Smooth Muscle-Specific SM22 Protein Is Expressed in the Adventitial Cells of Balloon-Injured Rabbit Carotid Artery

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Abstract—During the “response-to-injury” process after a mechanical insult to the porcine coronary arteries, the adventitial cells acquire the structural characteristics of myofibroblasts before being incorporated into smooth muscle (SM) layer. We assessed whether the SM-specific SM22 protein can be used as a tracer of adventitial cell-myofibroblast differentiation in the mild balloon injury of rabbit carotid artery. To achieve this goal, we used 2 monoclonal anti-SM22 antibodies (E-11 and 1-B8) and a molecular probe for the SM22α mRNA isoform in immunocytochemical and in situ hybridization experiments. The differentiation profile and the migratory and proliferative ability of activated adventitial cells were evaluated by a panel of antibodies to some SM and nonmuscle antigens and pulse- and end-labeling with bromo-deoxyuridine, respectively. In adventitial cells, SM22 antigenicity and SM22α mRNA were detectable at days 2 and 4 and, to a lesser extent, at days 7 and 21 after injury, particularly near the adventitia-media interface and mostly colocalizing with bromo-deoxyuridine–positive cells. The pulse-labeling experiments showed that the large majority of these cells penetrated the outermost layer of the tunica media without migrating to the subendothelial region. The phenotypic features of activated migrating and nonmigrating adventitial cells resembled those of vimentin-actin myofibroblast subtype and fetal-type SM cells. These findings indicate that a direct exposure of adventitia to the lumen is not required for phenotypic changes and proliferation/migration of these cells. After comparison of the SM22 expression in arterial vessels during early stages of development, we hypothesize that in the injured carotid artery the mural incorporation of adventitial cells and the spatiotemporal activation of SM22 expression are reminiscent of the vascular morphogenetic process and suggest the existence of a stem cell-like reservoir in adventitia. The early adventitial upregulation of SM22 expression in the injured vessel might be related to a multistep transition process in which nonmuscle cells are converted to myofibroblasts and, possibly, to SM cells. (Arterioscler Thromb Vasc Biol. 1999;19:1393-1404.)

Key Words: smooth muscle cell ■ adventitia ■ differentiation ■ endothelial injury ■ SM22

A basic aspect of the pathogenetic mechanism of atherosclerosis is the process of proliferation and migration of arterial smooth muscle cells (SMC) from the media to the subendothelial region.1–3 A similar phenomenon is observed in the endothelial-injured vessel, in which accumulating neointimal SMC derive essentially from the underlying medial tissue.2,4 The adventitial layer has so far been considered as just a site of perivascular inflammation and tissue healing, which might be involved in wall remodeling, but without any contributing role to the atherosclerotic lesion or neointimal formation.1,2,5

Recent works have, however, suggested that the adventitia may play an important role in atherosclerosis or neointima formation after endothelial injury. In fact, microcircums of vasa vasorum6–8 and activation of resident9–12 or blood-derived13 nonvascular cells have been implicated in the arterial wall lesion formation. According to the former proposed mechanism, in thick-walled large vessels containing thrombotic vasa vasorum, hypoxia produces foci of necrosis in the medial smooth muscle (SM) tissue, which in turn activate an SMC regeneration process. As for the latter proposed mechanism, the adventitia of injured coronary artery displays a cellular transition from fibroblasts to myofibroblasts,12 ie, a cell phenotype with hybrid or intermediate structural and functional properties between fibroblast and the SMC.14,15 There is now convincing evidence that these adventitial myofibroblasts possess directional (centripetal) migratory ability and can be, to a certain extent, incorporated in the tunica media and, possibly, in the neointima.16

If adventitial myofibroblasts derive from locally recruited fibroblasts or fibroblast-like cells,12 one might consider the possibility that the cellular conversion process described above might just be an expression of the differentiable plasticity of fibroblasts as occurs in villous placental stro-
ma.\textsuperscript{17} in some experimental models,\textsuperscript{18–20} and in some human pathological settings.\textsuperscript{21} Alternatively, such cell phenotypic transition might represent a reactivation of the mesenchymal cell pathway, which during development is involved in the morphogenesis of arterial wall.\textsuperscript{3} In this latter circumstance, it is plausible that a sort of embryonic remnant may persist in the adult at the level of adventitia, ie, in a region where during vasculogenesis the precursors of differentiating SMC (undifferentiated mesenchymal cells surrounding the endothelial tube) are thought to be localized.\textsuperscript{3}

In light of these hypotheses, it is important to ascertain in more detail the differentiable potential of activated adventitial cells in a model of endothelial injury of carotid artery. We have undertaken this study, taking into account the SM22 protein\textsuperscript{22,23} as a principal marker, to study the phenotypic changes in the adventitial cells. This calponin-related protein is composed of 4 electrophoretically distinguishable isoforms ($\alpha$, $\beta$, $\gamma$, and $\delta$),\textsuperscript{22,24–26} $\alpha$ being the most thoroughly investigated.\textsuperscript{26–28} The SM22 expression in adult is restricted to SM tissues,\textsuperscript{22–24} whereas in the early stages of development SM22$\alpha$ is also present in skeletal and cardiac muscle.\textsuperscript{29} In muscle tissues other than SM tissue, SM22 is switched off during birth or hatching.\textsuperscript{23,28,29}

The distribution patterns of SM22 found at the protein level in normal and injured rabbit carotid artery at various times of injury ($2, 4, 7$, and $21$ days) were compared with that found in situ hybridization (ISH) experiments for SM22 mRNA. Immunophenotyping of adventitial cells was accomplished by a panel of antibodies specific for SM lineage-related (SM-type $\alpha$-actin and calponin), SMC-specific (SM-type myosin heavy chain [MyHC] and $h$-caldesmon) proteins, cytoskeletal (vimentin, desmin, and nonmuscle [NM]-MyHC) and extracellular matrix (ECM; EIIIA-fibronectin) markers. Experiments of pulse- and end-labeling of proliferating SMC with bromo-deoxyuridine (BrdU) were also designed to evaluate whether phenotypic modifications were related to the migratory and proliferating ability of adventitial cells and SMC.

**Methods**

**Animals**

Aortic specimens to be used in 2-dimensional gel electrophoresis (2D-EF) and carotid samples to be processed in immunocytochemistry experiments were taken from 3-month-old male New Zealand White rabbits obtained from C & C Farm (Padna, Italy) (weighing about 3 kg), and day 12 rabbit fetuses. Forty-six adult rabbits were used in the endothelial injury experiments: 16 underwent surgery and were pulse labeled; see Figure 1) and euthanized at 2, 4, 7, and 21 days after surgery (see Figure 1). For each time point animal groups comprised 3 operated or 4 operated (pulse labeling, or operated + end labeling) rabbits. Rabbits were housed in the Department of Biomedical Sciences and quarantined for 7 days before use. Maintenance of animals was done in accordance with the recommendations stated in “Principles of Animal Care” (NIH publication No. 85 to 23, revised 1985) and the “Guidelines of the Animal Care Advisory Committee” of the Italian Ministry of Public Health. Euthanasia of all rabbits was performed by sodium pentothal injection.

**Endothelial Lesion**

Animals were anesthetized with sodium pentothal (30 mg/kg; Abbott Laboratories) and urethane (70 mg/kg), administered via a marginal ear vein. A 2F Fogarty embolectomy catheter (Baxter Healthcare Co) was introduced through an aseptic neck incision produced in the facial branch of the external left carotid artery and positioned approximately at the origin of the common carotid. An acute balloon injury was performed by inflating the balloon with 0.2 mL saline solution and then gently pulling it back along the entire length of the common carotid with constant rotation. The procedure was repeated 3 times. The catheter was then removed, the artery branch ligated, and the surgical wound closed. The animals were allowed to recover under observation before being placed in their cages. Evans blue dye injection 2 hours after surgery confirmed the near homogeneous removal of endothelium from injured carotid. Histological examination of this area revealed a marked structural alteration of SMC in the presence of a substantially intact wall organization. Because the lesion did not apparently involve disruption of the medial integrity (ie, the elastic membranes) and contact of the blood with the adventitia, we define the impact of the lesion on the carotid wall as mild injury.

After sacrifice, the left common carotid artery and the contralateral vessel were excised with great care to avoid any damage to the adventitial layer. The vessel segments were then perfused with OCT (Tissue Tek, Miles Inc) and inserted into the lumen of the thoracic aorta from the same animal. The mounted block was immersed in liquid nitrogen and stored at $-80^\circ$C. Contralateral carotid artery was used as a control.

**Two-Dimensional Gel Electrophoresis**

2D-EF for basic proteins at nonequilibrium pH condition was performed as outlined by O’Farrell\textsuperscript{30} and Gimona et al\textsuperscript{25} with the following modifications: ampholytes (Amersham) covered pH ranges 5 to 8 (2.5%), 4 to 6 (1.6%), 3 to 5 (1.6%), 7 to 9 (1.6%), and 2 to 11 (0.8%). Isoelectric focusing was performed according to the following protocol: 400 V (7 minutes), 500 V (7 minutes), and 750 V (75 minutes), without a preliminary run. The second-dimension electrophoresis was done in a 15% SDS-slab gel.

Western blotting of electrophoresed protein bands was done using parallel gels and following the standard conditions used in our laboratory\textsuperscript{31,32} with some modifications. Transfer of proteins from 2D gels to the nitrocellulose paper was performed for 90 minutes at 300 mA. Free binding sites were saturated in 5% fat-deprived milk, and E-11 or 1B8 primary antibody was applied to the paper for 30 minutes at $37^\circ$C. The secondary antibody was an IgG anti-mouse IgG coupled with horseradish peroxidase (Dako) applied to the paper at the same conditions as the primary antibody. Bound primary-secondary antibody complex was revealed by chemiluminescence using the Amersham ECL kit.

**Pulse- and End-Labeling**

Injured animals treated with 30 mg/kg of BrdU, dissolved in PBS, pH 7.2, were injected IP 12 and 24 hours after surgery (pulse labeling; see Figure 1) and euthanized at 2, 4, 7, and 21 days after injury. Another group of injured rabbits injected with the same dose of BrdU 12 and 24 hours before killing (end labeling; see Figure 1)
were euthanized at 2, 4, 7, and 21 days after injury. Three animals not operated on were also treated with BrdU and used as control group.

Antibodies
A panel of monoclonal antibodies was used to characterize immunocytochemically the adventitial cell phenotype at various times from injury induction. The specificity of the antibodies is reported in Table 1.

Immunocytochemistry
Primary antibodies were applied to freshly cut fixed or unfixed cryopreserved sections as previously described. Cryopreserved sections were taken from the middle part (about 1 cm) of the common carotid artery. For immunophenotyping of adventitial cells, 15 carotid cryopreserved sections from each control and injured animal were used. For cell proliferation/migration study, 10 carotid cryopreserved sections from each time point from control, injured pulse-labeled, or injured end-labeled rabbits were analyzed. For E-11 and 1-B8, anti-caldesmon and anti–endothelin-1 antibody, cryopreserved sections were fixed in 3.7% formaldehyde in PBS, pH 7.2, for 5 minutes. Anti–phosphoglucomutase-related protein (PGM) antibody and anti-laminin were applied to methanol- or acetone-fixed cryopreserved sections, respectively. In these procedures, the secondary antibody was the IgG anti-mouse IgG coupled with horseradish peroxidase. Bound IgG were revealed by incubation in aminohexylcarbazole solution. Counterstaining was performed with Harris’ hematoxylin. Controls were made by omitting the primary antibody and using nonimmune IgG followed by the secondary antibody. The double immunofluorescence assays were performed using a monoclonal anti–SM-type α-actin (Sigma) directly coupled with fluorescein isothiocyanate and the anti-BrdU antibody indirectly revealed with the anti-mouse IgG conjugated with rhodamine isothiocyanate.

BrdU-incorporating nuclei in normal and injured vascular tissue from pulse and end labeling were detected using a specific anti-BrdU antibody (Dako) according to the procedure reported by Faggian et al. Negative and positive BrdU-incorporating tissues (myocardium and small intestine, respectively) from the same animals used for BrdU labeling of injured vessels were used as controls.

Variability of immunophenotyping and BrdU-incorporation patterns from section to section were approximately on the order of ±10% of positive cells. The intensity of immunostaining in the carotid cryopreserved sections, especially at early stages of the response-to-injury process, was generally limited and apparently depended on the existence and size of the thrombus over the injured region (Chiavegato A, Franch R, Sartore S, in preparation, 1998).

In Situ Hybridization
The pBluScriptSKII+ vector containing a 275-bp fragment of rat SM22α-cDNA (a generous gift of Dr Joseph M. Miano, Cardiovascular Research Center, Department of Physiology, Medical College of Wisconsin) was linearized with the appropriate restriction enzymes. Sense and antisense RNA probes labeled with 35S-UTP were transcribed with T3 and T7 polymerases according to the manufacturer’s instructions (GibcoBRL). ISH was carried out on 10-μm cryopreserved sections collected onto 3-aminopropyl-triethoxysilane–treated slides. First ISH cryopreserved sections were fixed for 30 minutes with 4% formaldehyde in PBS, pH 7.2, and then processed following the protocol of Sassoon et al and DeNardi et al with some modifications. After rinsing in PBS, the cryopreserved sections were subjected to 4 minutes of digestion with 10 μg/mL proteinase K at room temperature, followed by 0.1 mol/L triethanolamine-acetic anhydride acetylation and dehydration. For hybridization, riboprobes were heated for 3 minutes at 90°C in 50% formamide, 0.3 mol/L NaCl, 0.3 mol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA (pH 8.0), 10 mmol/L NaH2PO4 (pH 8.0), 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mg/mL yeast tRNA, and 10 mmol/L DTT, and placed on ice. Approximately 60 μL of probe was applied to each slide, a coverslip was applied, and probes were hybridized in a humidified chamber at 50°C for at least 16 hours. The remainder of the ISH procedure was identical to that of DeNardi et al.

Results
Specificity of Antibodies
Isoform specificity of the 2 anti-SM22 antibodies in the vascular SM tissue is shown in Figure 2. Two electrophoretically distinct spots of 22-kDa, corresponding to the α and β isoforms of SM22, can be seen in the gel (Figure 2A). 1-B8 and E-11 antibodies react strongly with the α isoform, whereas E-11 is weakly positive with the β isoform (Figure 2B). No cross-reactivity of the 2 antibodies with the calponin is detectable (Figure 2B and 2C).

Immunophenotypic Characterization of Adventitial Cells in Normal and Injured Carotid Artery
Figure 3 shows the immunocytochemical reactivity of adventitial cells and the overlying SMC in normal and injured
carotid artery at various times from endothelial lesion with anti-SM22 antibodies. Because the SM22 immunostaining of the vascular tissues with the 2 antibodies is similar, only data pertaining to E-11 will be presented here.

In the adventitia from normal wall, there is almost no reactivity with resident cells (Figure 3A) except for rare vasa vasorum (not shown), whereas a marked staining is visible with the medial SMC. In uninjured carotid artery, there is no histological evidence for a tunica intima or intimal cushions. In injured vessel, SM22-positive adventitial cells are clearly identified at all postinjury times examined: from day 2 (Figure 3B) to day 21 (Figure 3E). However, at day 2, some staining is visible in the adventitial region close to the external elastic lamina (eel, dotted line), whereas at later postinjury times positive cells are randomly dispersed in the whole adventitia (asterisks in B through E). Medial SMC are homogeneously reactive soon after injury (B), then they become negative in the inner part of the wall (star in C). The medial area negative for SM22 is reduced at day 7 (star in D) and subsequently disappears completely at day 21. The neointima (it) is initially negative for SM22 except for rare cells (arrowheads in D), then cells in this tissue accumulated at day 21 after injury regain SM22 expression (E). iel indicates internal elastic lamina. (Bar=60 μm.)
found. In the media, there is a downregulation of SM22 cellular content, particularly around day 4 after injury (Figure 3C), which is reversed at later stages (Figure 3D and 3E), although SMC heterogeneity can persist in some regions (see for example Figure 4G). Neointimal cells are almost negative for SM22 at day 7 (Figure 3D), but become markedly positive at day 21 (Figure 3D) with some slight differences in the intensity level from region to region.

The presence of SM22-containing adventitial cells in injured carotid artery was also confirmed at the mRNA level. Figure 4C shows the result of ISH experiments performed on day 2 balloon-injured vessel in comparison with the uninjured carotid (Figure 4B) and the corresponding sense probe (Figure 4A). In concordance with data obtained at the protein level (Figure 3B), small foci of SM22α mRNA are identified in the adventitial region proximal to the external elastic lamina. Similar results were also found with day 4 injured carotid artery (not shown). In contrast, some differences exist between expression of protein and mRNA levels in the media. As might also be inferred by comparing Figure 3C with Figure 4C, there is in this tissue an apparent discrepancy between a relatively decreased SM22 expression at day 4 and an increased level of SM22α mRNA at day 2. To study this problem in detail, cryopreserved sections from day 4 (Figure 4D and 4E) and day 21 (Figure 4F and 4G) injured carotid arteries, which appeared heterogeneously stained with the anti-SM22 antibody, were taken and compared with parallel cryopreserved sections prepared according to the ISH protocol. Areas highly reactive for both SM22 protein and SM22α mRNA coexist along with areas in which the protein is more or less detectable in comparison with SM22α mRNA. The fact that the SM22 expression at the protein level is low and the SM22α mRNA is high might indicate that a regulatory mechanism controls the SM22 expression at the mRNA level. The opposite relationship between the relative protein and mRNA levels might be attributable to the upregulation of non-SM22α isoform expression recognized by the anti-SM22 antibodies used in this study.

Figures 5 and 6 and Table 2 show the results of the immunophenotyping experiments performed to characterize the normal and activated adventitial cells. In normal carotid artery the resident adventitial cells (not including nerves, vasa vasorum, and lymphatics) can be identified as fibroblasts, ie, vimentin and NM-MyHC positive cells (Table 2). On vessel damage, part of the cells near the adventitia-media interface subsequently express a cytoskeletal and cytocon-
tractile marker repertoire typical of VA (vimentin-actin)-myofibroblasts, ie, reactivity for vimentin, actin, and NM-MyHC, along with SM22 (Figure 5C). Small foci or isolated cells in this region are labeled for SM myosin (Figure 5E), the SM-related protein PGM (Figure 5F), fibronectin (Figure 6G), and, rarely, calponin (Figure 5B). Other SM-specific markers, such as caldesmon (Figure 5D) and desmin (Figure 6B) are not reactive. The outermost layer of the adventitia is composed of vimentin- (Figure 6A) and NM MyHC- (Figure 6C and 6D) positive fibroblasts. Endothelin-1 expression also appears (Figure 6E). Inflammatory cells identified by RAM11 (Figure 6H) are barely visible at 2 days after injury. Cells positive for the various antibodies do not morphologically resemble cells of nerve, vasa vasorum, or lymphatics. Taken together, these results are compatible with the presence of a 2-layer organization in adventitia of day 4 injured carotid artery: the outer region is composed of SM22-containing myofibroblasts and SMC.

The medial SMC in the injured artery display predominantly the phenotypic features of fetal SMC, namely positive for SM myosin, NM MyHC, calponin (Figure 5B), SM22 (Figure 5C), caldesmon (Figure 5D), desmin (Figure 6B), and laminin (Figure 6F), and increased staining for fibronectin (Figure 6G). Endothelin-1 expression is also evident (Figure 6E). Interestingly, in some medial regions facing the lumen there are NM cells, positive for vimentin (Figure 6A) and NM-MyHC (Figure 6C and 6D), which can be identified only in part as inflammatory RAM11-reactive cells (Figure 6H) or lymphocytes (not shown).

**Proliferation/Migration of Adventitial Cells**

Pulse- and end-BrdU labeling were used in injured carotid artery. The former procedure allows for monitoring the waves of migratory cells that might be induced at various times from injury in the wall compartments assuming that cells incorpor-
rating this thymidine analog become almost completely arrested in the S-phase of the cell cycle without being blocked in their migratory capability.\textsuperscript{37–40} Generally, the intensity of BrdU immunostaining is quite high and homogeneous, suggesting that a diluting effect consequent to BrdU incorporation and associated with cell proliferation is scarce. We are aware of the possibility, however, that BrdU-positive cells may maintain a relatively high immunocytochemical signal in the daughter cells after the first division. The second assumption is that the end labeling furnishes information about the localization of proliferating cells during the 24-hour window preceding euthanasia at 2, 4, 7, and 21 days after injury.

In uninjured vessel, only rare BrdU-positive cells are present in the media and adventitia (not shown), whereas numerous BrdU-incorporating cells are evident at the different postinjury times examined (see Figure 7). In the pulse labeling, the large majority of reactive cells are confined to the adventitia layer (see Figure 7A through 7G), although a limited number of cells, especially at 2 and 4 days after injury, can also be seen in the media just near the external elastic lamina (Figure 7A and 7C). Interestingly, numerous adventitial BrdU-positive cells appear to be localized proximal to the external elastic lamina, remarkably at the early stages after injury.

The end-labeling procedure gives rise to a more complicated picture than pulse labeling. At 2 days after injury (Figure 7B), the distribution of BrdU-positive cells is quite similar to that of the corresponding pulse-labeled artery (Figure 7A), inasmuch as the times of BrdU injections in the 2 protocols are close to each other (see Figure 1). Hence, the majority of BrdU-positive mural cells belong to the adventitial compartment (Figure 7B). At day 4 (Figure 7D), only very few BrdU-incorporating cells are visible in the adventitia, being mostly localized in the subendothelial region of the media. At the subsequent times, cells reactive with BrdU decline dramatically and at 21 days after injury (Figure 7H), no positive ones can be identified in the carotid wall. It is worth noting that a neointima is formed in the carotid artery from both types of BrdU protocols, but in contrast with the end-labeling procedure, in pulse labeling almost no BrdU-positive cells are seen in this tissue and the neointimal thickening is thinner (Figure 7E).

Our data are compatible with the idea that 2 temporally distinct waves of cell migration take place in the injured carotid artery wall: a first adventitial wave followed by a second medial SMC wave. In our experimental conditions, it seems that adventitial cells are short-range (directional) migratory cells and do not contribute substantially to neointima formation.

We have also sought to characterize the phenotypic profile of activated cells localized at both sides of the external elastic lamina. Figure 8 shows the result of double immunofluorescence experiments performed on carotid artery from a day 2 BrdU-pulse-labeled injured animal. The large majority of cells are stained for SM-type $\alpha$-actin and, thus, from the results shown in Figures 5 and 6 and Table 2, they can be identified as VA-myofibroblasts.

The fact that small groups of adventitial cells are reactive for some SM-specific markers (see Figure 5; in particular, SM myosin being the only reliable marker of SMC differentiation\textsuperscript{40}) poses the problem of evaluating their differentiation level using the combination of anti-SM myosin and anti-NM-MyHC antibodies.\textsuperscript{33} Secondly, it must also be verified whether SM22- and SM myosin-expressing cells have the ability to penetrate the adventitial-medial barrier, ie, if they possess a migratory ability. Figure 9 shows that the small foci of adventitial cells positive for SM myosin (Figure 9A) found at 2 days after injury are also labeled for NM-MyHC\textsubscript{pla1/2} (Figure 9B and 9C), thus indicating that these are fetal SMC.\textsuperscript{33} Secondly, in no circumstance are medial SMC proximal to the external elastic lamina negative for SM myosin and positive for NM-MyHC\textsubscript{pla1/2} (the expected pattern...
for a true NM cell), indicating that fibroblasts as such do not enter into media. In contrast, postnatal (SM myosin-positive, NM-MyHC pla2 -positive) and fetal (SM myosin-positive, NM-MyHC pla1/2 -positive) SMC are visible at the luminal side of the media, as expected from the inferred data of end labeling (ie, the second medial SMC wave described above). Serial cross-section immunostaining with anti-SM myosin and anti-SM22 antibodies show that BrdU-positive cells appearing at both sides of the external elastic lamina (Figure 9F and 9G) also display an SM myosin (Figure 9D) or SM22 (Figure 9E) content, suggesting that fetal SMC and SM22-containing VA-myofibroblasts are capable of penetrating the adventitial-media barrier.

Some Immunophenotypic Properties of Developing Aortic Wall

Because the directional migration of adventitial cells toward the lumen in the endothelial-injured model in adult rabbit might be reminiscent of the first stages of vascular morphogenesis (see Discussion), we have also looked at spatial distribution of SM22 antigenicity in developing aorta (12-day-old fetus; Figure 10). At this phase of vasculogenesis the wall organization is poor with no evidence for media-adventitia demarcation or an organized medial layer with the characteristic elastic laminae. It is generally accepted that in this period newly incorporated (in the developing vessel) SM a-actin-positive cells come from locally recruited mesoderm-derived mesenchymal precursors surrounding the hollowed endothelial tube. The parietal cells close to endothelium that we have examined are not yet composed of fully differentiated SMC as witnessed by the lack of reactivity with anti-SM myosin antibody (Figure 10C). By contrast, SM a-actin expression is visible both close to the lumen (as a relatively compact immunostaining) and in the surrounding cells (in a dispersed manner; Figure 10A). SM22 immunoreactivity is greatly expressed, in part overlapping that of actin but also encompassing a thicker wall layer (Figure 10B).

Discussion

In response to mild injury to rabbit carotid artery 2 important phenomena take place: (1) the adventitial cell activation accompanied by phenotypic changes related to the acquisition of SMC-related properties and (2) migration of adventitial myofibroblasts/SMC and medial SMC to the media and neointima, respectively.

Phenotypic Changes in the Adventititia

The results that support the first point are obtained by studying the expression of SM22, an SM-specific protein in adult avian23,25 and mammalian26–29 species, in comparison with a number of SM- and NM-specific antigens that have allowed the identification of 2 major cell types: the myofibroblasts and SMC. Myofibroblasts, which appeared during the wound repair process consequent to the application of a mechanical insult to the vascular wall, are characterized by the upregulation in the expression of SM-type a-actin in the preexisting vimentin- and NM myosin-containing adventitial

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**Figure 8.** Double immunofluorescence staining of day 2 injured carotid artery with anti-BrdU (A) and anti-actin (B) antibodies. Adventitial (a; single arrowhead) and medial (m; paired arrowheads) cells incorporating BrdU are also positive for actin. (Bar=40 μm.)

**Figure 9.** Immunoperoxidase staining of day 2 injured carotid artery with anti-SM myosin (A and D), NM-G2 anti-NM-MyHC Apla2 (B), NM-F6 anti-NM-MyHC Apla1 (C), anti-SM22 (E), and anti-BrdU (F and G) antibodies. Note that adventitial (a) cells positive for SM myosin are also labeled for NM myosins (red asterisk in A through C). Medial (m) SMC proximal to the external elastic lamina (dotted lines) are stained for SM myosin and variably with the anti-NM myosin antibodies. Medial SMC near the lumen can be stained for SM myosin+MyHC Apla2 (double black asterisk in A through C) or for SM myosin+MyHC Apla1/2+ (single black asterisk in A through C), corresponding to postnatal- and fetal-type SMC, respectively. In D through F, adventitial SM myosin- or SM22-positive cells are also labeled for BrdU (red arrowheads) as also happens with some medial SMC localized near the external elastic lamina (black arrowheads). (Bar=60 μm [A through C]; 40 μm [D through G].)
fibroblasts. This new cell phenotype can be classified as VA-myofibroblast on the basis of the nomenclature proposed by Gabbiani and coworkers. This new cell phenotype can be classified as VA-myofibroblast on the basis of the nomenclature proposed by Gabbiani and coworkers.14,15

Our immunophenotyping procedure has revealed a peculiar characteristic of adventitial myofibroblasts: the expression of the SM lineage-specific marker SM22. It is highly probable that at least the α isoform of SM22 is present in the activated adventitial cells, though we cannot exclude that upregulation of the β, γ, or δ variants22,24,25 may also occur during the arterial wall response-to-injury process. Because the expression of the calponin-related SM2223 or SM22α28,29 is generally confined to SM tissues, the presence of this protein might be quite unexpected. There are, however, some data that suggest that SM22 is also expressed in myofibroblasts from other experimental settings or in NM cell systems. In fact, serosal myofibroblasts from rabbit urinary bladder subjected to partial outflow obstruction19 or transforming growth factor-β1 infusion41 contain SM22-expressing VA-myofibroblasts. SM22 is also expressed in vitro in rat embryo42 and human senescent43 fibroblasts, in the former as an actin gelling protein, transgelin42. In addition, SM22 is present in cultured IMR-90 human fetal lung cells showing the structural characteristics of myofibroblasts.44 Altogether, the differentiation profile of activated adventitial cells resembles that of some SMC-like lines in which SM22, actin, and NM myosin are coexpressed.45,46

The fact that in our model of endothelial injury some adventitial cells in the region proximal to the external elastic lamina display an SM myosin content (a marker of fully differentiated SMC40) points to the existence of a differentiation pathway in which myofibroblasts can be converted into SMC. On the basis of our SMC immunophenotypic criteria,33,47 these SMC can be identified as fetal SMC (Figure 9A through 9C), ie, showing coexpression of SM and NM-MyHC-Apla1/2 isoforms as well as the fetal-fibronectin isoform.48,49 Thus, SM22 expression in adventitial cells can be considered as an indicator of the spatiotemporal cellular transition that brings about the myofibroblast to SMC transformation. This implies that the cellular conversion of myofibroblasts into SMC may occur independently from the migration into the media, possibly indicating that injured adventitia contains locally released factors that can allow this process to take place.

The presence of adventitial-derived myofibroblasts/SMC near the external elastic lamina and the tendency for the spatial-limited migration toward the lumen (see below) are reminiscent of the incorporation-differentiation process that some arterial vessels undergo during vascular morphogenesis.3 In fact, undifferentiated SMC precursors migrating from the mesodermal region surrounding the endothelial tube are incorporated into the developing vessel wall.3 Looking at Figure 10, showing the dorsal aorta from a day 12 rabbit fetus, it seems that a continuum of SM22-positive cells exists between the already established actin-containing aortic wall SMC and the surrounding tissue in which a real adventitia has yet to be formed. Although other hypotheses are possible, we put forward the idea that adult adventitia may contain a sort of a stem cell-like population that can be activated on demand, possibly differentiating according to an adventitial-medial axis resembling a vasculogenetic pathway.

Migration of Activated Vascular Cells
The results of pulse- and end-BrdU labeling experiments are compatible with the existence of 2 distinct migrating/proliferating cell responses in injured carotid artery wall, namely, adventitial cell proliferation-incorporation into the outer medial layer, and the proliferation-migration of medial/neointi-
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mal SMC. Pulse-labeling studies have shown that the large majority of adventitial cells and, to a lesser extent, cells in the outer layer of the tunica media incorporate BrdU soon after lesion. The time-course analysis of distribution of BrdU-incorporating cells also reveals that adventitial cell migration to the medial layer peaks around 2 days after injury and declines thereafter, possibly because of the decreased density of medial BrdU-positive cells (caused by increased total medial SMC).

Because there are almost no labeled cells in the neointima, according to the pulse-labeling procedure, it might be concluded that in our experimental model BrdU-positive cells accumulated in this tissue have mostly migrated or proliferated from the underlying medial SMC. In fact, the results of the application of end-labeling protocol at 4 and 7 days after injury indicate that the innermost medial region and neointima contain proliferating cells. Taken together our data suggest that 2 waves of migration/proliferation exist at the 2 interfaces of tunica media, ie, at the level of external and internal elastic lamina.

Some caution must, however, be taken about the conclusions drawn from the use of BrdU as a tracer of cell migration from 1 vascular compartment to another, particularly regarding the absence of BrdU in the neointima described above. Using the BrdU approach to this problem, it cannot be ruled out that the stability of this DNA-incorporated thymidine analog is maintained during cell migration. In addition, it might be that not all the activated cells are labeled by BrdU or that repeated cell division may cause some loss of the label. Future studies dealing with this problem must take into account other procedures, for example the use of a fluorescent dye applied to adventitial cells before injury to trace the fate of newly migrating cells.

**BrdU Incorporation and Cell Differentiation**

The 2 waves of proliferating/migrating cells in the injured carotid wall are accompanied by distinct time-dependent differentiable events: (1) differentiation of adventitial cells into myofibroblasts and then, in part, into SMC, and (2) dedifferentiation of adult-type into fetal-type SMC (or expansion of preexisting postnatal SMC). It is worth noting that in our model, in concordance with Shi et al, adventitial NM cells are not capable of migrating to the media as occurs with myofibroblasts or fetal SMC. It could be that SM22 expression in NM cells might confer some migratory advantage as inferred from this peculiar propensity that the SM22-positive NM cells (named type 250) from the adventitial surface of canine carotid artery show when grown in vitro. It would be of interest to investigate a possible correlation between the severity of injury and the level of upregulation of SM22 expression in adventitia.

Our data support the concept that competence for SMC migration from the media to the subendothelial space requires the dismantling of the differentiable apparatus of the cells, ie, an upregulation of the expression of NM cell markers (NM myosins, fibronectin, and vimentin) and downregulation of SM markers (SM myosin, caldesmon, calponin, PGM, and SM22). The neointima is initially poor of SM22-positive cells, but it gains a more abundant expression of SM22 when the proliferation level is scarce or negligible (Figure 3). Our results are in concordance with data from Shanahan et al, who found that SM22α expression is quite low in human atherosclerotic plaque, and with the in vitro differentiation-dependent expression of this polypeptide.

There is still an open question represented by the presence of NM cells in the inner medial layer that are negative for SM markers including SM22 (Figure 3) and cannot be accounted entirely for contaminating inflammatory cells. Further studies are needed to clarify the exact derivation of this cell type.

**Comparison With Adventitial Cell Activation in Overstretching Injury Model**

Using the overstretching model of coronary artery lesion, Scott et al and Shi et al have come to a similar conclusion about the phenotypic changes and the migratory properties of adventitial cells. Some differences, however, are noticeable. Contrary to the severe injury produced in the porcine coronary artery by these authors, the adventitia-to-media cell migration observed in our model is modest. In fact, in pulse-labeling tests only a few BrdU-positive cells can be seen dispersed in the media at day 4 or 7 (see Figure 7C and 7E). The neointima produced is scarce with rare BrdU-positive cells. In adventitia, the phenotypic changes from NM to myofibroblasts occur faster (2 days), and we have shown that SM22 differentiation can take place before adventitial cells migrate to the media. Although in the porcine injury, adventitial cell proliferation index peaks around 3 days after injury and the maximal level of phenotypic changes develops around day 7 to 14, in the rabbit model the waves of proliferation and phenotypic cell transition occur concomitantly.

To sum up, our data indicate that a direct exposure of adventitia to the lumen is not required for phenotypic changes and proliferation/migration of these cells. The difference observed in the porcine versus the rabbit model of injury might be because of the extent of lesion, the procedure to induce vessel damage, and the species used in the experiments.

**Perspectives**

Conclusive evidence for the fibroblast derivation and the factors potentially accompanying or inducing the NM cell to myofibroblast (to SMC) transition in the adventitia of injured carotid artery is lacking. Future studies must establish in detail whether other NM cell types in addition or alternative to fibroblasts are responsible for such phenomenon. In particular, pericytes, endothelial cells, SMC from vasa vasorum, and CD34 mononuclear blood cells should also be taken into account. Certainly, the transient perivascular inflammatory reaction and ischemia of vasa vasorum induced soon after endothelial injury may cause the release of a number of growth factors/cytokines, which may have a profound effect on the subsequent activation of local resident cells or blood- and SM-derived cells. Among the factors putatively involved, endothelin-1, as demonstrated in this study (Figure 6E and Table 2), transforming growth factor-β1, and platelet-derived growth factor seem to be good candidates. It remains to be established whether and how the process of activation-recruitment-incorporation of adventitial cells in the media (and neointima) is operative during atherogenesis.

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Smooth Muscle-Specific SM22 Protein Is Expressed in the Adventitial Cells of Balloon-Injured Rabbit Carotid Artery

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