nonmuscle cells (β-NM and γ-NM), where the α-, β-, and γ- designate variants distinguishable on the basis of their isoelectric points. In developing smooth muscle tissues nonmuscle actins predominate, whereas more than 70% of the actin in differentiated smooth muscle is of the α-SM and γ-SM types (39). The ratio of α-SM to γ-SM varies between different smooth muscle tissues (9). In rat aortic smooth muscle ~94% of the smooth muscle–specific actin is of the α-SM type (39).

In the present investigation, we explored the interrelationship between cytodifferentiation and growth in cultured rat aortic SMCs by using expression of α-SM actin as a marker for differentiation in these cells. Our specific objectives were: (a) to determine whether loss of α-SM actin was a prerequisite for initiation of proliferation in primary vascular SMC cultures, (b) to determine the time-course of changes in isoactin expression in relation to growth state in both primary and subpassaged SMC cultures, and (c) to explore whether growth arrest of cells in a defined serum-free medium (22) is associated with increased expression of α-SM isoactin.

Materials and Methods

Cell Culture

Rat thoracic aortic SMCs were isolated and cultured by a modification of the procedures described by Chamley-Campbell et al. (2). Male Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottdale, PA) weighing ~200-225 g were killed by CO_2 asphyxia, and the thoracic aorta from the descending thoracic aorta to the diaphragm aseptically excised and placed in Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY). Adhering fat and connective tissue were removed by blunt dissection. Vessels were then opened longitudinally and preincubated in HBSS containing 1 mg/ml collagenase (type CLS II, 146 U/mg, Worthington Biochemical Corp., Freehold, NJ), 0.5 mg/ml elastase (type I, 32 U/mg, Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 μg/ml) for 15-20 min at 37°C, in 95% air/5% CO_2. The adventitia was carefully removed under a dissecting microscope and the luminal surface scraped with forceps to remove endothelial cells. After dissection, aortas were placed in fresh enzyme solution, minced into 1-mm pieces, and incubated (37°C, 95% O_2, 5% CO_2) for an additional 1.5-2.0 h with tituration at 30-min intervals. The resulting cell-tissue suspension was filtered through a 85-μm stainless steel mesh. The filtrate was collected and 20% fetal calf serum (FCS, Gibco) added to inactivate enzymes. Cells were centrifuged (113 g, 8 min) and the cell pellet resuspended in medium-199 (Gibco) containing 10% FCS plus the above antibiotics. Cells were counted in triplicate in a hemocytometer, and cell viability assessed by trypan blue exclusion. Cells were then seeded at a density of 1.0×10^4 viable cells cm^{-2} in plastic culture dishes (Corning Glass Works, Corning, NY).

Cells were harvested for passaging at 2-wk intervals with a trypsin (0.01%, VMF, Worthington Biochemical Corp.), EDTA (0.02%, Sigma Chemical Co.) solution and plated at 10^4 cells cm^{-2}. Passaged cells were grown in either medium-199 containing 10% FCS plus antibiotics or in SFM (22) with or without 10% FCS supplementation depending on the experiment. SFM contained equal parts of Dulbecco's modified Eagle's medium (Gibco) and Ham's F-12 (Gibco) supplemented with insulin (10^{-6} M, Sigma Chemical Co.), trans-

ferrin (5 μg/ml, Sigma Chemical Co.), ascorbate (0.2 mM, Sigma Chemical Co.), and antibiotics. This SFM has been shown to maintain SMCs in a quiescent, noncatabolic, viable state for extended time periods (22). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air with media changes three times weekly.

Growth Curves

Cultures were washed, trypsinized, and monodispersions counted in a hemocytometer with four counts for each dish and triplicate dishes for each sample point.

[³H]Thymidine Autoradiography

Cells were pulse labeled for 1 h with [³H]thymidine (1 μCi/ml, 6.7 Ci/mmol, New England Nuclear, Boston, MA) in regular culture medium. Cultures were then washed twice with HBSS, fixed in 2% paraformaldehyde in HBSS for 5 min, dehydrated, coated with Kodak NTB$_2$ emulsion (diluted 1:1 with distilled water), and exposed for 7 d at 4°C. Dishes were developed in D-19 (Eastman Kodak Co., Rochester, NY), fixed with Rapid-Fix (Eastman Kodak Co.), and stained with hematoxylin. The percentage of labeled nuclei was determined by counting a random sample of at least 2,000 cells from each dish. Duplicate dishes were analyzed for each sample point.

For continuous labeling experiments, cells were pulsed at 24-h intervals with 0.01 μCi/ml [³H]thymidine. To ensure that the labeled thymidine was not degraded and could label cells over the entire 24-h pulse period, [³H]thymidine-containing medium was removed from cells at the end of a 24-h labeling period and placed on fresh cells for 1 h. Labeled cells were observed, indicating that labeled thymidine was biologically active. Growth curves of SMCs grown with and without 0.01 μCi/ml [³H]thymidine were not different, indicating that this dose of [³H]thymidine did not itself influence cell growth.

Antibody Staining

The specificity of the SM isoactin monoclonal antibody (designated CGA7) used in these studies has been reported previously (16). This CGA7 antibody is specific for the α-SM and γ-SM isoactins, but does not react with either cardiac or skeletal muscle α-isoactins or with β- and γ-NM isoactins.

Cells for combined immunocytological staining and autoradiography were grown in Bellco microslide culture chambers (Bellco Glass, Inc., Vineland, NJ) containing plastic slides cut from our regular culture dishes as a growth substrate. This facilitated immunocytological staining without altering cellular growth properties. At designated times cells were rinsed briefly with HBSS and fixed in methanol (4°C, three changes over 5 min). Slides were air-dried and stored desiccated at 4°C until used.

Antibody staining was done using an avidin–biotin–peroxidase procedure (Vectastain, Vector Laboratories, Burlingame, CA). Stained cells were examined by light microscopy and then processed for autoradiography as described elsewhere.

The following controls for specificity of staining were done: (a) substitution of the primary antibody with nonimmune mouse serum or with an antineurofilament monoclonal antibody; (b) exclusion of both antibodies; or (c) exclusion of the primary antibody alone.

Measurements of Contractile Proteins

Sample Preparation. Cells were removed from culture dishes by trypsinization, centrifuged (120 g, 10 min), washed with phosphate–buffered saline (PBS), recentrifuged, and cell pellets frozen immediately in a dry ice-acetone slush and stored at -80°C until assayed. The samples were then thawed and solubilized at 10^3-10^4 cells /μl in isoelectric focusing (IEF) medium as described by Driska et al. (7).

Isoactin Determinations: Two-dimensional Gel Electrophoresis. A modification of O'Farrell's (27) two-dimensional IEF/SDS electrophoretic technique described by Fatigati and Murphy (9) was used for resolving isoactins. This method uses a pH gradient of 4.0–6.5 (Pharmalyte, Pharmacia Fine Chemicals, Piscataway, NJ). Samples contained 3–20 μg of protein (23). After completion of electrophoresis in the second dimension, gels were either silver stained (26) and dried between two sheets of cellophane (40) for autoradiography, or stained with Coomassie Blue (8) for densitometric protein determinations. Identification of actins was done by: (a) co-electrophoresing our samples with purified skeletal muscle actin or endothelial cell lysates; (b) immunoblotting with actin antibodies; and (c) comparison to published results of others (9, 38).

Coomassie Blue–stained gels were scanned with a pinpoint light source at a wavelength of 525 nm as described by Fatigati and Murphy (9) for determinations of fractional isoactin contents. A Quick Scan Jr. densitometer, custom modified with high resolution optics (Helena Laboratories, Beaumont, TX),

was used. Multiple scans in both electrophoretic dimensions were made to determine maximal optical densities. The method was reproducible with repeated measurements on the same sample (i.e., the ratio of the standard deviation to the mean was 8.4%) and was linear over the range of protein loadings used.

Fractional Incorporation of [³⁵S]Methionine into Isoactins. Cells were pulsed with [³⁵S]methionine (986–1,103 Ci/mmol, New England Nuclear) for either 4 h (80 μCi/ml) or for 12 h (40 μCi/ml) depending on the experiment. Samples containing 10,000–60,000 trichloroacetic acid–precipitable cpm were applied per IEF gel and the isoactins resolved as described above. Kodak X-OMAT AR or K film, with exposure times of 24 h or 1–2 wk, respectively, was used and the autoradiographs scanned with an Optronics P-1000 densitometer. The fractional [³⁵S]methionine incorporation into each isoactin was quantitated using a nonlinear least squares Gaussian curve fitting algorithm described by Garrison and Johnson (14). Repeated evaluations of the same sample (including the use of both types of film) showed a standard deviation that was less than 5% of the mean.

Immunoblot Analyses of Isoactins. Isoactins were resolved by IEF/SDS gel electrophoresis as described above, transferred to nitrocellulose paper, and immunoblot analyses done as described by Gown et al. (16). Primary antibody titers used were between 2.5 and 20.0 μg protein ml⁻¹. Controls included exclusion of primary antibodies or substitution of primary antibody with an antineurofilament monoclonal antibody. The following actin antibodies were used: (*a*) CGA7, an SM-specific isoactin monoclonal antibody (16) (a gift from A. Gown, University of Washington); (*b*) B4, an SM-specific isoactin monoclonal antibody (21) (a gift from J. Lessard, University of Cincinnati); and (*c*) C4, a monoclonal that reacts with all isoactins (21) (also from J. Lessard).

Results

Cell Growth in Primary Vascular Smooth Muscle Cultures

A representative growth curve of rat aortic SMCs in primary culture is presented in Fig. 1. Cell viability, determined by trypan blue exclusion, was >95% in freshly dispersed cells. Plating efficiencies were between 40 and 60%. After plating, attached cells began to spread within 24–36 h and initiated DNA synthesis within 24 and 48 h after plating. (Fig. 3 *a*). Cells grew logarithmically for a period of 6–7 d during which the doubling time was ~1.8 d. Cells typically became confluent by 6–7 d and reached saturation density (1.25 × 10⁵ cells cm⁻²) by 9–11 d. The time to onset of growth, doubling times, and saturation densities were very similar between different primary culture preparations. Cells grew in the typical hill and valley pattern characteristic of SMCs and formed multiple cell layers based on both light and electron microscopic examination.
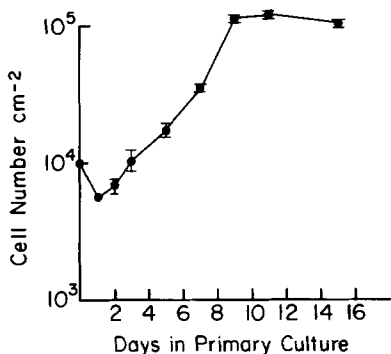
Figure 1. Growth curve of rat aortic SMCs in primary culture. Cells were grown in medium 199 + 10% FCS. Initial plating density was 10⁴ cells cm⁻². Cells were harvested using a trypsin-EDTA solution and counted in a hemocytometer. Each point represents the mean ± SEM from at least three dishes. At several points the SEM is less than the radius of the point and is not visible.
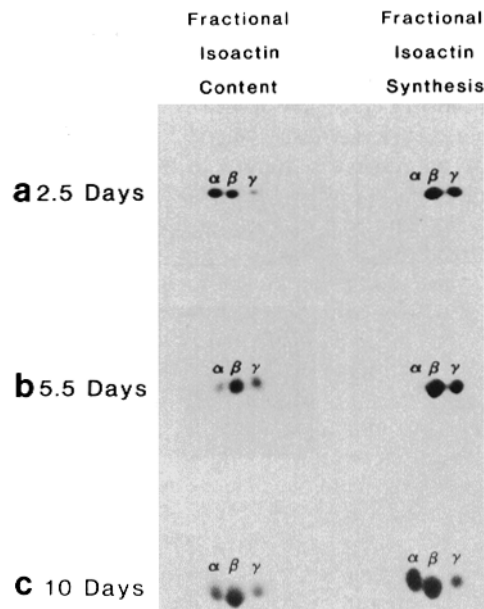

Figure 2. Two-dimensional gels showing the fractional content (Coomassie Blue–stained gel) and synthesis (autoradiographs) of isoactins in rat aortic SMCs at 2.5 d, 5.5 d, and 10 d in primary cell culture. Duplicate culture dishes were pulsed with [³⁵S]methionine (4 h, 80 μCi/ml). Samples were harvested, pooled, and isoactins resolved by two-dimensional IEF/SDS gel electrophoresis. Pooling of samples from replicate dishes was necessary to have sufficient sample for analysis. The total radioactivity loaded on each gel was approximately the same. Note that relative α-SM actin content was high but its synthesis extremely low in cells at 2.5 d. At 5.5 d, α-SM actin synthesis and content were low. At 10 d, cells were postconfluent and both α-SM actin content and synthesis were increased compared with earlier times in culture. Results of densitometric evaluations are shown in Fig. 3.

Changes in Isoactin Content and Synthesis in Relation to Cellular Growth in Primary Culture

Three isoelectric variants of actin were identified in cells by two-dimensional IEF/SDS gel electrophoresis (Fig. 2). Amino acid sequence analysis by others (38) has demonstrated that the α-isoelectric variant consists only of the α-SM type, that the β-NM actin is identical to that found in other nonmuscle cells, while the γ-isoelectric variant includes both the γ-SM and γ-NM actins. Thus, all references to γ-actin in this manuscript reflect both the muscle and nonmuscle components.

Major changes in fractional isoactin content and synthesis occurred when SMCs were grown in culture. Figs. 2 and 3 show results of a representative experiment, whereas Table I summarizes data from several different replicate experiments. SMCs in freshly isolated intact vessels contained ~70% α-SM actin with relatively small amounts of β-NM and γ-actin. Likewise, the major actin synthesized in intact vessels was α-SM. No detectable changes in actin content were observed within 36–48 h of plating (Fig. 3 *c*, and Table I, *B*). However, a dramatic decrease in the fractional synthesis of α-SM actin and an increase in β-NM and γ-actin synthesis ($P < 0.001$, analysis of variance) occurred within 36 h of plating (Fig. 3 *b*, Table I, *B*). Significantly, these changes preceded the onset of DNA synthesis as determined by [³H]thymidine autoradiography (Fig. 3 *a*). Thus, whereas cells at early time points
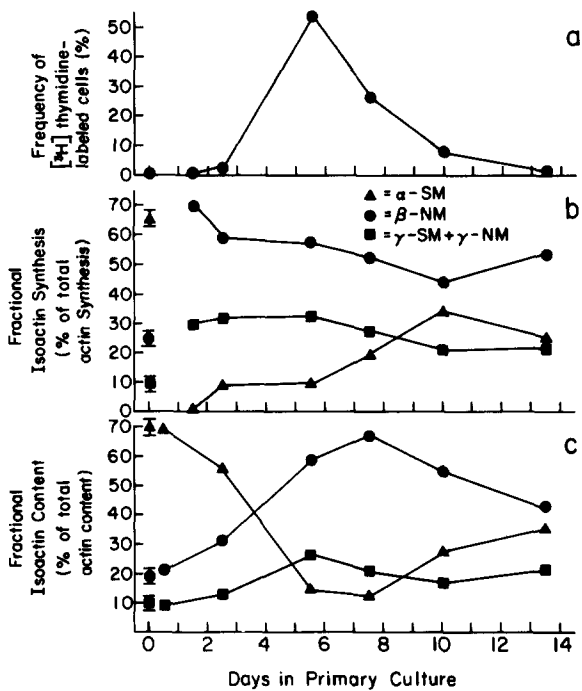
Figure 3. Changes in isoactin expression (Fig. 3, *b* and *c*) in relation to cellular growth rates (*a*) in primary rat aortic SMC cultures. Cells were plated at 1.0×10^4 cells cm^{-2} in medium 199 + 10% FCS. At various times, dishes were selected for determination of cellular growth rates (frequency of [^{3}H]thymidine-labeled cells) or fractional isoactin content and synthesis as described in Materials and Methods. Replicate dishes were pooled for gel electrophoretic analyses. The labeling index was determined on at least two dishes at each time point. The initial time point (i.e., 0 d) reflects values obtained in intact vessels. In this case, thymidine labeling indices were determined by injecting rats with 50 μCi/100 g [^{3}H]thymidine 1 h before they were killed and measuring the frequency of labeled SMCs in tissue sections by autoradiography. Relative isoactin content and synthesis was determined by incubating freshly obtained thoracic aortas in medium-199 containing 80 μCi/ml [^{35}S]methionine (3 h, 37°C, 95% air, 5% CO$_2$). Medial preparations of thoracic aortas were then prepared, homogenized, and gels run as described in Materials and Methods. Results from several replicate experiments are summarized in Table I.

contained significant α-SM actin, the fractional synthesis of this actin was extremely low. These early changes in fractional isoactin synthesis were followed by a corresponding drop in α-SM actin content and an increase in β-NM and γ-actin content. α-SM actin synthesis and content (Figs. 2*b* and *3*, and Table I, *C*) was low but not absent in subconfluent cells during exponential growth. α-SM actin synthesis increased in conjunction with a decrease in growth rate between day 5.5 and day 10 after plating as cells reached confluence (Figs. 2*c* and 3*b*) and was followed by a nearly threefold increase in the fractional content of α-SM actin between 7.5 and 13.5 d (Fig. 3*c*, and Table I, *D*). The increase in α-SM actin content and synthesis in postconfluent cells as compared with subconfluent cells was significant at the $P < 0.001$ level (Table I, *C* vs *D*). Density arrest of growth is thus associated with increased expression of α-SM actin and decreased expression of β-NM actin, while expression of γ-actin (i.e., γ-SM + γ-NM) was relatively unchanged. Significantly, these changes occurred in the presence of 10% FCS.

Simultaneous Evaluation of Actin Expression and DNA Synthesis in Individual SMCs: Combined Immunocytochemical and Autoradiographic Studies

To directly explore the interrelationship between growth state and actin expression on an individual cell basis the following experiment was done. Primary cultures were prepared and grown in the continuous presence of low levels of [^{3}H]thymidine (0.01 μCi/ml). Cells were harvested at 24 h, and then at 48-h intervals between 1 and 11 d. Cells were fixed, stained with the SM-specific isoactin antibody CGA7, processed for autoradiography, and observed by light microscopy.

At the 24-h time point, all cells appeared to stain positively with the CGA7 isoactin antibody. This indicates that non-SMC contaminants were rare and that there was not a subpopulation of SMCs deficient in SM isoactins. In addition, as illustrated in Fig. 4*a*, there were clearly cells present at early time points that had initiated DNA synthesis and were simultaneously stained with the CGA7 antibody. This and our gel electrophoretic analyses presented earlier (Figs. 2 and 3) demonstrate that total loss of SM isoactins was not a prerequisite for initiation of DNA synthesis.

The staining intensity with the CGA7 antibody appeared to be uniformly decreased in exponentially growing cells (days 5 and 7), suggesting that the SM actin present in rapidly growing cultures did not reside in a subpopulation of cells with a high SM actin content. Significantly, these observations also suggest that the major portion of the γ-actin present in subconfluent cultures is of the γ-NM type since the CGA7 antibody used recognizes only α-SM and γ-SM actin. This conclusion was supported by results of immunoblot analyses discussed below. A moderate increase in staining intensity with the CGA7 antibody occurred in postconfluent cells that likewise appeared to be uniform among cells.

Immunoblot Analysis of SM Isoactins in Primary Cultures

As noted previously, IEF/SDS gel electrophoretic analysis does not distinguish between the γ-SM and γ-NM actin forms. To determine whether the γ-SM actin is also expressed in cultured SMCs, Western blot analyses were performed on isoactins derived from subconfluent and postconfluent primary cultures, using several isoactin monoclonal antibodies (data not shown). While the CGA7 antibody (16) showed a high degree of cell specificity when staining whole cells, this antibody showed low reactivity in immunoblot analyses, suggesting that the antigenic determinant for this antibody is altered during electrophoretic and transfer processes. The C4 antibody (21), used as a positive control, reacted with all isoactins from both subconfluent and postconfluent cells. The B4 antibody (21) showed specific staining of α-SM and γ-SM actins from fresh homogenates of rat aortic smooth muscle, but did not react with β-NM or with γ-NM actin from several sources. As reported previously (21), B4 appeared to be selective for the γ-SM as compared with α-SM (21). There also appeared to be some loss of antigenicity with B4 in immunoblots since high antibody titers and protein loadings were necessary in our studies. Significantly, the B4 antibody showed little or no reactivity with γ-actin from subconfluent SMCs but showed reactivity with γ-actin from postconfluent SMCs. Thus, a portion of the γ-actin in these cells is of the SM type. However, due to the limited sensitivity and the

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Table 1. Fractional Isoactin Synthesis and Content* of Rat Aortic Smooth Muscle Cells in Intact Vessels and in Primary Culture

Group	α-SM Content	α-SM Synthesis	β-NM Content	β-NM Synthesis	γ-SM + γ-NM Content	γ-SM + γ-NM Synthesis
A Intact aortas	70.1 ± 2.1 n = 5	66.7 ± 1.6 n = 5	19.3 ± 1.1 n = 5	24.6 ± 1.7 n = 5	10.5 ± 1.1 n = 5	8.7 ± 1.9 n = 5
B Pre-growth cultures (1 d)	70.0 ± 0.2 n = 4 (3)	2.0 ± 2.0‡§ n = 2 (2)	20.2 ± 3.9 n = 4 (3)	68.8 ± 1.3‡§ n = 2 (2)	9.7 ± 0.2 n = 4 (3)	29.3 ± 0.8‡§ n = 2 (2)
C Subconfluent cultures (3–6 d)	17.1 ± 2.8‡∥¶ n = 4 (2)	13.3 ± 2.0‡∥¶ n = 6 (2)	58.3 ± 2.1‡∥¶ n = 4 (2)	54.9 ± 1.6‡∥¶ n = 6 (2)	24.4 ± 1.6‡∥¶ n = 4 (2)	31.8 ± 1.2‡§∥ n = 6 (2)
D Postconfluent cultures (10–14 d)	34.9 ± 4.0‡¶ n = 5 (3)	35.8 ± 4.5‡¶ n = 5 (3)	47.6 ± 2.5‡¶ n = 5 (3)	44.3 ± 2.1‡¶ n = 5 (3)	17.6 ± 1.7‡¶ n = 5 (3)	17.9 ± 2.3‡¶ n = 5 (3)

All values represent mean ± SEM. n, number of individual samples. Data from several independent replicate experiments are combined in this table. The number of separate experiments from which samples were derived is indicated in parentheses. A, Freshly obtained thoracic aortas were incubated in medium-199 containing 80 μCi/ml [^{35}S]methionine (3 h, 37°C, 95% air, 5% CO_2). Medial preparations of thoracic aortas were prepared homogenized, and gels run as described in Materials and Methods. n, number of animals. B, Primary cultures harvested at 1–2 d after plating and before initiation of DNA synthesis (see Fig. 3a). C, Subconfluent cultures harvested while growing exponentially. D, Cultures were 3–5 d postconfluent. Growth rates were extremely low and cells were at saturation density.

* Expressed as a percentage of total actin synthesis or content (see Material and Methods).

‡ Values are significantly different from that of intact vessels ($P < 0.001$, analysis of variance).

§ Fractional isoactin synthesis and content are significantly different ($P < 0.025$, analysis of variance).

∥ C is significantly different from D ($P < 0.05$, analysis of variance).

¶ Significantly different from B ($P < 0.05$, analysis of variance).
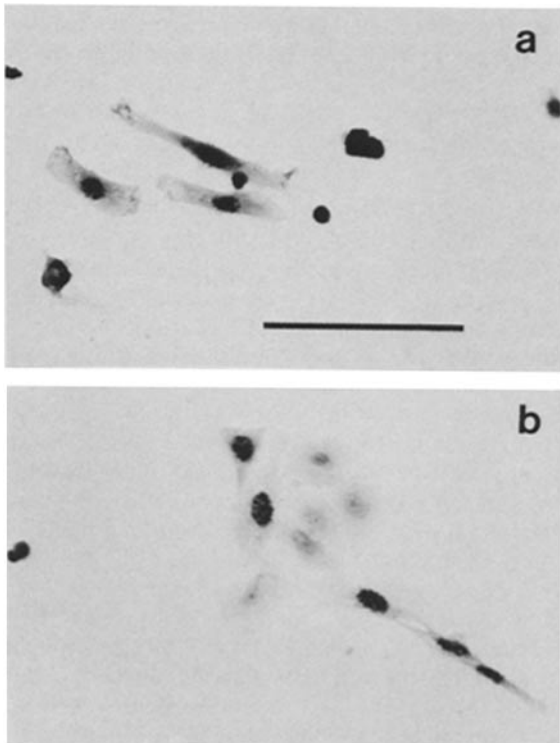
Figure 4. Simultaneous evaluation of [^{3}H]thymidine incorporation and isoactin expression in rat aortic SMCs after 3 d in primary culture using the CGA7 monoclonal antibody for SM isoactins (i.e., α-SM and γ-SM). Cells were isolated as described in Materials and Methods and grown in the continuous presence of [^{3}H]thymidine (0.01 μCi/ml) in medium-199 + 10% FCS. Cells were stained with either the CGA7 isoactin antibody (a) or control nonimmune antibody (b) using an avidin–biotin–peroxidase procedure. Cells were then fixed in 2% glutaraldehyde and processed for autoradiography. Note that several cells that stained with the CGA7 SM isoactin antibody (a), as well as control cells (b), have incorporated [^{3}H]thymidine. Bar, 25 μm.

qualitative nature of these analyses, we are reluctant to make any definitive statements regarding the relative abundance of γ-SM actin in subconfluent versus postconfluent cells.

Expression of Isoactins in Subpassaged SMCs

To determine whether the observed changes in isoactin expression were unique adaptations of primary cultures, primary cells at saturation density were harvested, plated at 1.0 × 10^4 cells cm^{-2} in SFM + 10% FCS, and fractional isoactin synthesis and content determined in subconfluent cells during exponential growth as well as in postconfluent density-arrested cells (Table II, A and B). As observed in primary cultures, the fractional synthesis and content of α-SM actin were significantly increased while β-NM and γ-actin were decreased in postconfluent versus subconfluent cells. In fact, the fractional levels of α-SM actin synthesis and content in subpassaged postconfluent cultures (Table II) were not significantly different from those of primary cultures ($P > 0.05$, analysis of variance).

The time-course of changes in fractional α-SM actin synthesis, relative to changes in cell number and replicative frequency, is illustrated in Fig. 5 (i.e., see the 10% FCS group). As in primary cultures, the increase in α-SM actin synthesis coincided with density-induced decreases in cellular growth rates that occurred as cells reached confluency (Fig. 6c).

Effects of Growth Stimulation and Growth Arrest on Actin Expression in Subpassaged SMCs

The preceding experiments have demonstrated that density-arrest of growth is associated with increased α-SM actin expression and that this occurs even in the presence of 10% FCS. The following series of experiments were designed to determine (a) whether growth arrest of subconfluent cells in SFM could evoke similar increases in α-SM actin expression, and (b) to explore whether subsequent growth stimulation of SFM-arrested cells with FCS was associated with decreased α-

Table II. Fractional Isoactin Synthesis and Content* in Passaged Rat Aortic Smooth Muscle Cells: Effects of Growth State

Group	α-SM Content	α-SM Synthesis	β-NM Content	β-NM Synthesis	γ-SM + γ-NM Content	γ-SM + γ-NM Synthesis
A Subconfluent cultures in 10% FCS	12.4 ± 1.2 n = 6 (3)	14.4 ± 1.3 n = 5 (2)	60.5 ± 1.2 n = 6 (3)	59.2 ± 1.6 n = 5 (2)	27.1 ± 0.8 n = 6 (3)	26.3 ± 1.4 n = 5 (2)
B Postconfluent cultures in 10% FCS	28.3 ± 1.4‡ n = 7 (3)	33.8 ± 1.5‡ n = 7 (3)	55.5 ± 1.5‡ n = 7 (3)	50.5 ± 1.3‡ n = 7 (3)	16.2 ± 0.3‡ n = 7 (3)	15.0 ± 0.8‡ n = 7 (3)
C Subconfluent cultures plated and grown in SFM for 2.5 d	—	27.2 ± 2.0‡§ n = 4 (2)	—	55.0 ± 0.4‡§ n = 4 (2)	—	17.8 ± 0.6‡ n = 4 (2)
D Cultures from C 12 h after stimulation with 10% FCS	—	7.8 ± 2.0ˡ n = 2 (2)	—	64.7 ± 9.4 n = 2 (2)	—	27.6 ± 7.3 n = 2 (2)
E Subconfluent cultures plated and grown in SFM for 5–7 d	—	32.6 ± 3.0‡ n = 2 (2)	—	49.8 ± 2.1‡ n = 2 (2)	—	17.6 ± 0.9‡ n = 2 (2)
F Subconfluent cultures grown in 10% FCS for 2.5 d and then switched to SFM for 5 d	—	33.3 ± 0.9‡ n = 2 (2)	—	50.3 ± 2.2‡ n = 2 (2)	—	16.6 ± 1.3‡ n = 2 (2)

All values represent mean ± SEM. *n*, number of individual samples. Data from several independent replicate experiments are combined in this table. The number of separate experiments which samples were derived is indicated in parentheses. In addition, as noted in Materials and Methods, it was necessary to pool replicate culture dishes for each sample point within a given experiment in order to have sufficient sample for analysis. *A*, Cultures were plated at 10^4 cells cm^{-2} and grown in SFM + 10% FCS. Cells were harvested when subconfluent while growing exponentially (2–4 d postplating). [^{3}H]Thymidine labeling indices (1-h pulse) were >30%. *B*, Cultures were plated and grown as in *A*. Cells were harvested between 4 and 6 d after reaching confluence or ~10–14 d after plating. At this time cells had reached saturation density and [^{3}H]thymidine labeling indices were (1-h pulse) < 2%. *C*, Cells were plated (1.0 × 10^4 cm^{-2}) and grown in SFM alone for 2.5 d. Cell number was unchanged over this time interval and the [^{3}H]thymidine labeling index was <5%. *D*, Cells were growth-arrested in SFM for 2.5 d (*C*) and then growth-stimulated by addition of 10% FCS. Cells were harvested 12 h after addition of FCS. *E*, Same as *C* but harvested 5–7 d after plating. *F*, Cells were plated (10^4 cells cm^{-2}) in SFM and 10% FCS for 2.5 d and then growth-arrested in SFM alone for 5 d. Thymidine labeling indices were <2%.

* Expressed as a percentage of total actin synthesis or content (see Materials and Methods).

‡ Significantly different from *A* ($P < 0.001$ for α- and γ-; $P < 0.05$ for β-, analysis of variance).

§ Significantly different from *B*, *E*, or *F* ($P < 0.05$, analysis of variance).

ˡ Significantly less than *A*, *B*, *C*, *E*, or *F* ($P < 0.05$, analysis of variance).

SM actin expression. In these experiments, cells were plated (1.0 × 10^4 cells cm^{-2}) and grown in either SFM or SFM + 10% FCS. At 2–3 d, some dishes were harvested from each group while others were switched to either SFM or SFM + 10% FCS and sampled at various times for determination of fractional isoactin synthesis, the frequency of [^{3}H]thymidine-labeled cells, and cell number. Results from a representative experiment are presented in Fig. 5, while results from several separate experiments are summarized in Table II. The relative cell densities present at various times are illustrated in Fig. 6.

Cells plated and maintained in SFM for 2.5 d had a fivefold lower labeling index (Fig. 5b) and a twofold lower cell number (Fig. 5c) than cells grown in SFM + 10% FCS. Significantly, fractional α-SM actin synthesis (Fig. 5a and Table II, C) was increased twofold ($P < 0.001$) in these growth-arrested cells, while the synthesis of β-NM ($P < 0.05$) and γ-actin ($P < 0.001$) was decreased. α-SM actin synthesis increased with time in cells maintained in SFM (Fig. 5a) and by 5–7 d was not significantly different from that of postconfluent cells in 10% FCS (Table II, B vs E). These results demonstrate that fractional synthesis of α-SM actin can be promoted in subconfluent SMCs by maintenance in SFM but the effect appears to require an extended period of growth arrest.

When cells plated in SFM + FCS were switched to SFM alone, fractional α-SM actin synthesis was initially moderately increased compared with cells that remained in FCS (Fig. 5a), but by 96 h α-SM actin synthesis was greater in cells in FCS than in cells switched to SFM (note that cell density was increased in the FCS group at this time). However, as observed

in cells plated directly in SFM, α-SM actin synthesis increased with time in SFM (Fig. 5a) and by 5–7 d after switching to SFM, α-SM actin synthesis was not significantly different from postconfluent cultures in 10% FCS (Table II, B vs F).

In contrast to the effects of growth arrest, serum stimulation of SFM-arrested cells induced a dramatic decrease in the fractional synthesis of α-SM actin within 12 h of stimulation (Fig. 5a, and Table II, D). Conversely, β-NM and γ-actin synthesis appeared to be increased although these changes were not statistically significant. It is important to note that the decreases in α-SM actin synthesis preceded a large increase in thymidine labeling index (i.e., from 3.4–28.2%) that occurred between 12 and 24 h after growth stimulation (Fig. 5b).

To determine whether withdrawal of FCS from cells at saturation density could further enhance fractional α-SM actin synthesis, cells were grown to saturation density in SFM + FCS and then switched to SFM alone (Fig. 7). No increases in fractional α-SM actin synthesis or content (data not shown) were observed in two separate experiments. Cell number was not different between the FCS and SFM groups in this experiment, and although the thymidine labeling index decreased in the SFM group, it was <1% for all groups.

Discussion

Chamley-Campbell et al. (2, 4) and others (37) have presented evidence suggesting that SMCs do not proliferate in cell culture until they have lost much of their contractile apparatus
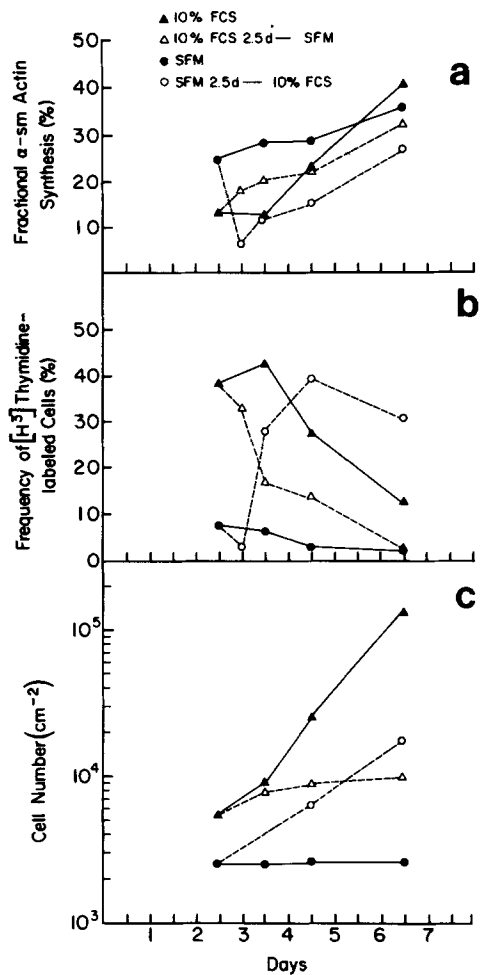
Figure 5. Representative experiment showing the effects of growth stimulation (i.e., SFM + 10% FCS) and growth arrest (i.e., SFM alone) on the fractional synthesis of α-SM actin (*a*) in cultured rat aortic SMCs. Fractional isoactin synthesis was measured as described in Materials and Methods. Cells were plated at 1.0×10^4 cells cm^{-2} in either SFM or SFM + 10% FCS and some dishes harvested at 2.5 d after plating. At 2.5 d some dishes from each group were switched to either SFM or SFM + 10% FCS and the fractional synthesis of isoactins determined at the times indicated. Replicate dishes (two per time point) were analyzed for cell number (*c*) and the frequency of cells undergoing DNA synthesis (*b*). Portions of this and replicate experiments are summarized in Table II.

as visualized by transmission electron microscopy and by immunocytochemical staining with an SMC-specific myosin antibody. These investigators have suggested that SMCs normally exist in a nonproliferating contractile state and that cells must phenotypically modulate as a prerequisite for cell proliferation. However, results of the present study demonstrate that in primary cultures, cells that had incorporated [^{3}H]thymidine simultaneously stained with a monoclonal antibody to the SM-specific isoactins. Furthermore, gel electrophoretic analysis showed that significant α-SM isoactin was present in cells at the time of onset of cell replication. Larsen et al. (20) demonstrated continued myosin expression in primary and established cultures of rat mesenteric SMCs using antimyosin antibodies and gel electrophoretic measurements. Thus, loss of cell-specific contractile proteins is not a prerequisite for initiation of DNA synthesis in primary cultures of

rat vascular SMCs. However, our studies do show that SMCs in both primary and subpassage undergo differential expression of SM- and NM-isoactins in relation to growth. Fractional synthesis and content of α-SM actin was low and NM actins high in sparse cultures during rapid growth, while density arrest of growth was associated with increased α-SM actin and decreased β-NM synthesis and content. Our results are similar to those of Strauch and Rubenstein (36) who observed that BC$_3$H$_1$ cells, a SM-like cell line derived from a mouse brain tumor, increase their synthesis of SM isoactins when serum-deprived. These results should be interpreted as evidence that expression of cell-specific characteristics and cellular growth are coupled rather than as evidence that phenotypic modulation is a prerequisite for SMC proliferation. Furthermore, these observations and those by others demonstrating that subpassaged cells continue to express a variety of characteristics of contractile responsiveness (5, 10, 13, 18, 24, 35) clearly demonstrate that the concept of contractile state and synthetic state is somewhat misleading and overly simplistic in describing the differentiated state of SMCs in culture.

Density arrest of growth in both primary and subpassaged SMCs in the present study was associated with an increase in the fractional synthesis and content of α-SM actin that occurred before attainment of saturation density and in the presence of serum. While results of Strauch and Rubenstein (36) are qualitatively very similar to ours, density-induced increases in α-SM actin in the presence of serum appeared to be attenuated in their BC$_3$H$_1$ cells. This may reflect, in part, a decreased sensitivity of BC$_3$H$_1$ cells to density-induced decreases in cellular growth and subsequent induction of α-SM actin expression. Strauch and Rubenstein (36) found that α-SM actin synthesis could be induced much sooner in BC$_3$H$_1$ cells by growth arresting confluent cells by withdrawal of serum, but no changes in α-SM actin synthesis were observed if cells were serum-deprived when subconfluent, suggesting that cell confluence was a prerequisite for α-SM actin induction. In contrast to their findings, the following observations in our studies demonstrate that α-SM actin expression can be promoted by growth arrest of subconfluent rat aortic SMCs. (*a*) Plating and maintenance of cells in SFM increased α-SM actin synthesis compared with cells in SFM + FCS even though cell densities were very low. (*b*) Extended growth arrest of low density cells in SFM was associated with further increases in α-SM actin synthesis without changes in cell density. (*c*) Growth arrest of low density rapidly dividing cells by switching to SFM enhanced α-SM actin synthesis. The apparent differences in our results and those of Strauch and Rubenstein may relate to differences in the cells studied or in the culture conditions used. The SFM used in our studies has been shown to maintain SMCs in a viable, nongrowing, positive protein balance state for extended periods of time (22). In contrast, cells maintained in unsupplemented Dulbecco's modified Eagle's medium as used by Strauch and Rubenstein (36), may have been in negative protein balance (22). An alternative explanation is that one or more of the components of SFM had a direct effect on α-SM actin expression. This seems unlikely, since there appear to be no differences in relative isoactin expression between cells grown in SFM + 10% FCS as compared with medium-199 + 10% FCS (data not shown). We want to emphasize that whereas our studies did not indicate that cell confluence was an absolute
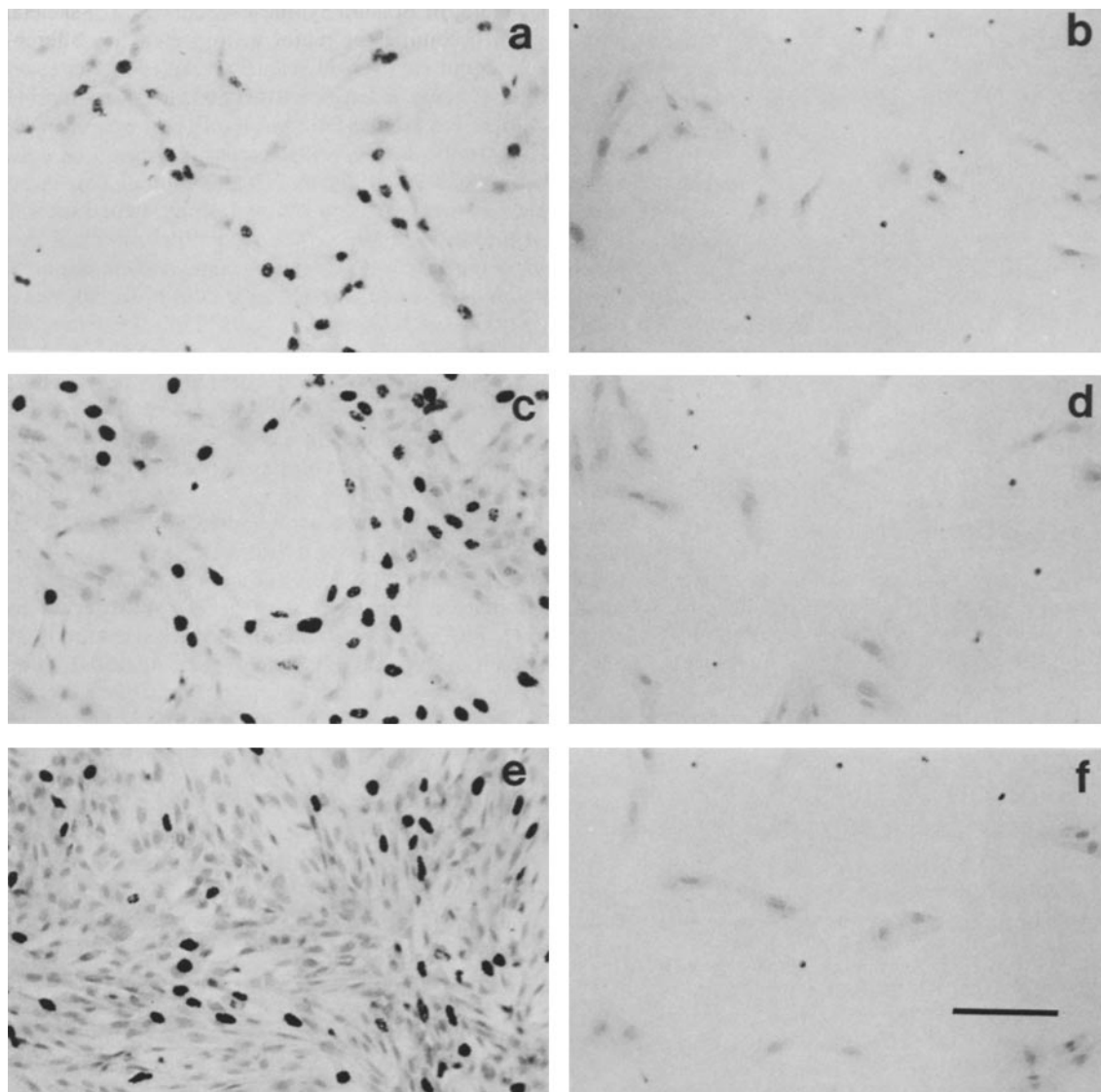
Figure 6. Light micrographs of cultures used for autoradiographic determinations of [³H]thymidine labeling indices in the experiments presented in Fig. 5: (*a*) 2.5 d in FCS; (*b*) 2.5 d in SFM; (*c*) 4.5 d in FCS; (*d*) 4.5 d in SFM; (*e*) 6.5 d in FCS; (*f*) 6.5 d in SFM. Marked increases in cell density occurred with time in cells grown in SFM + 10% FCS. In contrast, both the frequency of labeled cells and cell density were extremely low at all time points examined in cells maintained in SFM alone. Cells were stained with hematoxylin. Bar, 25 μm.

prerequisite for α-SM actin induction, they do support the suggestion of Strauch and Rubenstein that cell–cell contact has an important permissive effect in that induction was more rapid in density-arrested cultures than in SFM-arrested subconfluent cultures. Furthermore, the density-induced increases in α-SM actin expression occurred even at fairly high rates of cell replication (i.e., thymidine indices >20%, Fig. 5).

The studies presented here have focused on expression of α-SM actin since this is the major actin present in differentiated vascular smooth muscle and indeed comprises >25% of the total protein in these cells in vivo (9, 39). However, there is some evidence that induction of α-SM actin expression by growth arrest is accompanied by coordinate induction of a variety of other muscle-specific proteins and functions. In the present study results of immunoblot analyses indicate that γ-SM isoactin is also expressed in density-arrested postconfluent SMCs. Consistent with these findings, Franke et al. (10) demonstrated that the mRNA for both α-SM and

γ-SM is present in an established SMC line derived from the rat inferior vena cava although they did not specifically examine the influence of growth state. Larsen et al. (20) have presented gel electrophoretic and immunocytochemical data showing that myosin is present at all times in cultured SMCs from rat mesenteric arteries. Although Larsen et al. (20) did not observe any differences in total myosin heavy chain content between subconfluent and postconfluent cultures, they did observe changes in antigenic expression based on staining patterns. Furthermore, we have preliminary evidence (unpublished observations) suggesting that different myosin heavy chain isotypes may be present under these two conditions. Myosin from postconfluent SMCs was immunoreactive in Western blots with an SM-specific polyclonal myosin antibody (17, obtained from G. Stewart), whereas myosin from subconfluent cells was not. Schubert et al. (35) demonstrated an increase in the specific activity of creatine phosphokinase and myokinase associated with increased cell density and the
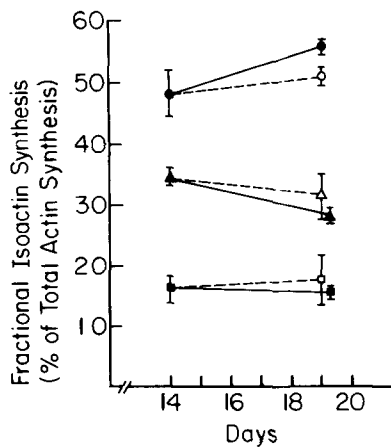
Figure 7. Representative experiment showing the effects of SFM on the fractional synthesis of isoactins in SMC cultures at saturation density. Cells were plated at 1.0×10^4 cells cm^{-2} in SFM + 10% FCS. On day 14 after plating, some cells were switched to SFM alone for 5 d while the remaining dishes remained in SFM + 10% FCS. Duplicate dishes were analyzed for isoactins at each time point. Values represent the mean ± SD. Replicate dishes from each group were pulsed for 1 h with [^{3}H]thymidine for determination of growth rates. Cell number was not different among groups ranging between 1.20×10^5 and 1.30×10^5 cells cm^{-2}. The frequency of [^{3}H]thymidine-labeled cells was 0.64% on day 14, 0.74% on day 19 for cells in SFM + 10% FCS, and 0.15% on day 19 for cells in SFM alone. Similar results were obtained in a separate experiment. ▲ and △, α-SM; ● and ○, β-NM; ■ and □, γ-SM + γ-NM; △, ○, and □, switched to SFM on day 14.

cessation of cell division in BC$_3$H$_1$ cells. Subsequently, Olson et al. (28) demonstrated that the induction of creatine phosphokinase in these cells occurred at the transcriptional level. Chamley-Campbell and Campbell (3) presented morphological evidence suggesting that the loss of contractile myofilaments in primary smooth muscle cell cultures could be inhibited by plating cells at a density that inhibited cell growth. Taken together, these cited studies and the present studies suggest that expression of a variety of SMC-specific characteristics can be promoted in cultured SMCs by inhibition of cellular growth. Nevertheless, it is also clear that many characteristics of differentiated SMCs are altered in cultured cells, including contractile capability, morphology, and biosynthetic properties (2, 37, 41) and that further studies are needed to determine what additional factors, other than growth control, are important in promoting cytodifferentiation in these cells.

The functional significance of changes in isoactin synthesis during SMC growth are unclear, but may reflect the physiological requirements of cells for specialized actin molecules for specialized cellular functions. For example, the dramatic decrease in α-SM synthesis and increase in the relative synthesis of β-NM and γ-actin before onset of DNA synthesis in serum-stimulated SMCs in the present study may reflect a need for specialized actin necessary for cell division. While we did not measure total actin synthesis in our studies, Riddle et al. (32) have shown that an increase in total actin synthesis precedes onset of DNA synthesis in growth-stimulated fibroblasts. Our results suggest that in SMCs, this increase occurs primarily in the nonmuscle isoactin forms. Analogous changes occur in skeletal muscle. Whereas prefusion skeletal myoblasts contain principally nonmuscle isoactins, after fu-

sion a switching in isoactin synthesis occurs with α-skeletal muscle actin becoming the major actin present in differentiated skeletal muscle (1, 6). Concurrent with these changes in isoactin expression, extensive changes occur in the structure and function of actin in skeletal muscle cells. Increased expression of SM-specific actins and decreased expression of nonmuscle actins has also been shown to occur during avian gizzard development (34) and during developmental growth of the rat thoracic aorta (29), although it is less clear what structural and functional changes accompany these alterations. Immunocytological studies by Pardo et al. (30) demonstrating differential subcellular localization of muscle and nonmuscle actins in skeletal muscle cells provide further support for the idea that isoactins have specialized roles within the cell. However, the precise nature of purported functional differences in isoactins are unclear, since muscle and nonmuscle actins appear to have similar physical properties (31) and actin binding sites (25).

Currently, there is much interest in alterations in contractile and cytoskeletal protein expression that occur in vascular SMCs in experimental models of atherosclerosis and in human atheromatous lesions (11, 13, 15, 19). With regard to actin, recent work (13, 15, 19) has demonstrated that intimal SMCs in either experimental or human atheromatous lesions contain predominantly β-NM and γ-NM actins rather than the α-SM type characteristic of medial SMCs. Interestingly, in a balloon-injury model in rats, Kocher et al. (19) observed that in older lesions (i.e., 75 d after injury), which had re-endothelialized, the ratio of SM- to NM-actins had returned to levels similar to that of normal medial SMCs. Results of the present study suggest that the changes in actin expression during lesion development were related to cellular proliferation, and suggest that studies of factors regulating isoactin expression in cultured vascular SMCs may provide useful information on the cell biology and pathology of vascular SMCs in vivo.

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