Rat Carotid Neointimal Smooth Muscle Cells Reexpress a Developmentally Regulated mRNA Phenotype During Repair of Arterial Injury

Mark W. Majesky, Cecilia M. Giachelli, Michael A. Reidy, and Stephen M. Schwartz

Smooth muscle cells (SMCs) cultured from the neointima of injured rat carotid arteries have a different shape and organization in vitro than SMCs from the uninjured media. The morphology of neointimal SMCs from adult rats strongly resembles that of a subset of medial SMCs from 12-day-old rat pups. In the present study, we show that adult carotid neointimal SMCs in vitro express the platelet-derived growth factor (PDGF)-B gene but have little or no PDGF α-receptor mRNA. In contrast, medial SMCs from contralateral uninjured carotids, grown and passaged under identical conditions, contain abundant PDGF α-receptor mRNA but little or no PDGF-B mRNA. Transcript levels for PDGF-A or PDGF β-receptor were not different in neointimal versus medial SMC cultures. The PDGF mRNA phenotype of adult neointimal SMCs strongly resembles that of an aortic medial SMC subset from newborn rat pups. Although intriguing, the differences in SMC phenotypes we observed in cell culture may depend on unique conditions in vitro and do not necessarily mean that analogous SMC diversity also exists in vivo. To address this question, we constructed and screened a SMC cDNA library for additional molecular markers of the common "pup-intimal" SMC phenotype. Two cDNA clones were identified whose cognate mRNA levels were developmentally regulated in rat aorta in vivo and were present at high levels in the adult carotid neointima formed 2 weeks after balloon catheter injury. Importantly, elevated levels of these two cognate mRNAs in carotid neointima compared with underlying media were maintained in cultures of neointimal versus medial SMCs in vitro. DNA sequence analysis indicated that the cDNA clones encoded rat tropoelastin and α1 procollagen (type I). These results provide further evidence that neointima formation in the adult rat carotid artery depends on reexpression of an SMC phenotype or subpopulation with special properties characteristic of earlier stages of artery wall development. Our studies to date indicate that two of these special properties are paracrine growth factor production and extracellular matrix synthesis. (Circulation Research 1992;71:759–768)

Key Words • platelet-derived growth factor • tropoelastin • smooth muscle cell diversity • extracellular matrix

We have reported that two distinct and stable smooth muscle cell (SMC) populations can be identified in cell cultures derived from 12-day-old rat aorta.1,2 One SMC type, termed rat pup SMC (P-SMC), has an epithelioid shape, secretes a platelet-derived growth factor (PDGF)-like mitogenic activity, and expresses both the PDGF-A and PDGF-B genes in vitro.2,3 The other SMC type, typical of SMC from adult rat aorta (A-SMC), has an elongated bipolar shape, does not secrete detectable amounts of PDGF, and expresses the PDGF-A but not the PDGF-B gene in vitro.2,4 Aortic SMC cultures from adult rats yielded only A-SMCs when isolated and passaged in the presence of whole-blood serum. However, when selected for the ability to replicate in culture media containing plasma-derived serum, SMCs with the morphological features of P-SMCs were obtained from adult rats.5

In a separate study, Walker et al6 reported that SMCs isolated from adult rat carotid neointima 2 weeks after balloon catheter injury had an epithelioid morphology in vitro that strongly resembles P-SMCs. In contrast, SMCs obtained from the contralateral uninjured carotid media were elongated bipolar cells that were indistinguishable from A-SMCs. Moreover, cultured neointimal SMCs secreted increased amounts of a PDGF-like activity when compared with parallel cultures of medial SMCs from uninjured arteries.6 The morphological and functional similarities of SMCs from adult neointima and the newborn aorta suggest that formation of a neointima in vivo may require the reexpression of a smooth muscle developmental sequence, perhaps including expansion of a distinct SMC subpopulation with special properties.7,8 The present studies were carried out to further explore this possibility.

Materials and Methods

Arterial Injury Model

Male Sprague-Dawley rats (500 g, 5 months old) (Tyler Laboratories, Bellevue, Wash.) were aneste-
tized, and an acute injury to the left common carotid artery was produced with an inflated balloon catheter as previously described.9 Two weeks later, SMCs from carotid neointimal and contralateral uninjured medial tissues were obtained by collagenase and elastase digestion as previously described.6

**Cell Culture**

Neointimal SMCs from injured left carotid arteries and medial SMCs from uninjured right carotid arteries of the same animals were routinely isolated and grown in Waymouth’s MB752/1 medium (GIBCO/BRL, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah) as previously described.6 For these studies, SMC cultures were used between the first and the 10th passage. Aortic SMC cultures from newborn and adult rats were isolated and maintained as previously described.1,2 All SMC cultures were tested and found to be negative for mycoplasma contamination.

**RNA Isolation and Transfer Blot Analysis**

Total cellular RNA was isolated, and RNA transfer blots were prepared and hybridized as previously described.10 DNA probes used for RNA hybridization analysis were as follows: PDGF-A, a 1.3-kb human cDNA fragment released from pD11; PDGF-B, a 2.1-kb Sac I–Sac II human cDNA fragment from pSM-112; PDGF α-receptor, a 6.4-kb EcoRI rat cDNA from p802E/BS13; PDGF β-receptor, a 4.7-kb EcoRI–Xba I human cDNA fragment from phPDGF-R14; actin, a 1.3-kb Pst I bovine cDNA fragment from pBA-115; rat tropoelastin, a 24-mer oligonucleotide probe (5'-ATACCTGGCAGCCTTTGGCAGCAGC-3') complementary to a sequence coding for a cross-link domain in rat tropoelastin15; and glyceraldehyde-3-phosphate dehydrogenase, a 1.2-kb Pst I human cDNA fragment released from pHcGAP,17 The tropoelastin oligomer was end-labeled with [γ-32P] ATP and T4 polynucleotide kinase (Pharmacia). The labeled oligomer was purified on a 15% polyacrylamide gel, eluted into TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and hybridized at 60°C as described.16

**Construction and Screening of an Aortic SMC cDNA Library**

Oligo dT–primed cDNA was synthesized from twice-selected poly(A)+ RNA isolated from cultured P-SMCs with a Pharmacia cDNA synthesis kit according to the manufacturer’s instructions. Double-stranded cDNA was size-selected (>1 kb) on a sucrose density gradient and then cloned into the EcoRI site of lambda gt11 (Lambda-Zap II, Stratagene Inc., La Jolla, Calif.). Three hundred thousand recombinants were screened by making replicate plaque lifts (Colon/Plaque Screen membranes, NEN, Boston) from each plate and hybridizing one filter of a pair with first-strand 32P-cDNA from P-SMCs and hybridizing the other filter with first-strand 32P-cDNA from A-SMCs. Phage plaques that produced at least 10-fold stronger hybridization signals using cDNA from P-SMCs compared with A-SMCs were isolated and plaque-purified, and insert sequences were obtained by phagemid rescue using R408 helper phage according to the manufacturer’s instructions. Twenty-six cDNA clones were obtained that represented nine unique sequence classes based on cross-hybridization analysis. Representative clones from each class were further screened for those sequences expressed in cultured adult carotid neointimal SMCs in greater abundance than in cultured adult medial SMCs from uninjured carotids. Finally, remaining positive cDNA clones were screened for those expressed in greater abundance in adult carotid neointima in vivo at 2 weeks after balloon catheter injury than in underlying media from the same injured arteries. Two cDNA clones that remained positive after being screened in this manner were subjected to DNA sequence analysis by the dideoxy-nucleotide chain termination method (Sequenase, United States Biochemical Corp., Cleveland, Ohio) by using forward and reverse primers complementary to pBluescript vector sequences. The identity of the cDNA sequences was determined by computer search (PC Gene, IntelliGenetics, Mountain View, Calif.).

**In Situ Hybridization**

Two weeks after a balloon catheter injury,9 rats were killed and immediately perfused for 5 minutes with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Arteries were removed and immersed in the same fixative for 2 hours. Rings of artery were dehydrated and embedded in paraffin. Sections were deparaffinized and dehydrated through graded ethanols on the day of hybridization and then briefly rinsed in 0.5× standard saline citrate (SSC). Sections were immersed in 4% paraformaldehyde at 4°C for 10 minutes, followed by incubation with 1 μg/ml proteinase K (Sigma Chemical Co., St. Louis, Mo.) for 8 minutes at 37°C. After a 10-minute wash in 0.5× SSC, sections were prehybridized with 25 μl per section of hybridization buffer consisting of 50% formamide, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, and 10 mM dithiothreitol. Slides were placed in airtight boxes containing blotting paper soaked with 4× SSC and 50% formamide and incubated for 2–4 hours at 55°C. 35S-labeled riboprobes were prepared as previously described,18 and riboprobes at 3×106 cpm along with 0.5 mg/ml yeast tRNA and hybridization buffer in a volume of 25 μl were added to the 25 μl already on the section and mixed by pipetting. Sections were hybridized overnight in the humidified boxes at 55°C. Slides were washed twice for 10 minutes each in 2× SSC with 10 mM β-mercaptoethanol and 1 mM EDTA and were then immersed in RNase A solution (20 μg/ml in 0.5 M NaCl and 10 mM Tris, pH 8.0) for 30 minutes at 37°C. Slides were then washed for 2 hours in 0.1× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA at 55°C, rinsed in 0.5× SSC, dehydrated in 50%, 70%, and 90% ethanols, each containing 0.3 M ammonium acetate, and quickly dried in a 50°C oven. For autoradiography, slides were dipped in Kodak NTB-2 nuclear track emulsion and exposed at 4°C for 3–15 days.

**PDGF Radioreceptor Assay**

Levels of PDGF-like activity in SMC-conditioned medium were estimated by radioreceptor assay as described.19 Serum-free culture medium was conditioned for 48 hours by confluent cultures of neointimal or medial carotid SMCs. Cultures of diploid human fibroblasts were incubated with various amounts of conditioned medium for 3 hours at 4°C, rinsed once with cold phosphate-buffered saline, and then incubated with
PDGF Ligand and Receptor Gene Expression

We previously reported that P-SMCs express both the PDGF-A and PDGF-B genes in vitro, whereas A-SMCs contain PDGF-A but not PDGF-B transcripts. To examine the PDGF mRNA phenotype of adult carotid neointimal SMCs, paired cultures of neointimal SMCs and contralateral medial SMCs were examined at the sixth passage in vitro. Both neointimal and medial SMC cultures contained similar levels of PDGF-A mRNA (Figure 1A). In contrast, only neointimal SMCs had detectable levels of PDGF-B gene expression.

The PDGF receptor mRNA phenotypes were determined for the SMC isolates shown in Figure 1A. Similar levels of PDGF β-receptor mRNA were found in all SMC isolates examined (Figure 1B), whereas PDGF α-receptor mRNA was much more abundant in medial SMCs than in paired neointimal SMCs (Figure 1B, lanes 1 and 3). The PDGF receptor mRNA phenotype for neointimal SMCs resembled that for P-SMCs rather than A-SMCs (Figure 1B). Although PDGF α-receptor mRNA was not detected in 15 μg total RNA from neointimal SMCs (Figure 1B, lane 1), a 6.5-kb PDGF α-receptor transcript was clearly present in 15 μg poly(A)+ RNA from these cells (not shown). Therefore, neointimal SMCs do express the PDGF α-receptor gene in vitro but at much lower levels or in many fewer cells than the paired medial SMC isolates examined here. Considerable variation in the levels of PDGF β-receptor transcripts was found among the different isolates, including variations within the same isolate examined at different times in culture. Since PDGF β-receptor gene expression is serum and growth factor responsive (References 21 and 22 and authors’ unpublished observations), we tried to make the comparisons shown in Figure 1B at similar cell densities, serum concentrations, and days after media change.

It should be pointed out that the analysis of PDGF receptor gene expression described above was confined to steady-state levels of the respective receptor mRNAs. Since we have not measured levels of PDGF receptor protein, ligand binding activity, or receptor coupling to signaling pathways, we emphasize that levels of mRNA reported here may not reflect cellular responsiveness to PDGF isoforms in intact SMCs.

Effects of Passage Number on PDGF-B Gene Expression

We previously observed that PDGF-B mRNA levels in newborn rat aortic SMC cultures were very low (sometimes undetectable) in primary culture and increased in successive subcultures. This may be due to

FIGURE 1. Platelet-derived growth factor (PDGF) ligand and receptor gene expression in adult rat carotid smooth muscle cells. Panel A: Fifteen micrograms of total cellular RNA from paired cultures of neointimal (lanes 1 and 2) and medial (lanes 3 and 4) smooth muscle cells was electrophoresed, blotted, and hybridized for PDGF-A (lanes 1 and 3) and PDGF-B (lanes 2 and 4) transcripts. Neointimal and medial smooth muscle cell cultures were used at the sixth passage. A similar analysis was performed for aortic smooth muscle cell cultures from newborn (12-day-old, lanes 5 and 6) and adult (3-month-old, lanes 7 and 8) rats for PDGF-A (lanes 5 and 7) and PDGF-B (lanes 6 and 8) mRNAs. Panel B: The blots shown in panel A were reprobed for PDGF α-receptor (lanes 1, 3, 5, and 7) and PDGF β-receptor (lanes 2, 4, 6, and 8) transcripts. The PDGF mRNA phenotype of adult carotid neointimal smooth muscle cells in vitro resembles that of newborn rather than adult aortic smooth muscle cells.

[125I]PDGF at 0.5 ng/ml in binding medium for 1 hour at 4°C. The cultures were rinsed three times at 4°C with binding rinse (phosphate-buffered saline, pH 7.4, 1 mM CaCl₂, and 0.1% bovine serum albumin), and cell-bound [125I]PDGF was solubilized with 2% Triton X-100. Purified human platelet PDGF was used as the standard. The human fibroblast target cells express both PDGF α-receptors and PDGF β-receptors; the latter are approximately 20-fold more abundant than the former on these cells.
selection of an SMC subpopulation (i.e., P-SMC) with a growth advantage in our culture system.

A similar analysis of adult carotid neointimal and medial SMC cultures was carried out as follows: Total cellular RNA was isolated at each passage from primary culture to the 10th subculture and subjected to RNA blot analysis. Both neointimal and medial SMC cultures expressed the PDGF-A gene at all passages studied. No consistent difference was observed that could be attributed to passage number. The variation in PDGF-A mRNA levels found in neointimal SMC cultures overlapped with the variation found in medial SMC cultures.

In contrast, PDGF-B transcripts in neointimal SMCs increased in abundance over the first four passages (Figure 2), whereas no expression of the PDGF-B gene was detected in paired medial SMC cultures isolated from the same animals (not shown). From passages 6 to 10, neointimal SMC cultures continued to express the PDGF-B gene at an elevated level. However, in three of five medial SMC isolates, variable levels of PDGF-B gene expression could be detected in passage numbers >6. We do not attribute this to contamination with neointimal SMCs because carotid medial SMC cultures were established from the uninjured contralateral vessel. Moreover, A-SMC cultures passed at the same time and schedule showed no evidence of PDGF-B gene expression.

Effects of Passage Number on PDGF Secretion

Levels of PDGF-like molecules in conditioned media were determined by radioreceptor assay. No significant increase in PDGF-like activity was found in conditioned media from neointimal compared with medial SMC cultures beyond the primary passage (Table 1). Thus, secretion of PDGF-like molecules correlates best with PDGF-A rather than PDGF-B mRNA levels in passaged rat carotid SMCs. These results are consistent with a previous report showing that little or no PDGF-B is secreted into conditioned medium by carotid neointimal SMCs. They also suggest that increased secretion of PDGF-like activity by carotid neointimal versus medial SMCs observed in primary culture (Table 1 and Reference 6) is not a stable property of these cells in vitro but rather may relate to the activated expression of PDGF-A in neointimal SMCs in vivo before placing the cells in culture.

Molecular Cloning of cDNA Sequences Selectively Expressed by Neointimal SMCs

The results described above suggest that adult carotid neointimal SMCs in vitro reexpress a developmentally regulated PDGF phenotype. However, we and others have shown that expression of PDGF ligand and receptor genes by passaged SMCs in vitro is considerably different from that observed for SMCs within intact arteries in vivo. Moreover, the levels of PDGF ligand and receptor mRNAs in SMC cultures can vary greatly in response to different culture conditions and passage numbers. Therefore, the PDGF phenotype differences we observed between cultured neointimal and medial SMCs, or between P-SMCs and A-SMCs, do not necessarily mean that analogous SMC diversity exists within the corresponding intact artery walls.

To address this question, we prepared a size-selected (>1kb) cDNA library from poly(A) RNA isolated from P-SMCs and screened it for sequences selectively expressed by carotid neointimal versus medial SMCs (see “Materials and Methods” for details of the screening procedure). The screening strategy was designed to satisfy a sequence of three objectives as follows: to identify cDNA clones that detected mRNAs that were 1) present in greater abundance in P-SMCs than A-SMCs, 2) also more highly expressed in neointimal smooth muscle cells. Values are mean ± SD.

The culture medium from paired isolates of adult carotid neointimal and medial SMCs was conditioned for 48 hours and assayed for PDGF-like molecules by radioreceptor competition binding assay as previously described. Two independent isolates of carotid neointimal and medial SMCs were examined by duplicate determinations at each of the indicated passage numbers. The paired isolates of carotid neointimal and medial SMCs were obtained 2 weeks after balloon catheter injury. The PDGF-like molecules produced by rat carotid neointimal or medial SMCs have been shown to consist of PDGF-AA and/or PDGF-AB but not PDGF-B.

Neointimal SMCs produce significantly more PDGF-like molecules (p < 0.05) than paired medial SMCs at first passage but not thereafter.

Table 1. Production of Platelet-Derived Growth Factor–Like Molecules by Rat Carotid Neointimal and Medial Smooth Muscle Cells

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<thead>
<tr>
<th>Passage</th>
<th>Neointimal SMCs</th>
<th>Medial SMCs</th>
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<tr>
<td>1</td>
<td>0.57 ± 0.15*</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.44 ± 0.06</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>4</td>
<td>0.19 ± 0.12</td>
<td>0.30 ± 0.06</td>
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<tr>
<td>6</td>
<td>0.27 ± 0.16</td>
<td>0.44 ± 0.14</td>
</tr>
<tr>
<td>8</td>
<td>0.21 ± 0.16</td>
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PDGF, platelet-derived growth factor; SMCs, smooth muscle cells.

FIGURE 2. Effect of serial passage on platelet-derived growth factor-B (PDGF-B) gene expression in adult carotid neointimal smooth muscle cells in vitro. Smooth muscle cell cultures from the injured left carotid neointima formed 2 weeks after balloon catheter injury were serially passaged for 12 subcultures. At the passage number indicated, total cellular RNA (15 µg per lane) was isolated, electrophoresed, blotted, and hybridized for PDGF-B transcripts. A replicate analysis of a second independent neointimal SMC isolate obtained 2 weeks after injury gave similar results. Neointimal smooth muscle cells at passage 10 contained levels of PDGF-B mRNA that were similar to the level shown for passages 6 and 8.

PDGF-like molecules (ng equivalents of PDGF per 10⁴ cells per 48 hr)
SMCs than in medial SMCs in vitro, and 3) also more abundant in carotid neointimal tissue than in underlying medial tissue in vivo. The isolation of cDNA clones that identified genes whose expression in intact arteries satisfied this screening protocol was our primary goal.

We initially identified 26 cDNA clones that represented nine different (non-cross-hybridizing) genes expressed by P-SMCs>A-SMCs in vitro. A computer search of the DNA sequence database (GenBank) indicated that the nine different cDNAs included five known and four unknown sequences. Four of these nine genes were found to also be expressed by intimal SMCs>medial SMCs in vitro. Finally, two of these remaining four cDNA clones were found to fulfill the complete screening criteria described above. DNA sequence analysis confirmed that these two cDNAs were derived from the transcripts of different genes (see below). The cDNA inserts from each of the two clones were labeled with $[^{32}P]$dCTP and used to assess cognate mRNA levels in the intact artery wall during normal postnatal development and during arterial wound repair (Figure 3). As shown in Figure 3A, clone p56A3 hybridized to a single mRNA species of 4.5 kb that was developmentally regulated in rat aorta. This transcript species was greatly increased after balloon catheter injury, particularly within the developing neointima, in both adult rat carotid artery (Figure 3B) and adult rat aorta (data not shown). The difference in 4.5-kb transcript abundance in the carotid neointima versus the underlying media in vivo was maintained in carotid neointimal versus medial SMCs isolated from the regenerating artery and grown under identical conditions in cell culture (Figure 3C). The latter results argue that an intrinsic difference between neointimal and medial SMCs, rather than a difference in exogenous factors present in the local environment, is responsible for maintaining unequal levels of p56A3-related mRNA.

Clone p171A3 hybridized to two major transcript species of approximately 5.7 and 4.6 kb in size that were also developmentally regulated in the rat aorta (Figure 3D). These two transcript species were strongly increased in abundance in adult rats during neointimal formation after arterial injury in the carotid artery (Figure 3E) or in the aorta (data not shown). Carotid

<table>
<thead>
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<th>p56A3</th>
<th>p171A3</th>
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<td>A.</td>
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<td>B.</td>
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**FIGURE 3.** Smooth muscle cDNA clones recognize developmentally regulated mRNAs that are also increased after arterial injury. Panel A: p56A3-related (tropoelastin) mRNA levels in 12-day-old rat pup aorta (lane 1), 3-month-old adult rat aorta (lane 2), cultured aortic smooth muscle cells from 12-day-old rats (lane 3), and cultured aortic smooth muscle cells from 3-month-old rats (lane 4). Panel B: p56A3-related mRNA levels in adult rat carotid artery at the indicated times after balloon catheter injury. W, weeks; I, intima; M, media. Panel C: p56A3-related mRNA levels in smooth muscle cells cultured from the neointima (lane I) or contralateral media (lane 2) of adult rats 2 weeks after a balloon catheter injury. Panels D–F: Same RNA samples as described in panels A–C, respectively, that were hybridized for p171A3-related mRNAs. For each lane shown, 15 µg total RNA was analyzed. In all cases, equal loading and transfer of total RNA per lane was ensured by visualizing the ribosomal RNA bands transferred to the membrane under ultraviolet light. In most cases, the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were also determined. Both approaches verified that the signals obtained using the smooth muscle cell cDNA inserts as probes cannot be attributed to variations in RNA sample loading or transfer. DNA sequence analysis confirms that p56A3 encodes rat tropoelastin, whereas p171A3 encodes α1 procollagen (type I).
neointimal SMCs in vitro retained a higher level of expression of sequences recognized by p171A3 than did paired cultures of carotid medial SMCs (Figure 3F).

Although PDGF-B was found to be selectively expressed by P-SMCs and intimal SMCs in vitro (Figure 1), it was not among the cDNA clones we obtained. Using a PDGF-B cDNA probe, we could show that PDGF-B cDNAs were indeed present in the library. The explanation for not detecting PDGF-B cDNA clones with the differential colony screening protocol used here most likely lies in the relative amount of PDGF-B mRNA in the initial poly(A)+ RNA used to make the cDNA library. In contrast to the very abundant tropoelastin and procollagen mRNAs, PDGF-B is a rare transcript. Since the representation of a given gene in a cDNA library is proportional to the abundance of its mRNA in the total poly(A)+ RNA pool, genes expressed at low levels (like PDGF-B) would be much less likely to be identified in the cDNA library with the differential screening approach used here.

**In Situ Hybridization Analysis of Rat Carotid Neointima**

To examine in greater detail the expression of p56A3-related sequences in developing rat carotid neointima, we employed in situ hybridization analysis. As shown in Figure 4A, antisense 32P riboprobes transcribed from p56A3 hybridized strongly within the developing carotid neointima but at much lower levels in the underlying media. In contrast, control sense-strand riboprobes produced only low background signals in neointimal and medial tissue (Figure 4B).

**Identification of SMC cDNA Clones as Tropoelastin and α1 Procollagen (Type I)**

DNA sequence analysis revealed that cDNA clone p56A3 encoded rat tropoelastin. The 5' sequence of p56A3 that we obtained is identical to nucleotides 293–494 of the rat aortic tropoelastin sequence reported by Pierce et al26 with the exception of codon 460–463, at which we found GCT (Ala) instead of GGT (Gly). Moreover, the 3' end of p56A3 is 99% identical in sequence over 232 nucleotides to that reported by Deak et al16 for the 3' untranslated region of a tropoelastin cDNA isolated from a neonatal rat aorta cDNA library.

Despite this near sequence identity, p56A3 identified a single transcript species in rat aorta (4.5 kb) that was clearly larger than the 3.5-kb transcript reported by Deak et al.16 To verify the identity of p56A3 as rat tropoelastin, we used a 24-mer oligonucleotide sequence encoding a cross-link domain from the C-terminus of rat tropoelastin to probe RNA blots of cultured SMCs from neonatal rat aorta. The 24-mer oligonucleotide probe was identical to the one used by Deak et al. Both the 24-mer probe and the cDNA clone (p56A3) identified a single 4.5-kb transcript in RNA blots from neonatal aortic SMCs (data not shown). Further evidence that p56A3 encoded rat tropoelastin was obtained by using a bovine tropoelastin cDNA clone (pcBE46)27 that also identified a single 4.5-kb transcript in cultured rat aortic SMCs (data not shown).

Clone p171A3 was identified as α1 procollagen 1 by DNA sequence analysis. A computer search revealed a 92% nucleotide sequence identity of p171A3 to murine α1 procollagen 1 at both the 5' and 3' ends of p171A3. Moreover, the transcript sizes we detected in rat arteries are consistent with those reported previously for α1 procollagen 1 mRNA.28

**Discussion**

Walker et al6 reported that SMCs derived from the neointima of rat carotid arteries have a different shape and organization in vitro than paired SMC isolates from uninjured media of the contralateral carotids. We have extended those findings here by showing that neointimal SMCs in vitro also display a different mRNA phenotype.

**Figure 4.** Photomicrographs showing localization of p56A3-related mRNA (tropoelastin) in carotid neointima by in situ hybridization. Panel A: Hybridization of antisense riboprobes to rat carotid artery 1 week after balloon catheter injury. The hybridization signal for p56A3-related mRNAs (rat tropoelastin) is abundant in the developing neointima but low or absent in the underlying media. The lumen is at the top left. Panel B: Hybridization of sense-strand control riboprobe to injured carotid artery. Cross section shows the signal due to nonspecific background binding of the riboprobe to the sectioned arterial tissue.
SMCs Diversity in Artery Wall Development and Repair

Repair of balloon catheter injury to adult rat carotid artery results not only in healing of the media but also in the formation of a new layer of artery wall, a neointima. Many of the properties required of adult SMCs to form a neointima are also found in SMCs of developing arteries in the embryo and neonate. SMC migration, division, extracellular matrix production, and remodeling are prominent features of developing blood vessels and of injured arteries during wound repair. Indeed, cytoskeletal and contractile protein isoforms characteristic of SMCs in fetal and newborn arteries are frequently reexpressed during neointima formation in adult blood vessels. Since the SMC population of the arterial media has generally been regarded as homogeneous, it is commonly thought that SMCs must revert from a highly differentiated contractile state to a more primitive synthetic state so that new cell functions (e.g., migration and replication) can be expressed. In contrast to this process of “phenotypic modulation,” the differences we observed in morphology and gene expression between paired carotid neointimal and medial SMCs are maintained over multiple cell generations and in a variety of growth conditions in vitro. The concept of phenotypic modulation may account for differences we describe in carotid SMC properties in vitro compared with the intact artery, but it does not predict the differences in cell shape and gene expression that we observed between paired neointimal and medial SMC isolates grown and passaged under identical conditions in culture. Thus, the mechanism by which SMCs with the neointimal phenotype are produced after arterial injury appears to be qualitatively different from the changes in SMC properties produced by phenotypic modulation. Since the phenotype that we described previously for P-SMCs strongly resembles that of adult carotid neointimal SMCs, we suggest that neointima formation in the adult arterial wall depends on renewed expression of a “tissue-forming” SMC phenotype specialized for growth factor production, cell proliferation, and extracellular matrix synthesis in vivo. The simplest interpretation of these data is that the artery wall is composed of a mixture of different SMC types, analogous to slow-twitch and fast-twitch skeletal myofibers in a muscle bundle. Thus, neointimal SMCs may originate by the expansion of a preexisting SMC subpopulation specialized for the repair of arterial injury. Alternatively, some fully differentiated SMCs may be converted to a more primitive state that is maintained in vitro.

We found that carotid neointimal SMCs examined at the sixth passage expressed the PDGF-B gene, whereas paired cultures of medial SMCs did not. However, in a previous study we found no difference in low levels of PDGF-B mRNA in vivo between carotid neointima versus underlying media either by RNA blot analysis or by in situ hybridization. These results are reminiscent of the pattern of PDGF-B gene expression that we observed previously for P-SMCs versus A-SMCs. Despite large differences in PDGF-B mRNA levels in passaged P-SMCs compared with A-SMCs, we found only similar low levels of PDGF-B transcripts in intact aorta from newborn and adult rats. Indeed, primary cultures of carotid neointimal SMCs or P-SMCs had low levels of PDGF-B mRNA that were similar to those in the intact artery. This might mean that increased levels of PDGF-B mRNA seen in passaged neointimal SMCs represent selective expansion of a distinct SMC subpopulation with a growth advantage in our culture system. Alternatively, accumulation of PDGF-B mRNA could represent stimulation of gene expression in vitro.

FIGURE 5. Drawing showing the diversity of arterial smooth muscle cell phenotypes. Aortic smooth muscle cells from newborn rat pups (P-SMCs) or adult rats (A-SMCs) were examined in cell culture. P-SMCs have an epithe-lioid shape and form a monolayer in vitro, secrete a platelet-derived growth factor (PDGF)-like activity (PDGF*), express the PDGF-B gene (BmRNA*), but contain little or no PDGF α-receptor mRNA (PDGF-αR*). A-SMCs express the extracellular matrix genes for tropoelastin, α1 procollagen (type I), and osteopontin at high levels. After normal postnatal development, A-SMCs were found to be spindle-shaped and form overlapping “hills and valleys” in vitro, did not secrete PDGF or express the PDGF-B gene, but did contain abundant PDGF α-receptor mRNA (PDGF-αR*). A-SMCs express the extracellular matrix genes described above at much lower levels than P-SMCs. The phenotype of smooth muscle cells cultured from adult rat carotid neointima 2 weeks after balloon catheter injury closely resembled that of P-SMCs rather than A-SMCs.
In either case, SMC cultures derived from the carotid neointima behave differently from those isolated from uninjured media despite being grown and passaged under identical conditions in vitro. Although intriguing, the differences in SMC phenotypes we observed in cell culture may depend on unique conditions in vitro and do not necessarily mean that such differences also exist in the intact artery wall in vivo.

To test whether the unique phenotype of passaged neointimal SMCs in vitro was representative of some property of these cells in injured arteries in vivo, we constructed and screened a rat SMC cDNA library for additional molecular markers of the “pup-intimal” SMC phenotype. The criteria we used for screening the cDNA library were designed to identify sequences that were overexpressed by neointimal SMCs both in vitro and in vivo when compared with corresponding medial SMCs. We identified two cDNA clones, derived from the products of different genes, that were expressed at much higher levels in developing carotid neointima than in underlying media 2 weeks after balloon catheter injury and whose differential expression in vivo was maintained in neointimal versus medial SMCs in vitro. Similarly, both of these cDNA clones recognized transcripts that were developmentally regulated in the rat aorta and were expressed at much higher levels in P-SMCs than in A-SMCs. DNA sequence analysis confirmed the identity of these cDNA clones as encoding rat tropoelastin and α1 procollagen I.

In addition, a further screening of the SMC cDNA library by use of a subtracted probe enriched in pup-intimal phenotype-specific sequences has identified a third extra-cellular matrix protein, osteopontin, that is also expressed in a pattern similar to that described above for tropoelastin and α1 procollagen I. Taken together, these findings strongly suggest that the unique properties of cultured SMCs from newborn rat aorta and adult carotid neointima do indeed reflect distinctly different cell potentials (possibly subpopulations) that also exist in vivo (Figure 5).

Further Evidence for SMC Heterogeneity

Several other laboratories have reported isolating two aortic SMC types very similar to the ones we describe here. Blaes et al.45 isolated SMCs from medial explants of thoracic aortas from 7–8-week-old Wistar rats. On passaging, two SMC populations were obtained. One SMC type was spindle-shaped and overlapped extensively, whereas the other SMC type formed a monolayer of cobblestone-shaped cells.45 Dreher and Cowan46 obtained SMCs by enzyme digestion of thoracic aortas from young Sprague-Dawley rats (150–200 g) and transfected into these cultures a plasmid expressing the neomycin resistance gene under the control of a metallothionein promoter. After selection with G418, four SMC clones were established that fell into two morphological groups: spindle-shaped (two clones) and epithelioid (two clones). Western blot analysis indicated that all four clones expressed smooth muscle myosin and α-smooth muscle actin at similar levels, thus establishing their smooth muscle origin.46 These authors noted that both SMC types could be identified in cultures of first-passaged cells before transfection, similar to our previous findings.12 Chipman et al.47 have used aortic SMCs from 1–3-day-old Sprague-Dawley rats as a rich source of cells that maintain high levels of elastin synthesis in vitro. In monolayer cultures, epithelioid islands of cells are present that accumulate thick bands of elastin-containing fibers at confluence.48

On the other hand, Sjolund et al.4 were unable to detect PDGF-B mRNA in aortic SMC cultures from 10-day-old Sprague-Dawley rats or in intact rat aorta. The differences between our results2 and those of Sjolund et al may, in part, be a question of RNA detection limits. We2,4 and others25–49–51 have reported detecting low levels of PDGF-B transcripts in normal rat, human, and baboon arterial wall. We reported that newborn rat aortic SMC cultures contained low levels of PDGF-B mRNA, similar to the intact artery, in the first two to four passages in vitro.2 Sjolund et al examined newborn aortic SMCs only in the first two passages in culture. However, Hultgardh-Nilsson et al.52 were unable to detect PDGF-B gene expression in early or late passage aortic SMC cultures from 6–10-day-old rats. Despite a lack of PDGF-B expression, their findings that newborn rat aortic SMCs were deficient in PDGF α-receptors and did not respond mitogenically to PDGF-AA, whereas paired adult aortic SMCs both bound and responded mitogenically to PDGF-AA, are similar to our results reported here. Variations in cell culture conditions and passaging schedules between different laboratories may be important in determining how different SMC phenotypes or subpopulations are sorted out during long-term growth in cell culture.

Summary and Implications

The studies reported here indicate that the phenotype of an SMC population that is present in newborn but not adult rat aorta is reexpressed during the active phase of neointima formation after arterial injury in adult rats (Figure 5). It is important to point out that the differences in cell shape, organization, and gene expression for SMCs isolated from rat arteries at different stages of development and wound repair that we have described here and elsewhere1–3,4,6,8,29,43 are displayed over many cell generations grown under identical conditions in vitro and thus differ from the changes in SMC properties usually attributed to phenotypic modulation.44 Our findings suggest that the view of the arterial media as being composed of a single homogeneous population of SMCs with identical potentials for cell division, growth factor production, and neointima formation may be too simplistic. The possibility that the SMC population of a normal artery wall may consist of cells that differ in their potential to proliferate, to synthesize and respond to growth factors and growth inhibitors, or to produce a neointima8 needs to be considered. This may have important implications for intimal SMC proliferation and luminal narrowing in vascular disease.

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