Cell Cycle Versus Density Dependence of Smooth Muscle Alpha Actin Expression in Cultured Rat Aortic Smooth Muscle Cells

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Abstract. Cultured smooth muscle cells (SMC) undergo induction of smooth muscle (SM) alpha actin at confluency. Since confluent cells exhibit contact inhibition of growth, this finding suggests that induction of SM alpha actin may be associated with cell cycle withdrawal. This issue was further examined in the present study using fluorescence-activated cell sorting of SMC undergoing induction at confluency and by examination of the effects of FBS and platelet-derived growth factor (PDGF) on SM alpha actin expression in postconfluent SMC cultures that had already undergone induction. Cell sorting was based on DNA content or differential incorporation of bromodeoxyuridine (Budr). The fractional synthesis of SM alpha actin in confluent cells was increased two- to threefold compared with subconfluent log phase cells, but no differences were observed between confluent cycling ($Budr^+$) and noncycling ($Budr^-$) cells. In cultures not exposed to Budr, confluent cycling S + G_2 cells exhibited similar induction. These data indicate that cell cycle withdrawal is not a prerequisite for the induction of SM alpha actin synthesis in SMC at confluency. Growth stimulation of postconfluent cultures with either FBS or PDGF resulted in marked repression of SM alpha actin synthesis but the level of repression was not directly related to entry into S phase in that PDGF was a more potent repressor of SM alpha actin synthesis than was FBS despite a lesser mitogenic effect. This differential effect of FBS versus PDGF did not appear to be due to transforming growth factor-beta present in FBS since addition of transforming growth factor-beta had no effect on PDGF-induced repression. Likewise, FBS (0.1–10.0%) failed to inhibit PDGF-induced repression. Taken together these data demonstrate that factors other than replicative frequency govern differentiation of cultured SMC and suggest that an important function of potent growth factors such as PDGF may be the repression of muscle-specific characteristics.

THE abnormal growth of vascular smooth muscle cells (SMC)[1] is an important feature of atherosclerosis and hypertension and, as such, has been the subject of vigorous investigation. A body of evidence now exists which suggests that the state of differentiation of smooth muscle (SM) influences its growth responsiveness and that the loss of proliferative suppressor mechanisms associated with differentiation and maturation, rather than just stimulation with positive regulators of cell growth, may comprise an important component of SMC growth control (Chamley-Campbell et al., 1979, 1981; Schwartz et al., 1986). It is well established that the expression of SMC differentiated characteristics are altered in intimal thickenings and in the atherosclerotic lesions of man and animals (Gabbiani et al., 1981, 1984; Kocher et al., 1984; Mosse et al., 1985). Lesion cells exhibit, among other changes, a reduction in SM-specific actins and alterations in the expression of intermediate filaments (Gabbiani et al., 1981, 1984; Kocher et al., 1984). However, whether these changes in differentiated state of SM are a prerequisite for cell proliferation as has been proposed by Chamley-Campbell et al. (1979, 1981) is controversial.

The development of in vitro systems for studying SMC differentiation, as well as the identification of SM-specific proteins for use as markers of differentiation, have facilitated closer examination of the relationship between growth and differentiation in SMC. Several investigators have demonstrated that serially subpassaged arterial SMC express SM-specific contractile proteins in vitro (Franke et al., 1980; Larsen et al., 1984; Owens et al., 1986; Rovner et al., 1986). Owens et al. (1986) have characterized the expression of SM alpha actin in cells cultured from rat thoracic aortic media and reported an inverse relationship between cell proliferation and SM alpha actin expression. Complete loss of SM alpha actin did not appear to be required for the onset of DNA synthesis in primary cultures. However, both SM alpha actin content and synthesis were threefold higher in postconfluent cultures than in log phase cells. Alpha actin ex-

1. *Abbreviations used in this paper*: Budr, bromodeoxyuridine; PDGF, platelet-derived growth factor; SFM, serum-free medium; SM, smooth muscle; SMC, smooth muscle cells; TGF-β, transforming growth factor-beta.

pression was likewise induced by growth arrest of subconfluent cells in a serum-free medium (SFM), but only after extended periods of growth arrest. Strauch and Rubenstein (1984) have reported a similar induction of alpha actin synthesis in postconfluent growth-arrested BC_3H1 cells, a SMC-like line derived from mouse brain tumour. Additionally, Rovner et al. (1986) demonstrated the expression of two SM-specific myosin heavy chains in contact-inhibited postconfluent but not in logarithmically dividing subconfluent rat aortic SMC.

While these data appear to establish an important relationship between growth and cytodifferentiation in vascular SMC, they also leave important issues unresolved. For example, the density induced expression of SM-specific contractile proteins may not result directly from cell cycle withdrawal but may merely occur concomitantly. One possibility is that cellular changes resulting from cell-to-cell contact at confluency rather than contact inhibition of growth may be responsible for the induction of SM-specific proteins. This is an issue of broad interest in the field of cell biology but is very poorly understood, in part because of a lack of appropriate cell culture models and an inability to dissociate the effects of cell–cell contact from contact inhibition of growth.

In the present study we have explored the effects of cell cycle status versus cell density on control of SM alpha actin expression by SMC by: (*a*) using fluorescence-activated cell sorting to determine whether withdrawal from the cell cycle is a prerequisite for the induction of SM alpha actin in cultured SMC at confluence, and (*b*) examining the effects of mitogenic stimulation of postconfluent SMC on expression of SM alpha actin.

Materials and Methods

Cell Culture

Smooth muscle cells were isolated from rat thoracic aorta and cultured by a modification of the methods of Owens et al. (1986) as reported by Geisterfer et al. (1988). Cells were plated at an initial seeding density of 3,500 cells/cm^2 in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) and grown to confluence in Medium-199 (Gibco, Grand Island, NY) containing 10% FBS (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. All SMC cultures used in these studies were of passage number 13 or less.

Cell Sorting

When cultures were confluent as assessed by phase microscopy, they were treated with bromodeoxyuridine (Budr; 10^{-4} M; Sigma Chemical Co., St. Louis, MO) or Budr vehicle for 28 h in the dark. The 28-h Budr-labeling period is approximately 1.4 cell cycle times and permits all cells that cycle at any time during the experiment to incorporate the label. At the time of the Budr pulse, replicate flasks were pulsed with [^{35}S]methionine (40 μCi/ml, >800 Ci/mmol; New England Nuclear, Boston, MA) for either the first 14 h (i.e., 14–28 h before harvest followed by a chase with an excess of unlabeled 1 mM L-methionine for 14 h) or the 14 h immediately before harvest.

Samples intended for sorting on the basis of DNA content were not treated with Budr and received [^{35}S]methionine (80 μCi/ml) for 4 h before harvest. Log phase cultures were treated similarly but were harvested while subconfluent.

After Budr and methionine pulses, cells were harvested in trypsin (0.05%; Gibco) and ethylenediaminetetraacetate (0.02%; Sigma Chemical Co.), collected in 50-ml centrifuge tubes containing 1.0 ml FBS, washed in PBS, and fixed in 8 vol of 100% methanol at 4°C for 15 min. Fixed samples were then resedimented and suspended in Hoechst 33258 (0.58 μg/ml in PBS; Sigma Chemical Co.) immediately before sorting. Stained SMC were sorted on a Epics V Fluorescence-Activated Cell Sorter (Coulter Electronics, Inc., Hialeah, FL) using a Coherent 90-5 argon ion laser with UV enhancement. The fluorochrome was excited at 351–363 nm and the emission screened by a 475-nm-long pass and UV laser block filters. Though the actual number of cells sorted into cell cycle subpopulations varied greatly between experiments, the lowest number collected in any subpopulation was approximately 400,000. Generally, between 0.5 and 1.5 × 10^6 cells were collected in Budr$^+$ or S + G_2 populations, while the quantity of G_1 and Budr$^-$ cells was usually much higher (2–5 × 10^6). The purity of sorted subpopulations was >99% as assessed by reassaying sorted populations in a limited number of cases. After collection in 15 ml centrifuge tubes, sorted cells were centrifuged, the supernatant discarded, and the pellet lysed and sonicated in an IEF buffer containing 1% SDS (Bio-Rad Laboratories, Richmond, CA), 10% glycerol, and 20 mM dithiothreitol (Sigma Chemical Co.) at a final concentration of approximately 5 × 10^6 cells/ml.

Growth Factor Studies

SMC were plated at 3,500 cells/cm^2 in medium containing 10% FBS in duplicate 60-mm petri dishes and replicate 24-well culture plates and grown until confluent. At confluency, medium was removed, cells were rinsed, and the cultures incubated with SFM containing equal parts of DME (Gibco) and Ham's F12 (Gibco) supplemented with insulin (1 μM; Sigma Chemical Co.), transferrin (5 μg/ml; Sigma Chemical Co.), ascorbate (0.2 mM; Sigma Chemical Co.), penicillin (100 U/ml; Gibco), and streptomycin (100 μg/ml; Gibco) for 5 additional days. This SFM formulation has been shown to maintain cultured SMC in an anabolic, quiescent state (Libby and O'Brien, 1983) and to maintain high levels of SM alpha actin expression in these cells (Owens et al., 1986).

After 5 d in SFM, the media was replaced and cultures were treated with appropriate mitogens: partially purified human PDGF (~0.4% pure; Raines and Ross, 1985), phenyl-Sepharose-purified PDGF (>95% pure; Raines and Ross, 1982) (both PDGF preparations were generously supplied by Dr. Russell Ross, University of Washington, Seattle, WA), PDGF vehicle (10 mM acetic acid in 2 mg/ml BSA), FBS, HPLC-purified transforming growth factor-beta (TGF-β) (supplied by Dr. Akira Komoriya, Rorer Biotech Inc., Rockville, MD), or TGF-β vehicle (1 mg/ml BSA) for 24 h. Parallel cultures were pulsed with either [^{3}H]thymidine (0.1 μCi/ml, 6.7 Ci/mmol; New England Nuclear) for 24 h or [^{35}S]methionine (40 μCi/ml) for 6 h before harvest.

[^{3}H]Thymidine Autoradiography

Multiwell plate cultures of SMC exposed to [^{3}H]thymidine were washed with PBS, fixed in 2% glutaraldehyde in PBS for 5 min, rewashed in PBS, dehydrated in 100% methanol, and processed for autoradiography as previously described (Owens et al., 1986). Labeling indices were determined by scoring at least 4,000 cells from quadruplicate titer wells.

Actin Isoform Synthesis

Actin isoforms were resolved by two-dimensional IEF/SDS-PAGE via a modification of the technique of O'Farrell (1975) as described by Fatigati and Murphy (1984) and Owens et al. (1986). Densitometric analysis of Coomassie Brilliant Blue-stained gels yielded linear results over the protein loading range used and a coefficient of variation for alpha actin of 8.4%. In these studies, IEF gels were loaded with 50,000–150,000 cpm of cell lysate per lane. Control studies (data not shown) demonstrated that methanol fixation of cells does not affect the determination of fractional SM alpha actin synthesis by two-dimensional IEF/SDS gel electrophoresis.

Determination of fractional isoactin synthesis was made on developed autoradiographs after exposure of dried gels to Kodak X-Omat K film for 1–10 d. Resultant images were digitized using an Eikonix 78/99 Digital Camera (512 × 512 pixel resolution). Determinations of integrated optical density were obtained using a Gould IP8500 Image Processor (Freemont, CA) by defining the spot area with an appropriate cursor outline; background density calculated on a pixel by pixel basis was determined in several adjacent areas and automatically subtracted. Values obtained from multiple exposures of each gel were routinely analyzed and compared to ensure operation within the linear range of the film. In addition, although the limited yields from sorting prohibited multiple determinations on all samples, this was done where possible.

Statistics

Fractional SM alpha actin synthesis values from sorted cell subpopulations were compared with that of log phase cells using the paired *t* test. Relevant comparisons within experiments (Budr$^+$ vs. Budr$^-$; S + G_2 vs. G_1) were

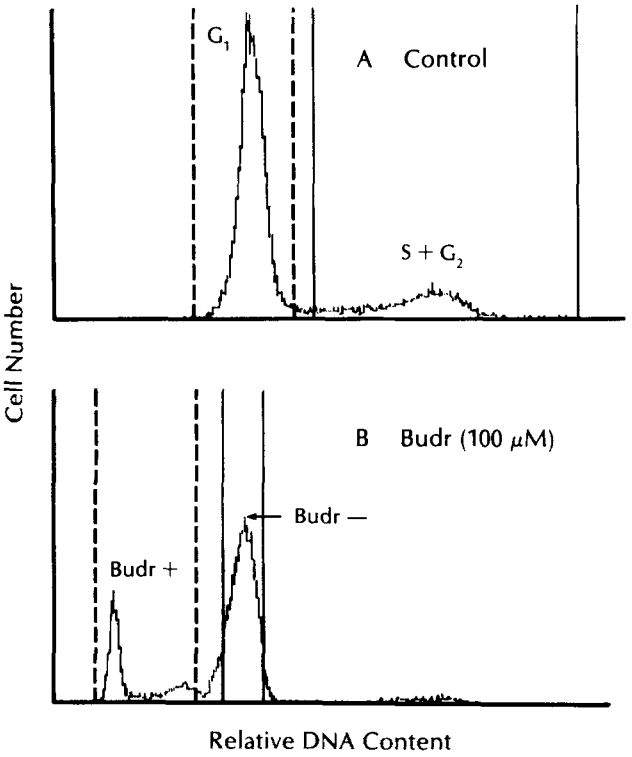

Figure 1. Representative flow cytometric DNA histograms of control and Budr-treated rat aortic SMC at confluency. Rat aortic SMC were plated at an initial seeding density of 3,500 cells/cm^2 in 75-cm^2 culture flasks and grown until cells were just confluent. At this time cultures were pulsed with vehicle or Budr (10^{-4} M) in the dark for 28 h. Cells were then harvested in trypsin, washed, fixed in 100% methanol, stained with Hoechst 33258, and sorted in an Epics V Fluorescence Activated Cell Sorter on the basis of either DNA content (*A*) or differential incorporation of Budr (*B*). Sorting of cell cycle subpopulations was carried out using gating windows similar to those depicted. The incorporation of Budr into the DNA of cycling cells results in a quenching of Hoechst 33258 fluorescence. Thus the fluorescence of cells which have synthesized DNA, i.e., cycling cells, is shifted to the left on the flow cytometric DNA histogram.

also conducted using paired *t* test. Mean thymidine-labeling indices and fractional SM alpha actin synthesis values from growth factor–stimulated cultures were first compared using analysis of variance. When analysis of variance led to rejection of the omnibus null hypothesis means were further compared using the Newman-Keuls test for multiple comparisons. All differences were considered statistically significant at $P < 0.05$ level.

Results

Fluorescence Activated Cell Sorting

Representative flow cytometric DNA histograms of control and Budr-treated rat aortic SMC at confluence are depicted in Fig. 1. When incorporated into the DNA of cycling cells, Budr results in a quenching of Hoechst 33258 fluorescence. Thus, the fluorescence of cells that have synthesized DNA during the 28-h Budr pulse (i.e., cycling cells) is shifted to the left on the flow cytometric DNA histogram. The following lines of evidence indicate that the rat aortic SMC used in these studies undergo only one additional cell doubling upon reaching confluency: (*a*) multiple Budr substitutions are not observed if Budr-labeling periods are extended (data not shown) and thus Budr$^+$ cells represent those cells completing their final transit of the cell cycle, and (*b*) cell number approximately doubles after establishment of confluency (Owens et al., 1986). Additional experiments have demonstrated that cells which are Budr$^-$ do not undergo DNA synthesis if the labeling period is extended to 56 h. It is therefore reasonable to conclude that this subpopulation is truly quiescent.

It was also necessary to demonstrate that Budr itself exerted no effects on SM alpha actin expression under these experimental conditions. Thus we tested the effects of Budr (10^{-4} M) on fractional alpha actin synthesis in unsorted cultures undergoing induction at confluency. Mean fractional alpha actin synthesis values for treated and untreated cultures were 20.69 and 20.25%, respectively. Statistical analysis of these data indicate that SM alpha actin synthesis is unaffected by treatment with 10^{-4} M Budr for 28 h ($P > 0.05$, paired *t* test).

Fractional SM Alpha Actin Synthesis of Cycling and Noncycling Cells

Fig. 2 presents autoradiographs of two-dimensional SDS gels showing the fractional SM alpha actin synthesis of cultured SMC subpopulations sorted on the basis of DNA content. It should be noted that, due to the prolonged period required for sorting cell cycle subpopulations, charge modifications of actin were sometimes observed in two-dimensional IEF/SDS gels. However, by comparing actin isoform expression between unsorted cells and those subjected to extended sorting we have found that resultant charge modifications do not affect fractional isoactin synthesis determinations. A summary of SM alpha actin synthesis values from densitometric analyses of autoradiographs is presented in Table I. Fractional beta and gamma actin values from sorted SMC subpopulations are depicted in Tables II and III, respectively. Results demonstrate that confluent cells from these sorts were induced to express SM alpha actin irrespective of their position in the cell cycle. Fractional SM alpha actin synthesis was two- to threefold greater in confluent cells than in subconfluent cells ($P \leq 0.05$) in log phase growth but there were no significant differences in this parameter (expressed as a percentage of log phase) between confluent cells in either G_1 (227.9%) or S + G_2 (219.5%). These results clearly demonstrate that SMC that continue to cycle as they reach confluency are induced to express alpha actin but do not provide comparison between definitively quiescent and cycling subpopulations since cells with 2C DNA content (i.e., sorted G_1) are comprised of both quiescent G_0 and cycling G_1 cells. Thus, we also examined actin isoform expression of SMC sorted on the basis of differential incorporation of Budr. Results demonstrate (Fig. 3 and Table I) that both Budr$^+$ (i.e., cycling cells) as well as Budr$^-$ (i.e., quiescent cells) have undergone induction of SM alpha actin synthesis relative to that of log phase cells ($P < 0.05$), and exhibit fractional SM alpha actin synthesis of 254.2 and 290.8%, respectively, compared with log phase cells.

To rule out the unlikely possibility that the induction of SM alpha actin synthesis occurs in subconfluent log phase S + G_2 cells as well as in confluent cycling cells, an additional experiment was performed in which log phase cells were

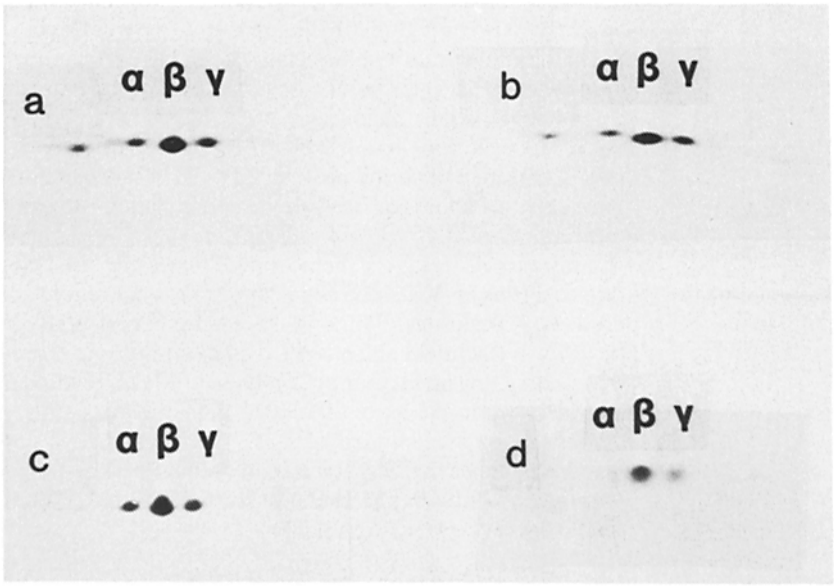

Figure 2. Autoradiographs of two-dimensional SDS gels showing the fractional isoactin synthesis of cultured SMC subpopulations sorted on the basis of DNA content: (*a*) confluent G_1; (*b*) confluent S + G_2; (*c*) confluent unsorted control; (*d*) unsorted log phase culture. Fractional isoactin synthesis of sorted cell populations was determined by densitometric analysis of autoradiographs as described in Materials and Methods. Note that confluent cells in the S and G_2 phases of the cell cycle (*b*) have undergone induction of SM alpha actin synthesis with respect to cells in log phase growth (*d*).

*Table I. Fractional Alpha Actin Synthesis of SMC Sorted on the Basis of DNA Content or Differential Incorporation of Budr**

	DNA			Budr	
	Confluent	Log phase		0–14-h preharvest‡	14–28-h preharvest§
G_1	227.9 ± 39.9 *n* = 4‖	91.6 *n* = 1	Budr$^+$	254.2 ± 30.6 *n* = 4	192.8 ± 24.0 *n* = 3
S + G_2	219.5 ± 51.7 *n* = 4	Undetectable *n* = 1	Budr$^-$	290.8 ± 34.6 *n* = 4	255.8 ± 48.7 *n* = 3
Unsorted	276.2 ± 66.6 *n* = 3	100.0 ± 6.1 *n* = 5	Unsorted	247.7 ± 36.5 *n* = 4	212.6 ± 18.4 *n* = 3

* Expressed as a percentage of log phase control; data presented as mean ± SEM.
‡ Cultures were pulsed with [^{35}S]methionine during the final 14 h of the 28-h Budr pulse.
§ Cultures were pulsed with [^{35}S]methionine during the first 14 h of the 28-h Budr pulse.
‖ *n*, number of independent experiments.

*Table II. Fractional Beta Actin Synthesis of SMC Sorted on the Basis of DNA Content or Differential Incorporation of Budr**

	DNA			Budr	
	Confluent	Log phase		0–14-h preharvest‡	14–28-h preharvest§
G_1	56.67 ± 3.15 *n* = 4‖	62.83 *n* = 1	Budr$^+$	55.12 ± 4.19 *n* = 4	58.52 ± 2.31 *n* = 3
S + G_2	54.56 ± 4.20 *n* = 4	65.77 *n* = 1	Budr$^-$	50.56 ± 2.98 *n* = 4	56.97 ± 4.41 *n* = 3
Unsorted	50.14 ± 5.48 *n* = 3	64.12 ± 0.80 *n* = 5	Unsorted	52.95 ± 2.63 *n* = 4	59.75 ± 1.58 *n* = 3

* Data presented as mean ± SEM.
‡ Cultures were pulsed with [^{35}S]methionine during the final 14 h of the 28-h Budr pulse.
§ Cultures were pulsed with [^{35}S]methionine during the first 14 h of the 28-h Budr pulse.
§ *n*, number of independent experiments.

sorted on the basis of DNA content. Fractional SM alpha actin synthesis of sorted G_1 and S + G_2 subpopulations was determined as above. Fractional SM alpha actin synthesis of log phase G_1 cells (91.6% of unsorted log phase control) was comparable to that of unsorted log phase cultures while fractional SM alpha actin synthesis of log phase S + G_2 cells was undetectable (Table I). These results eliminate the possibility that induction of SM alpha actin synthesis occurs in S + G_2 phases of the cell cycle, irrespective of culture density.

It should be noted that, for the purposes of procuring sufficient quantities of cycling cells, it was necessary in this

*Table III. Fractional Gamma Actin Synthesis of SMC Sorted on the Basis of DNA Content or Differential Incorporation of Budr**

	DNA			Budr	
	Confluent	Log phase		0–14-h preharvest‡	14–28-h preharvest§
G_1	19.38 ± 1.25 $n = 4$‖	27.34 $n = 1$	Budr$^+$	17.60 ± 2.44 $n = 4$	20.79 ± 2.63 $n = 3$
S + G_2	21.88 ± 2.40 $n = 4$	34.23 $n = 1$	Budr$^-$	17.74 ± 3.00 $n = 4$	15.57 ± 1.23 $n = 3$
Unsorted	20.23 ± 2.15 $n = 3$	25.12 ± 1.56 $n = 5$	Unsorted	20.50 ± 3.33 $n = 4$	17.43 ± 3.08 $n = 3$

* Data presented as mean ± SEM.
‡ Cultures were pulsed with [^{35}S]methionine during the final 14 h of the 28-h Budr pulse.
§ Cultures were pulsed with [^{35}S]methionine during the first 14 h of the Budr pulse.
‖ n, number of independent experiments.

study to sort cells early in the induction process. The subjective assessment of initial cell confluence by phase contrast microscopy undoubtedly contributed to high interexperiment variability in the degree of induction. Cultured SMC permitted to grow beyond this point underwent approximately one more doubling, reaching saturation density. The level of induction of SM alpha actin synthesis at saturation density is considerably higher than that reported in these studies for cells that had just reached confluency (Owens et al., 1986). To examine whether levels of induction in cell cycle subpopulations changed with time after initial establishment of confluency, the level of induction of SM alpha actin was compared between SMC cultures which were pulse labeled with [^{35}S]methionine during the initial 14 h of the 28-h Budr-labeling period and those labeled with [^{35}S]methionine during the latter 14 h of the Budr-labeling period. Induction of SM alpha actin synthesis was observed over both time intervals in both Budr$^+$ and Budr$^-$ cells, but the fractional SM alpha actin synthesis during the first half of Budr labeling was significantly lower in Budr$^-$ cells (255.8%) as compared with that of Budr$^-$ cells labeled later in the induction process (290.8%); $P < 0.05$; see Table I), indicating that increasing time of quiescence at confluence is associated with enhanced expression of SM alpha actin. No statistically significant differences were observed in Budr$^+$ or unsorted cells between the two pulse periods.

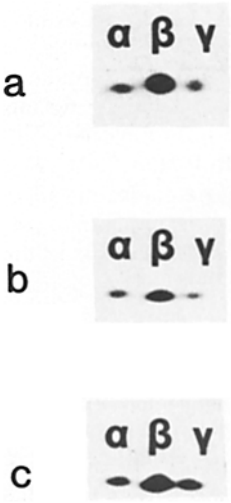

Figure 3. Autoradiographs of two-dimensional SDS gels showing the fractional isoactin synthesis of cultured SMC subpopulations sorted on the basis of differential Budr incorporation: (*a*) Budr$^+$; (*b*) Budr$^-$; (*c*) unsorted control. Isoactin synthesis was evaluated in cultures that had been pulsed with [^{35}S]methionine for 14 h before harvest. Results demonstrate that induction of SM alpha actin synthesis occurs in Budr$^+$ (cycling cells) relative to that of log phase cells (see Fig. 2 *d*), as well as in Budr$^-$ (quiescent) and unsorted cells.

Effects of Mitogenic Stimulation on SM Alpha Actin Expression in Postconfluent SMC

Since the preceding data strongly implicate a role for cell density in the expression of SM alpha actin that may be independent of cell cycle status, it was of further interest to ask whether postconfluent, quiescent SMC could be mitogenically stimulated, and if so, whether cell proliferation was associated with repression of SM alpha actin synthesis under these conditions. [^{3}H]thymidine-labeling indices and fractional SM alpha actin synthesis values of postconfluent SMC cultures exposed to FBS or PDGF are depicted in Fig. 4. (Autoradiographs of two-dimensional IEF/SDS gels are shown in Fig. 5.) Both PDGF and FBS were mitogenic for postconfluent SMC in SFM ($P < 0.05$), stimulating 31 and 61% of the cells to undergo DNA synthesis, respectively. Significantly, FBS, which promotes mitogenesis in the majority of cells was considerably less effective in repressing SM alpha actin synthesis (47% decrease) than was PDGF (75% repression), a less potent mitogen ($P < 0.05$).

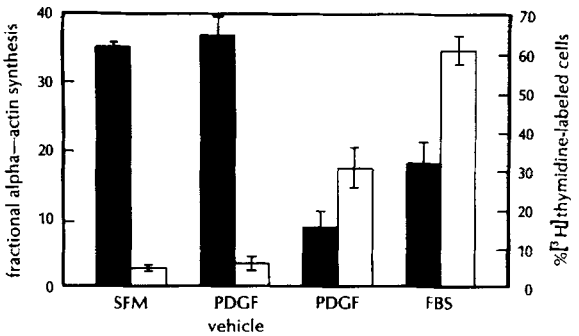

Figure 4. Effect of mitogens on S phase entry and SM alpha actin synthesis in postconfluent quiescent SMC cultures. SMC were plated as above and grown to confluency in the presence of 10% FBS and then switched to serum-free medium for 5 d. At the end of this period cultures were treated with either PDGF, serum, or vehicle for 24 h: parallel cultures were pulsed with either [^{3}H]thymidine for 24 h or [^{35}S]methionine for 6 h before harvest. Cultures pulsed with [^{3}H]thymidine were processed for autoradiography and the thymidine indices determined. These studies were carried out using a partially purified preparation of PDGF (6 ng/ml, 0.4% purity; a gift from Dr. Russell Ross). (*Solid bars*) Fractional alpha-actin synthesis; (*open bars*) % [^{3}H]thymidine-labeled cells.

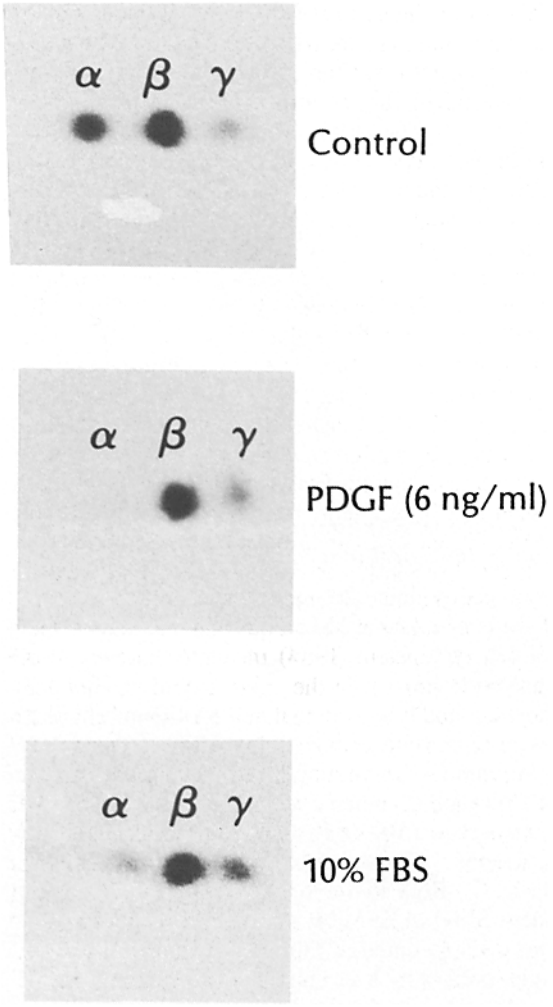

Figure 5. Autoradiographs of two-dimensional IEF/SDS gels from SMC treated with PDGF or FBS as described in Fig. 4.

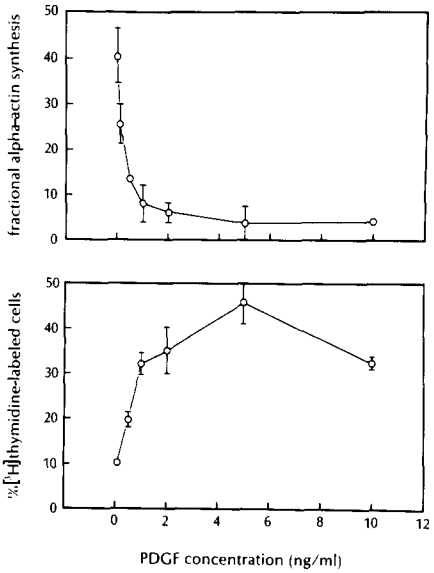

Figure 6. Concentration dependence of PDGF effect on SM alpha actin synthesis (*top*) and S phase entry (*bottom*). Cultures were treated identically to those in Fig. 4 with the exception that a highly purified PDGF (0.1–10.0 ng/ml; >95% purity) was used in this study.

To exclude the possibility that the observed effects of PDGF could be attributed to a contaminant of the partially purified PDGF preparation, it was necessary to test a highly purified preparation of PDGF on SM alpha actin expression under identical experimental conditions. Fig. 6 depicts the concentration dependence of both repression of SM alpha actin synthesis and [^{3}H]thymidine-labeling index using phenyl–Sepharose–purified human PDGF (>95% purity) from outdated human platelet-rich plasma (Raines and Ross, 1982). In this experiment, purified PDGF exhibited high potency and efficacy in repressing alpha actin synthesis (ED_{50} = 0.21 ng/ml) and stimulating S phase entry (ED_{50} = 0.63 ng/ml). ED_{50} values determined from an independent experiment in a different SMC line were 0.90 and 1.74 ng/ml, respectively (data not shown). Maximal effects of PDGF were seen at a PDGF concentration of 5 ng/ml which induced a 91% repression in fractional SM alpha actin synthesis and a 48-fold increase in the thymidine-labeling index.

The differential effects of PDGF versus FBS on repression of SM alpha actin synthesis and mitogenesis (Figs. 4 and 5) could be due to factors present in serum which inhibit mitogen-induced repression. To address this possibility we examined the ability of different concentrations of FBS to inhibit PDGF-induced repression of SM alpha actin synthesis (Fig. 7). Again PDGF and FBS exhibited differential effects on repression of SM alpha actin synthesis and thymidine indices ($P < 0.05$). However, co-treatment of SMC cultures with FBS (0.1–10.0%) and PDGF did not significantly affect the degree of mitogen-induced repression, though it should be noted that co-treatment of PDGF with 1 or 10% FBS resulted in an enhanced mitogenic response ($P < 0.05$). These results suggest that the differential efficacy of FBS and PDGF in repressing alpha actin synthesis cannot be accounted for by a serum inhibitor of PDGF-induced repression. However, due to inherent limitations in experiments involving undefined serum-containing media, these experiments are not definitive (see Discussion). Thus the effects of a possible serum-derived inhibitor, TGF-β, were examined in a defined serum-free medium. TGF-β was selected based on the following considerations: TGF-β is present in serum in relatively high concentrations and has been shown to affect SMC growth (Assoian and Sporn, 1986) and organization behavior (Majack, 1987), as well as myoblast differentiation (Massague et al., 1986; Olson et al., 1986). The results of a representative experiment are shown in Table IV. Results demonstrated that co-treatment with TGF-β partially inhibited the mitogenic response for both PDGF (24%; $P < 0.05$) and FBS (25%; $P < 0.05$) but did not significantly affect the repression of SM alpha actin synthesis by either mitogen. Interestingly, TGF-β alone elicited a 25% increase in the fractional synthesis of SM alpha actin.

Discussion

It has been established from previous studies that growth arrest of SMC is associated with induction of SM-specific contractile proteins (Strauch and Rubenstein, 1984; Owens et al., 1986; Rovner et al., 1986). At confluence, SMC exhibit

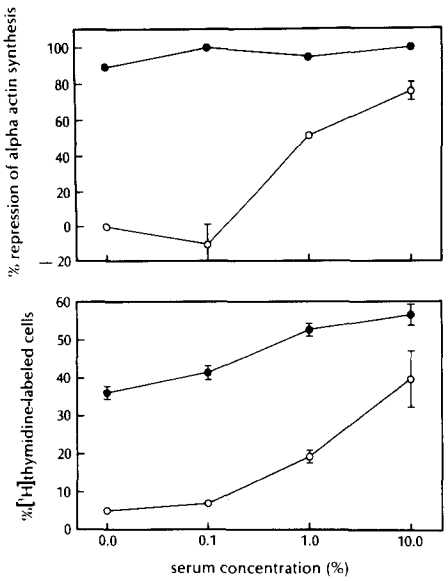

Figure 7. Effect of FBS on PDGF-induced repression of SM alpha actin synthesis (*top*) and stimulation of S phase entry (*bottom*). Postconfluent SMC cultures in SFM were treated with FBS (0.1–10%) with and without PDGF (10 ng/ml). Cultures were pulsed with [^{3}H]thymidine and [^{35}S]methionine and harvested as described in Fig. 4. Similar results were obtained in a separate independent experiment. (*Open circles*) FBS + PDGF vehicle; (*solid circles*) FBS + PDGF.

contact inhibition of growth and undergo rapid induction of SM alpha actin synthesis. Whereas these observations suggested a causal relationship between cell cycle withdrawal and the induction of SM alpha actin synthesis, results of the present study clearly demonstrate that cell cycle withdrawal is not a prerequisite for induction of SM alpha actin synthesis in cultured SMC at confluency since both definitively cycling (S + G_2) as well as Budr$^+$ cells are induced as compared with subconfluent cells. Results suggest an important role for cell density or cell-cell contact in control of SM alpha actin expression. This is consistent with observations by Strauch and Rubenstein (1984) who found that cell confluence was a prerequisite for the induction of alpha actin synthesis in the BC_3H1 cell line, although BC_3H1 cells show an attenuated induction of alpha actin synthesis at confluency in the presence of serum unlike rat aortic SMC used in the present studies.

*Table IV. The Effect of TGF-β on the Repression of Alpha Actin Synthesis and S Phase Entry Induced by PDGF and FBS**

Treatment	Percent repression of alpha actin synthesis‡	Percent [^{3}H]thymidine-labeled cells
SFM	0.00 ± 11.00§[b]	4.97 ± 0.37[d]
PDGF (10 ng/ml)	97.66 ± 1.09[a]	41.31 ± 2.47[b]
FBS (10%)	71.85 ± 4.95[a]	49.34 ± 0.64[a]
TGF-β (2.5 ng/ml)	−25.31 ± 4.92[c]	5.26 ± 1.34[d]
PDGF + TGF-β	90.37 ± 3.09[a]	31.58 ± 1.46[c]
FBS + FGF-β	86.06 ± 7.39[a]	37.11 ± 2.58[b]

* Data presented as mean ± SEM of one representative experiment. Actin synthesis determinations were performed in duplicate; [^{3}H]thymidine-labeling index in quadruplicate.
‡ Expressed as a percentage of appropriate control.
§ Means with different letter designations are significantly different (Newman-Keuls Test; $P < 0.05$).

Though our data indicate that cell cycle withdrawal is not a prerequisite for induction of SM alpha actin synthesis at confluency, we cannot rule out the possibility that induction of SM alpha actin synthesis in cycling cells at confluency is a "programmed" event associated with cell cycle withdrawal but preceding it by a fixed interval. The finding that induction precedes cell cycle withdrawal has precedent since other workers have discovered the expression of differentiated characteristics in erythroblasts before cell cycle withdrawal (Campbell et al., 1971). Furthermore, other studies have demonstrated the association of cell-cell contact at confluency with cytodifferentiation (Rodesch, 1973; Munson et al., 1982; Strauch and Rubenstein, 1984; Owens et al., 1986). However, the present study is to our knowledge the first to demonstrate the dissociation of contact inhibition of growth from contact-induced cytodifferentiation.

It should be noted, however, that our data, as well as those of Strauch and Rubenstein (1984) indicate that the growth state of SMC is important in the control of cytodifferentiation. In previous studies we found that induction of SM alpha actin also occurs in subconfluent cultures of rat aortic SMC subjected to prolonged growth arrest in a defined serum-free medium. Furthermore, in these previous studies, the level of induction increases with time in association with decreases in the fraction of replicating cells. Results of present studies indicate that quiescent, postconfluent SMC in culture can initiate DNA replication upon stimulation with mitogenic agents (FBS and PDGF), and that mitogenesis is associated with marked repression of SM alpha actin synthesis. PDGF is an extremely potent repressor of SM alpha actin synthesis, with half-maximal repression occurring at picomolar concentrations.

Interestingly, PDGF was substantially more effective in repressing SM alpha actin synthesis than was FBS for a given level of mitogenic stimulation. One possibility is that the lower potency of serum relative to its mitogenic activity may relate to certain as yet unidentified factors present in serum which promote differentiation in cultured SMC. The results of the present study, however, indicate that one putative factor that might have this activity, TGF-β, does not antagonize either the PDGF- or FBS-induced repression of alpha actin synthesis, though TGF-β alone elicits a small increase in the expression of SM alpha actin synthesis. Furthermore, results from FBS-PDGF co-treatment experiments suggest that serum does not contain an inhibitor of mitogen-induced repression in that 0.1–10.0% FBS failed to inhibit PDGF-induced repression. However, these experiments are not definitive in this regard, largely due to inherent limitations of experiments using serum-containing media. For example, the enhanced mitogenic response observed with FBS (1 or 10%) and PDGF, or the high dose of PDGF used, may have obscured the effects of a putative inhibitor. Alternatively, the principal mitogens in FBS may simply be less effective repressors of alpha actin synthesis than is PDGF. A final possibility is that PDGF may have been more effective than FBS in inducing a G_0–G_1 transition or "competence state" but not as effective in promoting progression to S phase, consistent

with observations that repression of muscle creatine phosphokinase by anionic brain FGF in BC_3H1 cells does not require mitogenesis (Lathrop et al., 1985). If the repression of SM alpha actin synthesis after mitogenic stimulation accompanies G_0–G_1 transition, this might be invoked to explain the differential effects of PDGF versus FBS on SM alpha actin synthesis.

Results of this study suggest that an important action of potent growth factors such as PDGF may be in the control of SM differentiation. The possible function of PDGF in the regulation of SM differentiation is suggested by its altered expression in developmental and pathological situations. Cultured SMC derived from young but not old rats express high levels of PDGF-like mitogens (Seifert et al., 1984). Walker et al. (1986) have reported that the production of a PDGF-like mitogen by SMC derived from intimal lesions is fivefold that produced by cells derived from normal media. Recently, Barrett and Benditt (1987) demonstrated increased *sis* gene transcript levels in human atherosclerotic lesions compared with normal arteries which suggests that increased production of the PDGF b-chain by lesion cells may play an important role in atherogenesis. Clearly, the emerging role of PDGF in the control of SM differentiation warrants further investigation of the mechanism by which mitogens regulate the expression of SM-specific gene products.

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