Arterial Smooth Muscle Cells in Vivo: Relationship Between Actin Isoform Expression and Mitogenesis and their Modulation by Heparin

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Abstract. Quiescent smooth muscle cells (SMC) in normal artery express a pattern of actin isoforms with α-smooth muscle (αSM) predominance that switches to β predominance when the cells are proliferating. We have examined the relationship between the change in actin isoforms and entry of SMC into the growth cycle in an in vivo model of SMC proliferation (balloon injured rat carotid artery). αSM actin mRNA declined and cytoplasmic (β + γ) actin mRNAs increased in early G_0/G_1 (between 1 and 8 h after injury). In vivo synthesis and in vitro translation experiments demonstrated that functional αSM mRNA is decreased 24 h after injury and is proportional to the amount of mRNA present. At 36 h after injury, SMC prepared by enzymatic digestion were sorted into G_0/G_1 and S/G_2 populations; only the SMC committed to proliferate (S/G_2 fraction) showed a relative slight decrease in αSM actin and, more importantly, a large decrease in αSM actin mRNA. A switch from αSM predominance to β predominance was present in the whole SMC population 5 d after injury. To determine if the change in actin isoforms was associated with proliferation, we inhibited SMC proliferation by ~80% with heparin, which has previously been shown to block SMC in late G_0/G_1 and to reduce the growth fraction. The switch in actin mRNAs and synthesis at 24 h was not prevented; however, αSM mRNA and protein were reinduced at 5 d in the heparin-treated animals compared to saline-treated controls. These results suggest that in vivo the synthesis of actin isoforms in arterial SMC depends on the mRNA levels and changes after injury in early G_0/G_1 whether or not the cells subsequently proliferate. The early changes in actin isoforms are not prevented by heparin, but they are eventually reversed if the SMC are kept in the resting state by the heparin treatment.

Actin exists in six different, highly conserved isoforms that are expressed in unique patterns depending upon the type and growth state of the individual cell (35, 38, 40). It is known that arterial smooth muscle cells (SMC)[1] from mature animals express predominantly the α-smooth muscle (αSM) actin isoform (15, 41). This pattern is altered to β actin predominance when SMC proliferate under a variety of normal and pathological conditions as well as in culture (1, 14, 20–23, 31, 32, 34, 37–39).

The relationships between growth state and expression of actin isoforms in SMC is presently not clear. Although proliferating SMC in vivo eventually re-express αSM actin as they return to quiescence (22), cultured SMC growth arrested by serum starvation show slight re-expression of αSM actin and usually do so only if they are also in a postconfluent state (32, 34). Even so, the expression of αSM actin is never as great as it is in vivo. Owens et al. (32) have reported that after the addition of serum to passaged growth-arrested SMC, αSM actin synthesis declines before the cells enter S phase; Skalli et al. (34) observed that among primary cultured SMC entering the growth cycle for the first time, only those in S phase show a decrease in αSM actin.

To examine the regulation of actin isoform expression in arterial SMC stimulated to enter the growth cycle, we have made use of an in vivo model of SMC mitogenesis. In the adult rat carotid, SMC proliferation is barely detectable (0.06% per day; 9); between 24 and 27 h after endothelial removal by the passage of an inflated intraluminal balloon catheter, ~30% of the cells enter S phase as a synchronous wave (7, 8, 27).

We now demonstrate that changes in actin isoform mRNA level and synthesis occur long before SMC enter S phase and can be dissociated from entry into S phase with the growth inhibitor heparin. In the absence of heparin, changes in actin isoform mRNA level and expression are mostly seen in SMC

1. *Abbreviations used in this paper*: αSM, α-smooth muscle; SMC, smooth muscle cells.

entering the S and G_2 phases but not in those remaining in G_0 or G_1. Furthermore, arteries in which SMC proliferation is partially inhibited by heparin exhibit re-expression of the differentiated phenotype as early as 5 d after injury.

Materials and Methods

SMC Mitogenesis in Vivo

SMC were induced to proliferate in the left carotid arteries of male Wistar rats (between 400 and 500 g) by denuding the endothelium and stretching the wall with a balloon catheter (9). In this arterial injury model, there is also some loss of SMC from the inner layer of the media (9). For the experiments involving protein, DNA, or RNA extraction, injured left carotids and uninjured right carotids were removed at various times after surgery, flushed with saline solution, stripped of adventitia and frozen in liquid nitrogen.

SMC proliferation was inhibited by the administration of heparin (6–8, 27). Subcutaneous miniosmotic pumps (model 2ML2; Alza Corp., Palo Alto, CA) connected to indwelling left jugular venous catheters were placed either 24 h before or just after carotid injury; heparin (type II; Sigma Chemical Co., St. Louis, MO) was delivered continuously and intravenously at the rate of 0.3 mg/kg/per h. Control animals received a continuous infusion of the carrier solution (normal saline).

Cell Sorting

Populations of quiescent (G_0/G_1) or proliferating (S/G_2) SMC were obtained by enzymatic digestion of injured carotids and cell sorting of the SMC at 36 h after surgery. At this time, cells committed to enter the cycle (20–30% of the SMC) are in S or G_2 phases but have not divided (27); as well, very few of the remaining G_0/G_1 cells (70–80% of the SMC) subsequently proliferate (7, 8). For each experiment, 10 injured carotids were excised, irrigated clear of blood, stripped of adventitia, and minced. The tissue was digested in medium containing collagenase and elastase for ~2 h at 37°C as described previously (34). FCS (final concentration 20%) was added and the solution filtered through nylon mesh. The isolated cells were centrifuged at 200 *g*, resuspended in PBS containing 1% albumin, and stained with Hoechst dye for 20 min. Less than 5% of the cells were stained positive with trypan blue. The cells were again pelleted, resuspended in PBS, and sorted into G_0/G_1 or S/G_2 populations (34). The isolated cells were pelleted, resuspended in 50 μl of sample buffer, and stored at −20°C for two-dimensional gel analysis.

SMC Proliferation

To quantitate SMC proliferation, animals received [^{3}H]thymidine (5.0 Ci/mM; Amersham Corp., Zürich, Switzerland), 0.5 mCi/kg i.p. at 17, 9, and 1 h before they were killed; carotids from these animals were fixed by perfusion in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned, and processed as described previously for autoradiography (9). Slides dipped in emulsion (NTB2; Eastman Kodak Co., Rochester, NY) were developed after 2 wk and the fraction of labeled nuclei determined.

RNA Extraction

Frozen carotid arteries were pooled (10–15 carotids per time point) and rapidly homogenized with a polytron (type PT 10/35; Kinematica, Lucerne, Switzerland) for 60 s in 3.5 ml of a sterile solution, pH 7.4, containing 4.5 M guanidinium thiocyanate, 50 mM EDTA, 25 mM sodium citrate, 0.1 M 2-β-mercaptoethanol, and 2% laurylsarcosine. The preparations were further homogenized with a syringe attached to a 21-gauge needle. The homogenates were centrifuged for 10 min at 5,900 *g* at 15°C and the supernatant further purified by ultracentrifugation through a cushion of 5.7 M CsCl as described by Chirgwin et al. (5). The RNA pellets were resuspended in 10 mM Tris-HCl, pH 7.4, 0.5% SDS, and 1 mM EDTA, extracted twice with saturated phenol-chloroform, and then extracted once with chloroform-isoamylalcohol (24:1; vol/vol). The RNA was ethanol precipitated, resuspended in sterile water, and stored at −70°C.

RNA from cytofluorometrically isolated SMC was purified as follows: SMC were suspended in PBS (2×10^6 cells/ml); 8×10^4 cells (40 μl) were lyzed in 0.1 M Tris-HCl buffer, pH 7.4, containing 360 μl of 4 M guanidine thiocyanate and 1 M β-mercaptoethanol. After addition of 20 μg of randomized *Escherichia coli* RNA as carrier, the nucleic acids were precipitated in ethanol and purified as described by Huarte et al. (18).

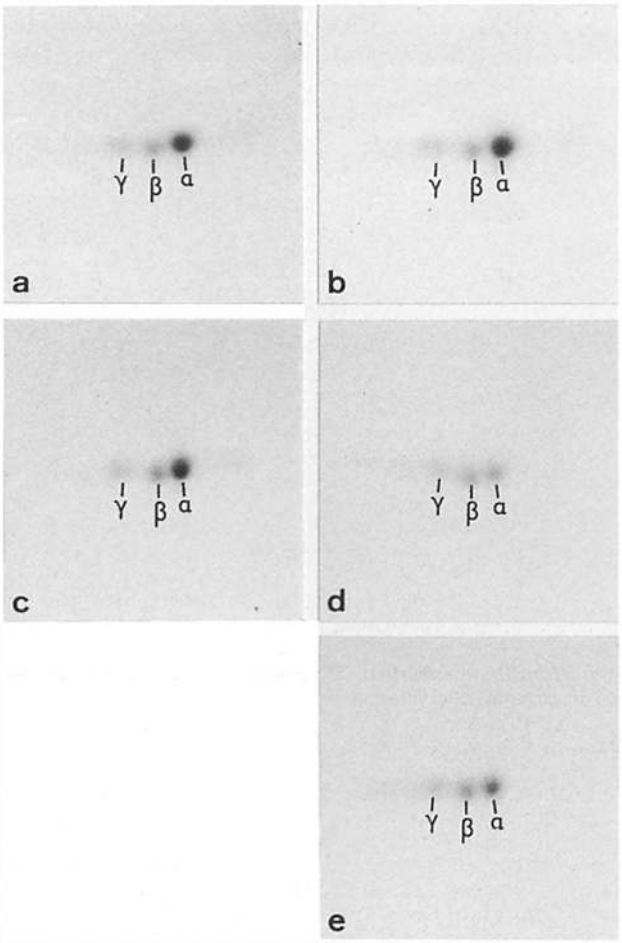

Figure 1. Two-dimensional gel electrophoresis of total protein extracts from injured rat carotids at 0 h (*a*), 8 h (*b*), 24 h (*c*), 5 d (*d*), and 5 d heparin-treated (*e*). Only the actin isoforms (α, β, γ) are shown. Note the decrease in α and increase in β at 5 d and the reversal in the heparin-treated vessel.

Northern Blot Hybridization

Total RNAs (2 μg/lane) were denatured with glyoxal and were subjected to electrophoresis in 1% agarose gels in 10 mM phosphate buffer, pH 6.8; gels were stained with acridine orange and examined under UV light. The RNAs were then transferred to Biodyne filters (Pall Corp., Glen Cove, NY); the filters were baked for 2 h under vacuum at 80°C. The Northern blots were prehybridized for 4 h at 58°C in 50% deionized formamide, 50 mM Na Pipes buffer, pH 6.8, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5× Denhardt's solution (28), and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out under the same conditions with SP6-RNA polymerase–transcribed ^{32}P-labeled cRNA probes according to Melton et al. (29). The probes used were derived either from the coding region (total actin probe [pRAoαA-C]: a 320-bp Bgl II–Ava II fragment corresponding to the sequence coding for the amino acids 185–291) or from the 3′ untranslated region of the αSM actin mRNA (αSM probe [pRAoαA-3′UT]: a 130-bp Dde I–Hind III fragment; 21). The Northern blots were washed twice for 20 min at 58°C in 3× SSC and 2× Denhardt's solution, and subsequently in 0.2× SSC, 0.1% SDS, and 0.1% Na-pyrophosphate, pH 7.0, for 20 min at the same temperature for the total actin probe or for three washes 20 min each at 78°C for the αSM actin probe. The filters were dried and exposed to Kodak X-Omat SO-282 film at −70°C between intensifying screens. Films were exposed between 1 and 3 d and analyzed by means of computerized densitometric scanning. We have previously shown that there is a good correlation between densitometry and scintillation counting (1, 20). The fractions of αSM and β actin + γ actin mRNAs as a percentage of total actin mRNA were obtained by calculating the ratio between the 1.7- or 2.1-kb band and

Table I. Actin Isoform Expression in Normal and in Injured Carotid Artery

Time after injury	Actin isoforms α	β	γ
Normal artery	81.2 ± 1.0	13.9 ± 0.8	4.9 ± 2.0
8 h	88.2 ± 2.9	9.2 ± 3.2	2.7 ± 0.3
24 h	79.7 ± 1.4	16.7 ± 1.3	3.7 ± 1.7
5 d	45.0 ± 3.0	39.3 ± 0.7	15.7 ± 2.6
5 d, saline-treated	44.3 ± 2.5	43.4 ± 4.7	12.3 ± 2.5
5 d, heparin-treated	65.3 ± 4.9	24.1 ± 8.5	10.7 ± 3.5

All values are mean (% of total actin) ± SD; $n = 2$.

the sum of the 1.7- and 2.1-kb bands on Northern blots hybridized with pRAoαA-C. The percentage of αSM and of β actin + γ actin mRNAs were calculated by arbitrarily defining 100 values of the respective bands of control at 0 h for each experiment.

In Vitro Translation of Total RNA

In vitro translation of total rat carotid RNA was conducted according to the protocol of Pelham and Jackson (33) using rabbit reticulocyte lysate (Genofit, Geneva, Switzerland) and ^{35}S-labeled methionine (Amersham Corp., Zürich, Switzerland; 1). The products were analyzed by one- and two-dimensional gel electrophoresis.

Protein Synthesis In Vivo

Six rats underwent left carotid injury and insertion of Alzet pumps containing either heparin or saline as described above. In addition, a percutaneous indwelling catheter was placed in the right jugular vein and connected to a syringe infusion pump. The animals were left unrestrained. 18 h after surgery, when the animals were awake and eating, an infusion of [^{35}S]methionine (3.0 mCi/ml in lactated Ringer's solution; prepared according to the method of Crawford and Gesteland [10]) was started and continued for 6 h at an infusion rate of 0.8 ml/h (1). At the end of 6 h blood was collected in heparinized tubes for determination of free plasma methionine (1). The carotids were excised, irrigated with saline, and frozen in liquid nitrogen. Previous studies (1) have shown that this synthesis protocol produces a stable blood level of [^{35}S]methionine for at least 5 h.

SDS-PAGE

Extracts of tissue were prepared by dissolving carotids in sample buffer containing 1% SDS and 1% dithiothreitol (DTT). These tissue extracts and the products of in vivo synthesis, in vitro translation, or cell sorting, were stored at −20°C. For SDS-PAGE, these extracts were diluted 1:2 in sample buffer containing 1% SDS, 1% DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na-*P*-Tosyl-L-arginine methylester in 0.0625 M Tris-HCl, pH 6.8, as previously described (23, 24), and 40 μg of protein were loaded on 10% gels with a 3% stacking gel under reducing conditions (24). For two-dimensional gel electrophoresis, the extracts were diluted 1:5 with buffer A according to the method of O'Farrell (30), and between 20 and 50 μg of protein were loaded. The pH gradient was established with 6% preblended ampholines, pH 4.0–6.5 (Pharmacia Fine Chemicals, Lucerne, Switzerland). The gels were focused at 1,000 V overnight. The second dimension was run on 10% polyacrylamide gels. For quantification, the gels of total carotid protein extract were stained with Coomassie Blue, and the relative proportions of actin isoforms were quantified by densitometry (23). Gels of products of in vivo synthesis or in vitro translation were dried and exposed to Kodak X-Omat SO-282 film. The relative percentage of actin isoforms was determined by densitometry (1, 23).

Results

In normal, uninjured carotid arteries the αSM isoform accounts for ~80% of the actin protein (Fig. 1; Table I). The proportion of αSM actin mRNA is even greater (90–95% of actin mRNA; Fig. 2). The proportion of αSM actin synthesized in vivo or obtained from in vitro translation of total RNA is markedly less than that for the proteins (Figs. 3 and 4; Table II). These results were reported previously for the rat aorta (1) and suggest that (*a*) some of the αSM messages are not translated, and (*b*) the turnover of the αSM isoform is slower than that of the other actin isoforms.

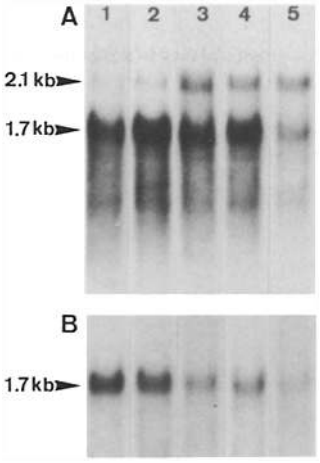

Figure 2. Autoradiogram of Northern blots of total RNA (2 μg/lane) from injured arteries at 0 h (lane *1*), 1 h (lane *2*), 8 h (lane *3*), 24 h (lane *4*), and 5 d (lane *5*). (*A*) After hybridization with pRAoαA-C (total actin probe); note increase in cytoplasmic actin mRNA band (2.1 kb) and decrease in α actin band (1.7 kb) at 8 h, 24 h, and 5 d. (*B*) After hybridization with pRAoαA-3′UT (α actin probe); note decrease in αSM actin at 8 h and after.

Although SMC in injured rat carotid artery start to synthesize DNA between 24 and 27 h after surgery (27), the change in actin isoforms from α to β predominance associated with proliferation occurred between 1 and 5 d (Fig. 1; Table I).

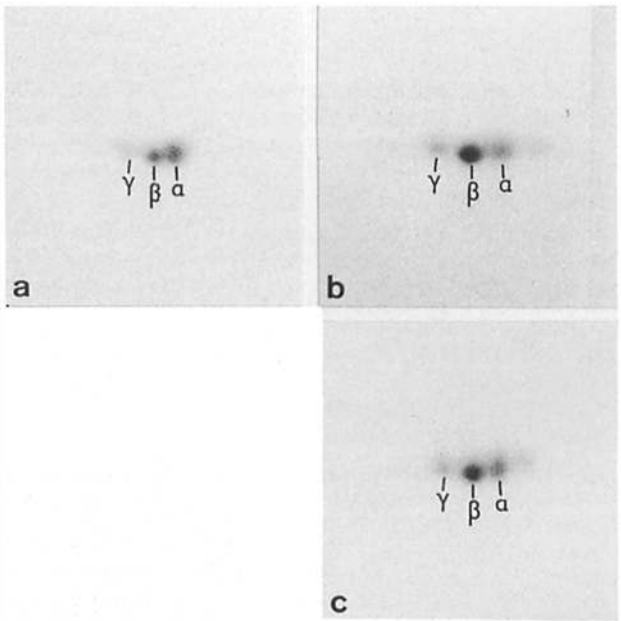

Figure 3. Autoradiogram of two-dimensional gel electrophoresis of total protein extracts from injured or normal carotid arteries of rats infused with [^{35}S]methionine and either saline or heparin. *a*, normal carotid; *b*, injured carotid from saline-treated rat at 24 h; *c*, injured carotid from heparin-treated rat at 24 h. Note the decrease in α and increase in β actin synthesis in the injured carotids. There are no significant differences between the saline- and heparin-treated injured carotids. To visualize γ actin, the gel in *a* has been slightly overexposed, thus resulting in some overlapping of α and β spots.

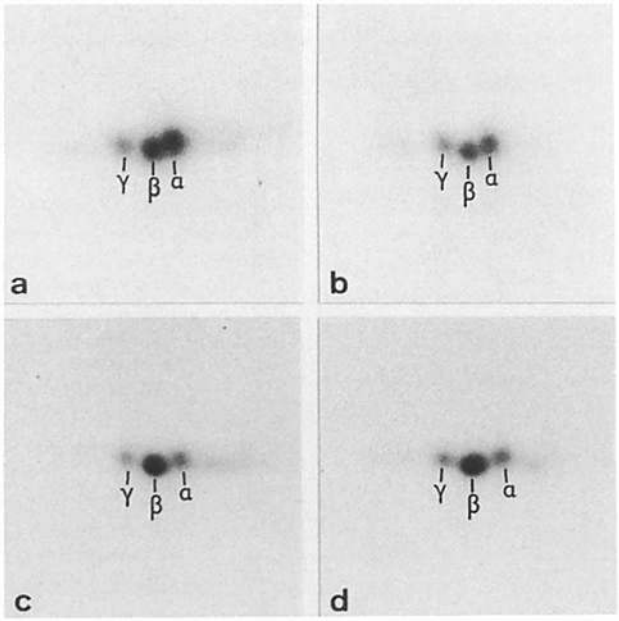

Figure 4. Autoradiogram of two-dimensional gel electrophoresis of products of in vitro translation of total injured carotid RNA. *a*, 0 h; *b*, 1 h; *c*, 8 h; and *d*, 24 h. Note the change in actin isoforms between 1 and 8 h after injury. To visualize γ actin, gels in *a* and *b* have been overexposed thus resulting in some overlapping of α and β spots.

During this period, no changes were observed in the total actin content on one-dimensional gels; actin remained between 12 and 15% of total protein (data not shown). Nevertheless, the relative synthesis of the αSM actin isoform decreased and that of the β actin isoform increased by 24 h (Fig. 3; Table II). Results of in vitro translation experiments (Fig. 4; Table II) confirmed the synthesis experiments. Northern blot hybridization showed that changes in actin isoform synthesis and in vitro translation corresponded to variation of the relative amounts of αSM and β actin + γ actin mRNAs (Fig. 2; Table III). The change in mRNAs occurred between 1 and 8 h (Fig. 2; Table III).

To know whether only those SMC committed to enter the cell cycle exhibit the change in actin isoform expression, we removed the injured carotids at 36 h and sorted SMC into G_0/G_1 and S/G_2 populations. The relative proportions of the actin isoforms were measured on two-dimensional gels and the content of actin mRNA was measured by hybridization of Northern blots with the αSM probe (Fig. 5; Table IV). The available evidence supports the view that SMC commit to enter the growth cycle shortly after injury and do so as a synchronous wave; those cells remaining in the resting state do not appear to start proliferating at some later time (7, 8, 27). Given these special characteristics of SMC hyperplasia in the injured carotid and in analogy to previous in vitro experiments (34), we selected 36 h as a moment when most of the cells committed to proliferation would be in S or G_2 but would not have reached G_0/G_1 again. We obtained an S/G_2 population between 20 and 30% that corresponds closely with the measured labeling index and the growth fraction (8, 27). Two-dimensional gel analysis demonstrated a slight but detectable decrease in the proportion of αSM actin in the

Table II. In Vivo Actin Synthesis and In Vitro Translation of Actin mRNA

Time after injury	α	β	γ
In vivo synthesis:			
Normal carotid	52.9 ± 1.5	34.9 ± 1.9	12.2 ± 0.4
24 h, saline-treated	31.5 ± 4.3	54.3 ± 4.1	14.1 ± 0.2
24 h, heparin-treated	37.4 ± 2.0	48.0 ± 1.6	14.6 ± 1.5
In vitro translation			
0 h	46.9 ± 5.2	38.7 ± 0.1	14.4 ± 5.4
1 h	43.1 ± 2.3	42.3 ± 1.2	14.5 ± 1.1
8 h	20.2 ± 1.3	65.6 ± 1.3	14.1 ± 1.9
24 h	21.5 ± 1.3	65.4 ± 1.8	13.1 ± 0.7

All values are mean (% of total actin) ± SD; $n = 2$ for translation experiments and $n = 5$ for synthesis experiments. All values are normalized according to the difference in methionine content between αSM (15 Met) and cytoplasmic (16 Met) actins (41).

S/G_2 cells compared to the G_0/G_1 cells (Fig. 5; Table IV) which had an actin isoform pattern identical to that of freshly isolated SMC (data not shown). Northern blot hybridization demonstrated that the content of αSM actin mRNA was lower in S/G_2 cells compared to the G_0/G_1 cells (Fig. 5 *c*).

To investigate whether the switch in the SMC program of actin isoform synthesis was tightly linked to entry into S phase, we inhibited G_0/G_1–S transition with heparin. Previous experiments with the balloon injury model have demonstrated that heparin blocks ~50% of the stimulated SMC in late G_0/G_1 and reduces the thymidine labeling index and the growth fraction (6, 7, 27). These results were confirmed in the present series of experiments and showed that the thymidine labeling index between 24 and 48 h was reduced by heparin treatment (control: 10.2 ± 2.3, $n = 4$; heparin: 1.2 ± 0.9, $n = 5$). However, the change in actin mRNAs and actin isoform synthesis (Table II) at 24 h was not prevented by a continuous infusion of heparin beginning before the time of carotid injury; for both control- and heparin-treated arteries, αSM mRNA declined by ~50% and cytoplasmic mRNA increased ~ twofold (Fig. 6; Table III). At 24 h, the synthesis of αSM actin declined and that of β actin increased in injured vessels of saline-treated and heparin-treated arteries compared to normal artery (Fig. 3; Table II). At this time, there was no significant difference between the levels of αSM and cytoplasmic actins in heparin-treated compared to saline-treated arteries. On the other hand, αSM actin mRNA and protein were reinduced by 5 d in arteries of heparin-treated

Table III. Expression of αSM and β Actin + γ Actin mRNAs in Normal and Injured Carotid Artery with or without Heparin Treatment

Time after injury	αSM*	β + γ*
1 h	100 ± 2	100 ± 30
8 h	60 ± 26	300 ± 130
24 h saline	54 ± 21	216 ± 22
24 h heparin	46 ± 12	180 ± 62
5 d saline	14 ± 4	160 ± 62
5 d heparin	46 ± 10	104 ± 67

* Calculated as percentage of the values at 0 h. All values are mean ± SD; $n = 3$.

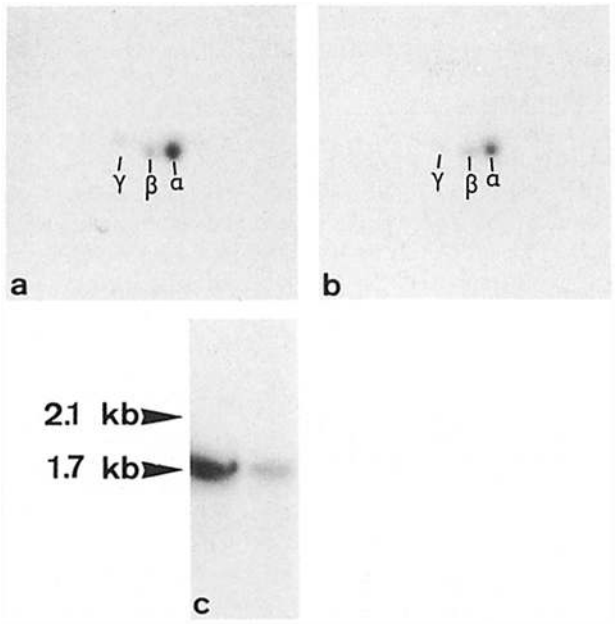

Figure 5. Two-dimensional gel electrophoresis of total protein extracts from SMC sorted into G_0/G_1 (*a*) and S/G_2 (*b*) populations at 36 h after injury. In *c*, an autoradiogram of a Northern blot of total RNA (2 μg/lane) from G_0/G_1 (*left*) and S/G_2 (*right*) SMC, 36 h after endothelial removal, has been hybridized with pRAoαA-3'UT (αSM probe); note the decreased α actin mRNA band in S/G_2 cells compared with G_0/G_1 cells.

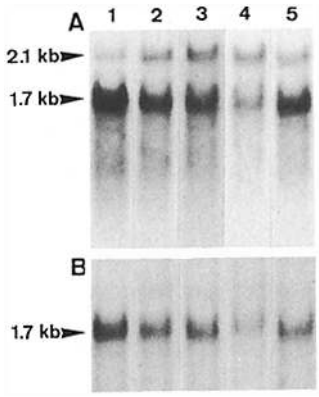

Figure 6. Autoradiogram of Northern blots of total RNA (2 μg/lane) from injured carotid arteries of saline- and heparin-treated rats after hybridization with pRAoαA-C (*A*) or pRAoαA-3'UT (*B*). Lane *1*, uninjured carotid; lane *2*, 24 h, saline-treated; lane *3*, 24 h, heparin-treated; lane *4*, 5 d, saline-treated; and lane *5*, 5 d, heparin-treated. Note decrease in α and increase in cytoplasmic actin mRNAs in both saline- and heparin-treated carotids at 24 h and a reinduction of α actin mRNA at 5 d in the heparin-treated carotids.

rats compared to saline-treated rats (Figs. 1 and 6; Tables I and III); in heparin-treated arteries, αSM mRNA increased ~ threefold over saline-treated rats, and cytoplasmic mRNAs declined slightly (20–50%).

Discussion

In the arterial wall of mature animals, SMC quiescence is characterized by a typical pattern of actin isoform expression (α predominance; 20, 31, 41). SMC proliferation is associated with a switch to a phenotype similar to that of SMC in developing arteries (β predominance; 20). Proliferating SMC show a decrease in smooth muscle–specific contractile proteins and an increase in nonmuscle actin and myosin isoforms (20, 31). Although it has been suggested that these changes are a prerequisite for proliferation (4), the biochemical evidence in support of this hypothesis has not been conclusive. In primary culture of SMC, αSM actin content declines only after the cells have entered S phase (34); αSM actin synthesis decreases before S phase (1) despite the fact that the relative proportion of αSM actin mRNA remains similar to that present in SMC of normal aortic media (21). Owens et al. (32) have reported that passaged rat aortic SMC growth arrested in serum-free medium synthesize more αSM actin than when they are proliferating. When growth-arrested SMC are stimulated with serum, αSM actin synthesis decreases before the cells enter S phase. These results suggest that early after stimulation but before DNA replication, αSM synthesis but not αSM mRNA and protein content decreases in cultured rat aortic SMC. A similar observation has been made by Wice et al. (43) in the SMC-like cell line BC3H1 stimulated by FCS or fibroblast growth factor. However Wang and Rubenstein (42) have found in the same cell line that epidermal growth factor inhibits the synthesis of αSM actin together with the expression of its mRNA. In summary, the in vitro studies of aortic SMC and BC3H1 cells suggest that after the addition of mitogen, the repression of αSM actin synthesis can occur at the transcriptional and/or translational levels.

Table IV. Actin Isoforms in SMC Sorted into G_0/G_1 and S/G_2 Populations at 36 h after Carotid Injury

SMC population	α	β	γ
G_0/G_1	86.1 ± 1.0	11.9 ± 1.0	2.0 ± 0.5
S/G_2	76.8 ± 1.5*	17.2 ± 1.5	6.0 ± 0.5

All values are mean (% of total actin) ± SD; $n = 2$.
* Significantly different ($p < 0.05$ using t test) from values of G_0/G_1 SMC.

In the present series of experiments, we decided to study the changes in SMC actin isoforms with proliferation in vivo because of some advantages of the balloon carotid model. For example, previous in vivo work has demonstrated that αSM actin expression is linked to quiescence (1, 20, 21, 31), but in vitro quiescence is difficult to define. It can be produced by withdrawal of serum or by overcrowding and postconfluence, but in every instance the actual level of thymidine labeling (1–5%) is greater than in vivo (~0.06%). Furthermore, although in normal artery SMC express predominantly αSM actin, in vitro quiescent SMC do not (20, 32). In addition to being truly quiescent at the outset, SMC in the in vivo arterial injury model proliferate as a synchronous wave. Proliferation takes place in three dimensions as opposed to two in vitro; this might be of some importance for pathological situations. For these reasons, we thought that the in vivo studies, although more difficult to conduct than in vitro ones, would give a clearer picture of how the actin isoforms are regulated as SMC enter the growth cycle.

In the injured rat carotid model of SMC mitogenesis, the content of αSM actin did not decline until 5 d after carotid injury, however αSM actin mRNA levels, αSM actin synthesis, and translation of αSM actin mRNA all declined before 24 h after injury, suggesting the possibility of transcriptional

or posttranscriptional control. Our in vivo experiments demonstrated a parallel decline in αSM actin mRNA and newly synthesized αSM actin contrary to what was previously observed in primary cultures of SMC (21). The decline in αSM actin mRNA corresponded to an increase in β and γ mRNA. The increase in β actin mRNA and synthesis seems to be a general property of not only arterial SMC but also other cells entering the growth cycle and might play a central role in the regulation of proliferation (11, 16, 17, 25, 43). It is possible, however, that the increased expression of the cytoplasmic actins is not linked to the down regulation of αSM actin, since Wice et al. (43) found that the addition of fibroblast growth factor to quiescent BC3H1 cells does not alter cytoplasmic actin synthesis although αSM actin synthesis is decreased. The first small but significant changes in the proportion of actin isoforms were detected in SMC committed to replicate (S/G_2) compared with cells remaining in G_0/G_1, and are linked to a clear relative decrease of αSM actin mRNA in S/G_2 cells. A similar observation has been made previously in primary cultured SMC (34). The pattern of actin isoforms was clearly modified in the whole population of SMC 5 d after endothelial injury, compared with the normal arterial wall.

From the foregoing it appears that the expression of actin isoforms in SMC undergoing a change of growth state is regulated at multiple levels. A similar situation has been observed in striated muscle during differentiation in which regulation occurs at the translational as well as the transcriptional and posttranscriptional levels (12).

Our results demonstrate that in vivo the changes in SMC actin isoform mRNAs and synthesis after endothelial removal occur before the cells leave G_0/G_1. These changes in actin isoform expression might be important for SMC commitment to cell cycle entry. Alternatively, they might be one of the consequences of cell cycle entry. The expression of actin isoforms and of their mRNAs is particularly altered in cells entering in the S/G_2 phase compared to those remaining in G_0/G_1.

To test the hypothesis that expression of actin isoforms is linked to growth state we measured actin synthesis and the levels of actin isoform mRNA and protein in injured arteries of heparin-treated animals. We have previously shown that heparin inhibits SMC proliferation in injured rat carotid artery by blocking the transition from G_0/G_1 to S phase but does not suppress the expression of ornithine decarboxylase, an activity characteristic of early G_1 (27). Similar findings have been made in vitro (2). We designed the experiments to answer two questions: (*a*) does heparin inhibit the program of actin isoform changes induced in injured artery, and (*b*) does it cause the early reinduction of the quiescent phenotype (αSM actin predominance) in association with growth inhibition? Our results demonstrate that although growth of SMC is inhibited by heparin, the G_0/G_1 changes in actin isoform mRNAs and synthesis are not prevented. On the other hand, by keeping a large fraction of the carotid SMC in the resting state with heparin treatment, we observed a re-expression of αSM actin mRNA and protein at a time after injury (5 d) when cytoplasmic actin mRNAs and protein are usually predominant. Expression of the quiescent phenotype is not observed under normal circumstances until 60 d after injury (20).

These results suggest that a switch from predominance of αSM to predominance of β and γ actin mRNAs and synthesis is an early change affecting the SMC after endothelial injury. It may be necessary but certainly is not sufficient for entry of SMC into the replicative state. Heparin does not affect the early change in actin isoform mRNAs and synthesis even though it does inhibit G_0/G_1 to S transition. Our findings are somewhat analogous, in a reverse sense, to what has been described in striated muscle cells stimulated to undergo fusion but blocked by various pharmacological agents (12); under some circumstances expression of muscle-specific mRNAs as well as fusion are inhibited while under others the muscle-specific mRNAs are still expressed despite the lack of fusion.

The finding that αSM actin is expressed by heparin-inhibited SMC suggests the possibility that the heparin-inhibited state might closely resemble true quiescence. This hypothesis has been proposed by several groups and is supported by the observation that heparin and heparan sulfate extracted from normal arteries or by cultured endothelium and quiescent SMC inhibit SMC growth and migration in vitro (2, 3, 13, 26, 27). In other organ systems heparin or heparin-like molecules promote functions characteristic of the differentiated state (36). These observations also support the possibility that quiescence in a normal artery is actively maintained. Alternatively, increased expression of αSM actin might be part of any program that keeps SMC from proliferating. The relationship between quiescence in a normal artery and the growth-inhibited state produced by heparin in an injured artery will be clarified when the patterns of expression of the actin isoforms and other muscle-specific proteins have been defined under a variety of conditions of growth arrest.

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