PDGF Ligand and Receptor Gene Expression during Repair of Arterial Injury

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Abstract. Smooth muscle cells (SMC) in rat carotid artery leave the quiescent state and proliferate after balloon catheter injury, but the signals for mitogenesis are not known. In this study, the possibility that cells within damaged arteries produce a growth factor that could act locally to stimulate SMC replication and repair was examined. We found that the genes for PDGF-A and -B (ligand) and PDGF receptor (α and β subunits) were expressed in normal and injured carotid arteries and were independently regulated during repair of carotid injury. Two phases of PDGF ligand and receptor gene expression were observed: (*a*) In the early stage, a large decrease in PDGF β-receptor mRNA levels preceded 10- to 12-fold increases in PDGF-A transcript abundance in the first 6 h after wounding. No change in PDGF α-receptor or PDGF-B gene expression was found at these times. (*b*) In the chronic phase, 2 wk after injury, neointimal tissue had lower levels of PDGF α-receptor mRNA (threefold) and higher levels of PDGF β-receptor mRNA (three- to fivefold) than did restored media. Moreover, in situ hybridization studies identified a subpopulation of neointimal SMC localized at or near the luminal surface with a different pattern of gene expression than the underlying carotid SMC. Luminal SMC were strongly positive for PDGF-A and PDGF β-receptor transcripts, while showing little or no hybridization for PDGF-B or PDGF α-receptor. Immunohistochemical studies showed strongly positive staining for PDGF-A in SMC along the luminal surface. These data show that changes in PDGF ligand and receptor expression occur at specific times and locations in injured carotid artery and suggest that these changes may play a role in regulating arterial wound repair.

VESSEL wall injury produced by passage of a balloon catheter through the lumen of an artery initiates a sequence of smooth muscle cell (SMC)[1] responses including proliferation, migration toward the damaged surface, and formation of a fibrocellular neointima (Bjorkerud and Bondjers, 1971; Stemerman and Ross, 1972; Spaet et al., 1975; Schwartz et al., 1975). The stimuli that signal these cellular responses in vivo are not known. Platelet degranulation products may play a role in initiation of this process (Ross, 1986), but lack of continued platelet interactions with the injured artery at later times (Groves et al., 1979; Clowes et al., 1986; Jorgensen et al., 1988) suggests that other factors, perhaps those produced by cells in the regenerating vessel wall itself, direct continued SMC proliferation during repair of arterial injury.

The concept of endogenous control of SMC wound repair by local growth factor production in vivo is supported by reports that a change in SMC growth properties accompanies the repair process. For example, explants of mechanically injured rat aorta show increased SMC migration from the explant with a greatly reduced serum requirement for growth than SMC outgrowth from explants of normal aorta (Rhee et al., 1977; Grünwald et al., 1984). This might be explained by the findings that, under certain circumstances, arterial SMC in vitro produce peptide growth factors that can stimulate their own growth (Seifert et al., 1984; Nilsson et al., 1985; Clemmons et al., 1985) and migration (Grotendorst et al., 1981). Indeed, SMC cultured from injured rat carotid arteries secreted increased amounts of a PDGF-like activity into conditioned media than did comparable isolates from uninjured carotids (Walker et al., 1986). However, it is not clear how behavior of cells in culture relates to the activity of SMC in regenerating vessel wall in vivo. This is of particular concern for PDGF-A and PDGF-B genes because it is known that their expression is greatly increased when artery wall cells are placed in culture (Barrett et al., 1984; Majesky et al., 1988; Sjölund et al., 1988).

PDGF can be composed of two distinct but related polypeptides (A and B) that are products of unique genes (reviewed in Ross et al. [1986]). All three dimeric combina-

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1. *Abbreviations used in this paper*: ODC, ornithine decarboxylase; SMC, smooth muscle cells.

tions (AA, AB, BB) have been purified from natural sources (Hart et al., 1990). Biological activities of the PDGF isoforms are often dissimilar on the same target cell type (Nistér et al., 1988). This may be due to different numbers of distinct binding sites available for each ligand on target cells (Kazlauskas et al., 1988; Seifert et al., 1989). Two separate PDGF receptor genes (α and β) have been identified (Yarden et al., 1989; Hart et al., 1988; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989; Lee et al., 1990). Products of these genes exist at the cell surface as monomers that can associate to form noncovalent dimers capable of binding dimeric PDGF molecules with high affinity (Seifert et al., 1989; Heldin et al., 1989; Williams, 1984). The β-subunit binds only the β-chain of PDGF whereas the α-subunit binds both A- and B-chains. This difference in ligand specificity of the receptor subunits determines the isoform specificity of high-affinity dimeric receptors: $\beta\beta$ receptors bind only PDGF-BB, $\alpha\beta$ receptors bind PDGF-AB or -BB and $\alpha\alpha$ receptors bind all three isoforms with high affinity. Since not all forms of PDGF receptor are capable of binding all isoforms of PDGF, the biological functions of PDGF in vivo could be regulated at the levels of receptor number (Terracio et al., 1988) and composition as well as ligand concentration and composition. This argues for a need to determine expression levels for both ligand genes and both receptor subunit genes before suggesting the existence of a PDGF-dependent endogenous regulatory mechanism.

In the studies reported here, we have explored the properties of cells repairing extensive injury to rat carotid artery in vivo with regard to the following questions. (*a*) Do cells in quiescent carotid artery express the genes for PDGF (A, B) or PDGF receptor subunits (α, β) before injury? (*b*) Is expression of the ligand genes (PDGF A or B) stimulated in vessel wall cells during repair of the wound? (*c*) Are the PDGF receptor genes (α, β) expressed in regenerating arteries and, if so, how does their expression vary in relation to that of the ligand genes? and (*d*) What do the patterns of PDGF ligand and receptor gene expression suggest about possible roles for locally produced PDGFs in cellular responses to arterial injury?

Materials and Methods

Arterial Injury Model

Male Sprague-Dawley rats (500 g, 5 mo old) (Tyler Laboratories, Bellevue, WA) were anesthetized and acute injury to the left common carotid artery was made with an inflated balloon catheter as previously described (Clowes et al., 1983). At the indicated times after injury, animals were killed and both injured (left) and uninjured (right) common carotids were retrieved and stripped of periadventitial fatty and connective tissues in PBS at 4°C. Endothelium of the right carotid was removed by gently scraping the luminal surface with the edge of a Teflon card. Efficacy of this procedure was verified by loss of hybridization signal for von Willebrand's factor mRNA. Arteries were then snap frozen in liquid nitrogen for subsequent RNA isolation. In some cases, thickened neointima from regenerating carotid arteries was separated from underlying media by careful dissection under magnification and RNA from the two tissue preparations was analyzed separately.

RNA Isolation and Blot Hybridization

Frozen arterial tissue was ground to a fine powder under liquid nitrogen and total cellular RNA was prepared by acid guanidinium thiocyanate-extraction as described (Chomczynski et al., 1987). Agarose gel electrophoresis and RNA transfer to nylon membranes (Zeta Probe; Bio-Rad Laboratories, Richmond, CA) were carried out as previously described (Majesky et al., 1988). After transfer, RNA blots were exposed to short-wave UV light both to cross-link RNA to the membrane and to visualize the major ribosomal RNA bands. At this point, photographs were taken and assurance was made that equal amounts of total cellular RNA had transferred to the membrane. Blots were hybridized as previously described (Majesky et al., 1988) using cDNA probes labeled with ^{32}P-dCTP by random primer extension (Amersham Corp., Arlington Heights, IL) and then washed at 60°C in two changes of 0.045 M NaCl/0.0045 M sodium citrate, pH 7.0/0.1% SDS for 10 min each and exposed to Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY) at −70°C.

^{32}P-Autoradiography

For quantitation of ^{32}P-autoradiographic signals, blots were exposed to preflashed film and exposures were collected at increasing intervals (two-fold) of time. Autoradiograms were scanned at 600 nm with a Beckman laser densitometer (Beckman Instruments Co., Fullerton, CA). The difference in exposure times required to produce signals of equal film density was determined for each comparison made. This difference was taken as the relative change in levels of a particular mRNA species. To compare signal intensities from replicate experiments, two types of controls were performed: (*a*) an identical amount of human osteosarcoma cell line U-20S RNA from a common original source was included on each blot run. Transcript levels for carotid samples run on different days were then compared when signal intensities for the U-20S transcripts were equivalent. (*b*) A sample of carotid RNA from a previous experiment was included each time a new experiment was analyzed. In this way, absolute signal intensities from different experiments could be made comparable by the U-20S controls and relative comparisons could be made of test samples analyzed on different days by using the carotid RNA controls. For these studies, transcript levels were normalized to total cellular RNA.

In Situ Hybridization

In situ hybridizations were carried out as previously described (Wilcox et al., 1988). Carotid arteries were rinsed in ice-cold PBS, a 5-mm portion from the middle of the common carotid was excised, placed in 4% paraformaldehyde-0.1 M sodium phosphate buffer, pH 7.4, at 4°C for 3 h and then transferred to sterile 15% sucrose/PBS overnight. The tissue was frozen in OCT (Miles Scientific Laboratories, Elkhart, IN) and stored at −70°C. Frozen sections (5 μm) were thaw-mounted on polylysine-coated slides, pretreated with proteinase K (1 μg/ml, 10 min), prehybridized for 2 h in 50 μl of prehybridization buffer (0.3 M NaCl/20 mM Tris, pH 8.0/5 mM EDTA/1× Denhardt's solution/10% dextran sulfate/10 mM DTT/50% formamide) and hybridized by addition of 300,000 cpm of ^{35}S-riboprobe in a small volume of prehybridization buffer. After hybridization, the sections were washed with 2× SSC/10 mM β-mercaptoethanol/1 mM EDTA (twice for 10 min each) (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), treated with RNase (20 μg/ml, 30 min at room temperature), washed in 2× SSC (as above) followed by a high stringency wash as 55°C. The remaining steps were carried out exactly as described (Wilcox et al., 1988). ^{35}S-riboprobe transcripts were prepared from the following plasmids: (*a*) pF9A5 contains a 0.9-kb mouse PDGF-A cDNA fragment and was a generous gift of M. Mercola and C. Stiles, Harvard University, Boston, MA; (*b*) pCB8BA1A2 contains a 500 bp mouse PDGF-B genomic fragment and was also provided by Mercola and Stiles; (*c*) p802E/B5 contains a full-length 6.4-kb cDNA insert for rat PDGF receptor α-subunit (Lee et al., 1990), and a 1.4-kb Bam HI fragment from the external domain subcloned into pGEM-3 was used, and (*d*) pmPDGFR carries a full-length 5.8-kb cDNA for mouse PDGF receptor β-subunit (Yarden et al., 1986) and was provided by L. T. Williams (University of California, San Francisco). A 1.2-kb Hinc II fragment subcloned into pGEM-3 was used.

DNA Probes

DNA probes used for RNA blot hybridizations were as follows: PDGF-A, a 1.3-kb Eco RI human cDNA fragment released from pD1 (Betsholtz et al., 1986); PDGF-B, a 2.1-kb Sac I–Sac II human cDNA fragment for pSM-1 (Ratner et al., 1985); PDGF β-receptor, a 4.7-kb Eco RI–Xba I human cDNA fragment from phPDGF-R (Gronwald et al., 1988); PDGF α-receptor, a 6.4-kb Eco RI rat cDNA from p802E/B5 (Lee et al., 1990); histone, a 1.7-kb Ava I–Sal I mouse genomic fragment from pH312 (Stimac et al., 1984); ornithine decarboxylase (ODC), a 1.0-kb Sal I–Hind III mouse cDNA fragment (McConlogue et al., 1984); *fos*, a 1.0-kb Pst I

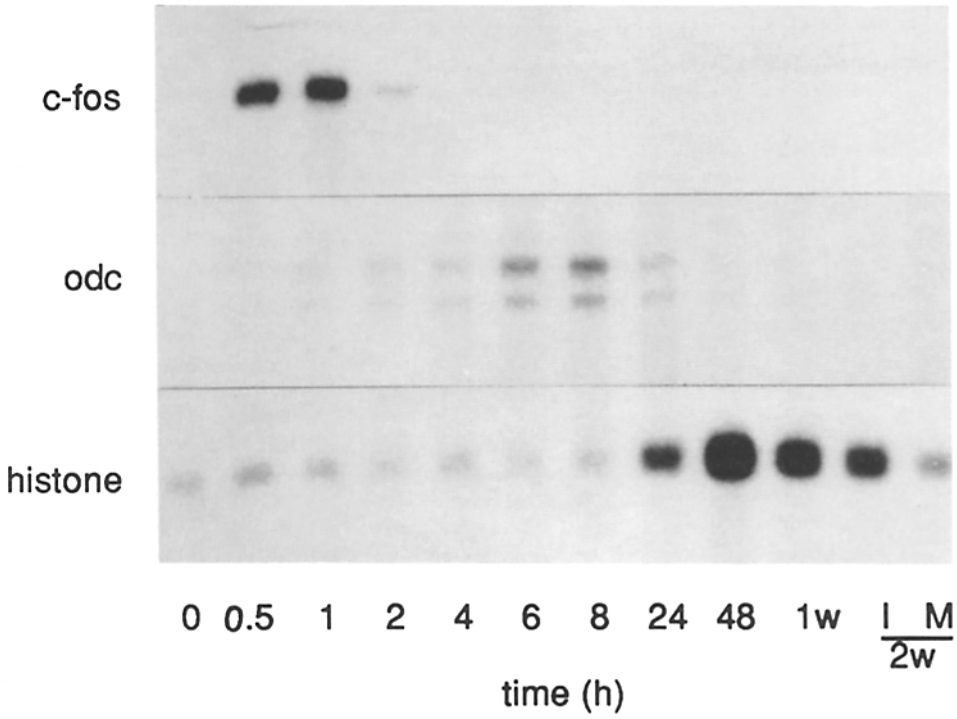

Figure 1. Expression of c-*fos*, ODC, and histone genes after carotid injury. Total cellular RNA was isolated from left carotid arteries at the times indicated after balloon catheter injury (10–12 vessels pooled per time point). 12 μg was electrophoresed, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA probes as indicated. At 2 wk (*W*), the neointima (*I*) was stripped off the underlying media (*M*) before RNA isolation. Blots were exposed to film for 16 h.

genomic fragment from p*fos*-1 (Curran et al., 1982); von Willebrand's factor, a 1.8-kb Sac I human cDNA from pUC18/r3SacI-1 (Sadler et al., 1985).

Immunohistochemistry

Segments from normal and balloon catheter-injured rat common carotid arteries were embedded in OCT (Tissue Tek) and frozen sections (5 μm) were thaw-mounted onto glass slides. Sections were incubated in 0.3% hydrogen peroxide in cold methanol for 30 min to block endogenous peroxidase activity and permeabilize the cells. Nonspecific binding of rabbit IgG was blocked by preincubation with normal goat IgG (1:50 in PBS/0.1% BSA). The sections were sequentially incubated at room temperature with rabbit anti-human PDGF-AA IgG (1:100) (Hart et al., 1990) for 1 h, biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, CA) for 30 min, and avidin-peroxidase (ABC kit, Vector Laboratories) for 30 min. Each incubation was followed by a wash in PBS and a 15-min incubation in PBS/0.1% BSA. Staining was visualized with 0.05% 3,3'-diaminobenzidine/0.03% hydrogen peroxide in 0.05 M Tris pH 7.6. Slides were rinsed in 0.05 M Tris pH 7.6 and counterstained with methyl green. The rabbit anti-PDGF-AA IgG used for this study was prepared against recombinant human PDGF-AA and recognizes PDGF-AA and PDGF-AB but not PDGF-BB, as previously described (Hart et al., 1990).

Results

Rapid Activation of Genes Associated with Cell Proliferation after Carotid Injury

We previously showed that a subset of SMC rapidly exit the quiescent state and proliferate after balloon catheter-injury to rat carotid artery (Majesky et al., 1987). To examine the kinetics of activation of genes whose expression may be critical for this transition, total cellular RNA was isolated at various times after carotid injury and examined by RNA transfer blot analysis.

c-fos

Rapid and transient increases in c-*fos* transcripts (2.2-kb) were found in injured left carotids (Figs. 1 and 3). Stimulated c-*fos* gene expression was evident at 30 min, maximal at 60 min (>40-fold above uninjured carotids) and diminished to barely detectable levels by 2 h. No further change in c-*fos* gene expression was seen in wounded carotids during the 2-wk period studied. Barely detectable levels of c-*fos* mRNA in uninjured right carotids remained unchanged at the times shown in Fig. 1 (data not shown).

ODC

In a previous study, we showed that ODC activity is transiently stimulated in wounded carotid arteries, reaching maximal values (23-fold above uninjured carotids) at 6 h (Majesky et al., 1987). We show here that levels of ODC transcripts (2.7 and 2.2 kb) rapidly increase and then decrease after carotid injury in a similar pattern (Fig. 1). Thus, ODC activity is probably determined by levels of mRNA available for active enzyme synthesis. Like c-*fos*, only a single peak of ODC transcript accumulation was seen during the 2-wk period studied, corresponding in time to transition of a majority of SMC from quiescence (G_0) into the prereplicative (G_1) phase.

Histone

SMC in injured carotid arteries enter S phase around 24 h after wounding with maximal rates of DNA synthesis observed at 33 h (Majesky et al., 1987). Histone transcripts were barely detectable in the first 8 h after injury (Figs. 1 and 3). Large increases in histone mRNA levels were found at 24 and 48 h, corresponding to the peak period of DNA synthesis detected previously by thymidine autoradiography (Clowes et al., 1983). Histone transcript levels declined by 1 wk, but remained elevated compared with uninjured carotids. By 2 wk, histone mRNA in neointima was five- to

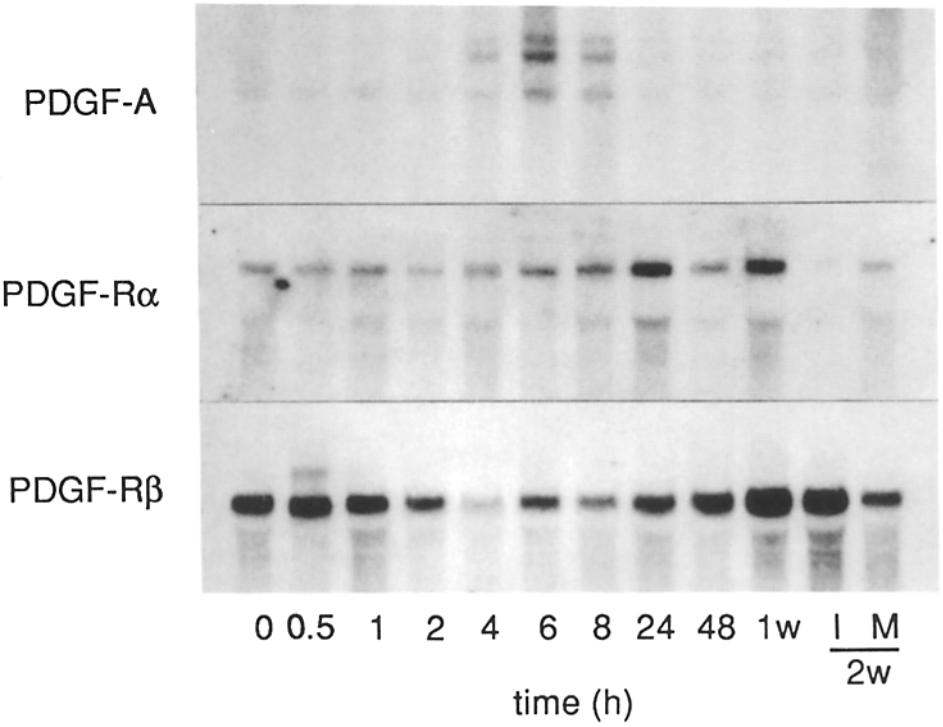

Figure 2. Expression of PDGF-A and PDGF receptor subunit (α and β) genes after carotid injury. Isolation of total cellular RNA, electrophoresis, blot transfer and abbreviations used are described in the legend to Fig. 1. 12 μg of total cellular RNA per lane was examined. The same blot was hybridized sequentially with PDGF-A, PDGF receptor β-subunit, and PDGF receptor α-subunit probes. Washed blots were exposed to film for 18, 12, and 48 h respectively.

eightfold more abundant than in underlying media of regenerating left carotid arteries. Histone mRNA levels were unchanged in uninjured right carotids during this same 2-wk period (data not shown).

Arterial Injury Stimulates Transient Gene Expression for PDGF-A and Rapid Loss of PDGF Receptor β mRNA

PDGF-A. A family of PDGF-A transcripts (2.9, 2.3, and 1.7 kb) was present at low levels in uninjured carotids (Figs. 2 and 3), similar to previously reported results for adult rat aorta (Majesky et al., 1988; Sjölund et al., 1988; Sarzani et al., 1989). Arterial injury evoked large and transient stimulation of PDGF-A gene expression in damaged left carotids, but not in uninjured right carotids (Fig. 2). Detectable increases in PDGF-A mRNA levels (3-fold) were observed 4 h after wounding and reached maximum (10- to 12-fold above uninjured carotids) at 6 h. Similar changes in PDGF-A gene expression after acute injury were seen in three separate experiments. By 24 h, when SMC enter S phase, PDGF-A transcript abundance declined to about fourfold above levels in uninjured carotids. Slightly increased amounts of PDGF-A mRNA were present at 1 wk after injury. No difference in PDGF-A mRNA levels was found between dissected portions of intimal or medial tissues from injured vessels at 2 wk when compared with uninjured carotids. All three size classes of PDGF-A mRNA coordinately increased and decreased in abundance.

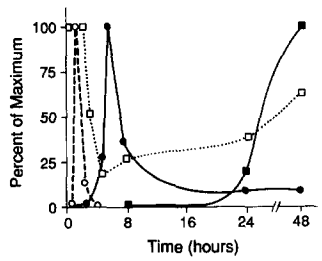

Figure 3. Rapid changes in gene expression during the first 48 h after carotid injury. Rats (n = 12 per time point) were killed at the indicated times after carotid injury. Autoradiographs of RNA transfer blots shown in Figs. 1 and 2 were scanned with a laser densitometer and relative signal intensities (expressed as a percent of the signals producing the greatest film density for each transcript shown) were plotted. Symbols used: (○) c-*fos*; (●) PDGF-A; (□) PDGF receptor β-subunit; (■) histone. Changes in gene expression similar to those plotted above were observed in two additional arterial injury experiments. Transcript levels for PDGF-B and PDGF α-subunit are not shown as they varied less than twofold during the above time period.

PDGF-B. A single 3.5-kb transcript for PDGF-B was found at low levels in uninjured carotids, similar to previously reported results for adult rat aorta (Majesky et al., 1988; Sorzani et al., 1989). This low level of PDGF-B gene expression was unchanged over the 2 wk studied after carotid injury (data not shown). PDGF-B mRNA levels were not significantly different between neointima and media of injured (left) carotids at 2 wk after wounding nor in uninjured (right) carotids at any of the times studied.

PDGF β-Receptor. Reports that PDGF-AA and PDGF-BB elicit contractions of freshly isolated rat aortic strips indicates that normal artery wall from adult rats contains at least some functional PDGF receptor α and β subunits (Berk et al., 1986; Block et al., 1989).

Consistent with these reports, readily detectable levels of a single 5.7-kb transcript for PDGF β-receptor were found in uninjured carotid arteries (Fig. 2). Rapid loss of PDGF β-receptor mRNA occurred in the first 4 h after injury (Figs. 2 and 3). PDGF β-receptor mRNA levels were reduced ~50% at 2 h, a change that preceded increases in PDGF-A mRNA in the same injured arteries. Further reductions in PDGF β-receptor mRNA levels were found at 4 h, resulting in ~80% loss of receptor transcripts compared with uninjured carotids. This acute loss of PDGF β-receptor mRNA in injured arteries was

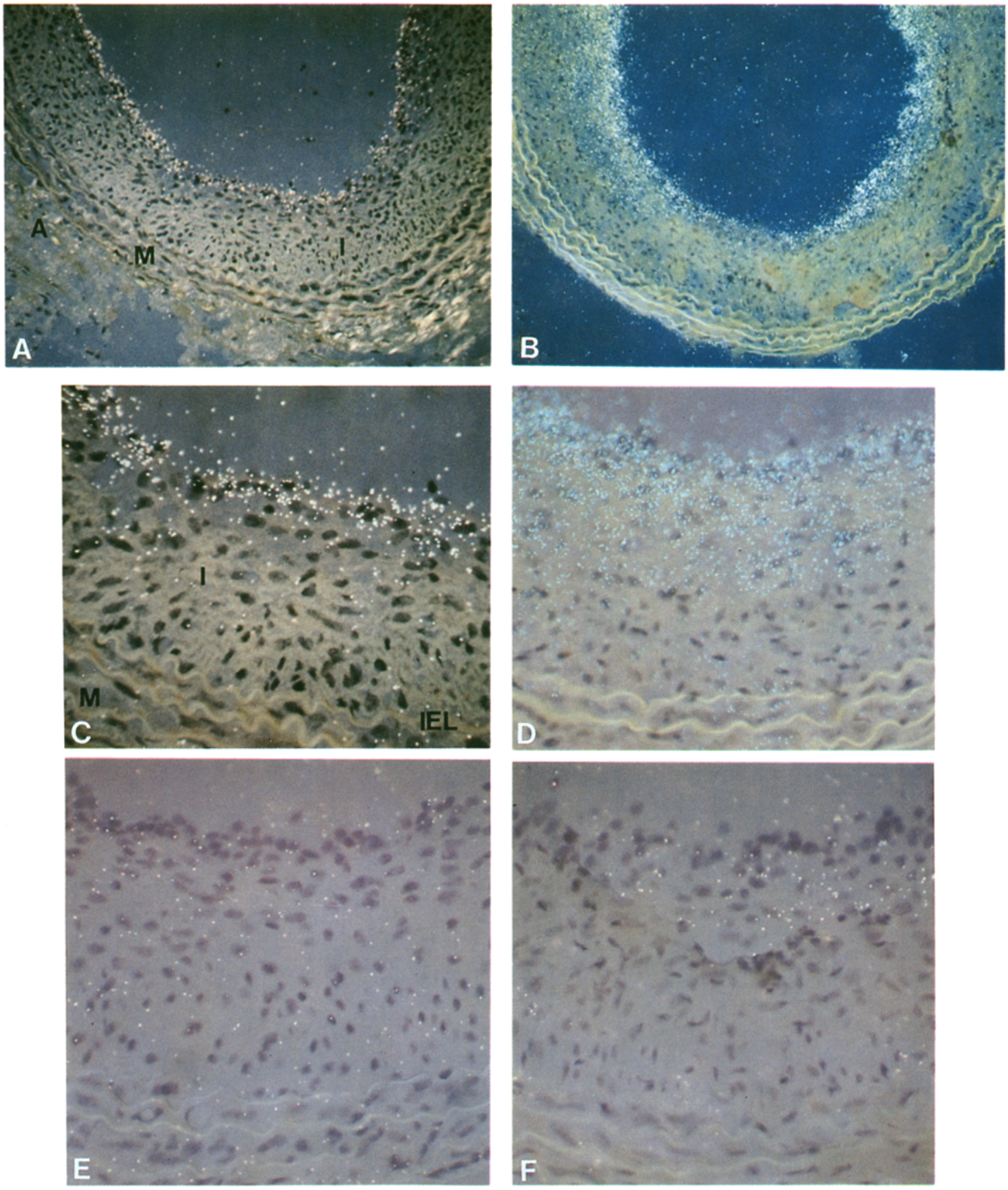

Figure 4. Localization of PDGF and PDGF receptor gene expression in carotid neointima 2 wk after injury. Serial sections were taken from the central portion of left common carotid artery 2 wk after injury. At this time, endothelial cell regeneration from the distal ends of the common carotid has not progressed into the central one-third of rat carotid artery (Reidy, 1985), therefore cells lining the luminal surface are neointimal smooth muscle cells. Sections were hybridized with the relevant ^{35}S-labeled cRNA probe as follows: (*A* and *C*) PDGF-A (×100 and ×310); (*B* and *D*) PDGF β-receptor (×100 and ×310); (*E*) PDGF-B (×310); (*F*) PDGF α-receptor (×310). Autoradiographic grains were not found accumulated at cut edges of the media or along adventitial tissue edges for any of the probes used. Photographs were made using a combination of polarized light epiluminescence and bright field illumination. The lumen of the artery is at top. (*I*) intima, (*M*) media, (*A*) adventitia, (*IEL*) internal elastic lamina.

observed in three separate experiments. Over the next week, gradual increases in PDGF β-receptor levels were observed so that about twofold greater amounts were present 1 wk after injury than in uninjured vessels. PDGF β-receptor mRNA levels were consistently three to fivefold higher in neointima than underlying media at 2 wk in three separate experiments.

PDGF α-Receptor. A single 6.5-kb transcript for PDGF α-receptor was detected in uninjured carotids (Fig. 2). No significant change in PDGF α-receptor mRNA levels was found in the first week after carotid injury. The variations in signal intensity for PDGF α-receptor transcripts between 24 h and 1 wk shown in Fig. 2 were not consistently found in a repeat experiment, and may reflect variations between animals. However, PDGF α-receptor mRNA was reproducibly found to be two to threefold more abundant in media than in neointima at 2 wk. No significant changes in either PDGF α or β-receptor transcript levels were found in uninjured right carotids during these 2 wk after left carotid injury.

In Situ Hybridization Analysis of Carotid Neointimal Thickening

Previous studies showed that rates of SMC proliferation are not uniform throughout the thickness of neointimal tissue during its formation and growth after carotid injury (Clowes et al., 1983; Clowes and Schwartz, 1985). Rather, a highly localized region of SMC proliferation is found at or near the luminal surface. To more closely examine the relation of PDGF ligand and receptor gene expression to ongoing SMC proliferation during carotid intimal thickening, we used in situ hybridization to resolve transcript distribution at the cellular level.

PDGF-A. Consistent with RNA blot hybridization results (Fig. 2), in situ hybridization analysis showed that a majority of cells in the left carotid media and neointima at 2 weeks after injury were negative for PDGF-A gene expression. However, a subset of neointimal SMC located in close proximity to the luminal surface was strongly positive for PDGF-A hybridization (Fig. 4 *A*). This finding was consistently observed in neointimal sections from four separate experiments. Since endothelial cell regeneration from the distal ends of the common carotid artery has not progressed into the central portion of the artery at this time (Reidy, 1985; Clowes et al., 1986), cells lining the luminal surface and strongly positive for PDGF-A mRNA are neointimal SMC not endothelial cells (Reidy, 1985). SMC deeper into the center of the neointima as well as the restored media appeared quite similar to the uninjured carotid with few, if any, cells positive for PDGF-A hybridization.

PDGF-B. RNA transfer blot analysis showed low levels of PDGF-B transcripts in carotid neointima 2 wk after injury (data not shown). Likewise, in situ hybridization detected no cells that were clearly positive for PDGF-B transcripts in carotid neointima (Fig. 4 *E*).

PDGF β-Receptor. Transcripts for PDGF β-receptor were abundant in neointimal SMC that line the luminal surface at 2 wk after injury (Fig. 4 *B*). Cells expressing the PDGF β-receptor gene were localized around the luminal surface so that at low magnification the pattern of silver grains appeared as a ring that traced the lumen of the artery. PDGF β-receptor transcripts were also present in SMC deeper within the neointimal thickening but at reduced levels. This pattern of PDGF β-receptor transcript localization was a consistent finding in four separate experiments. Thus a remarkably similar distribution of PDGF-A and PDGF β-receptor gene expression is evident in rat carotid neointima two weeks after injury. Given the apparent uniformity of silver grain distribution in the luminal SMC population for either of these two transcript species, it is highly likely that the same cells express both PDGF-A and PDGF β-receptor genes at the same time during neointimal thickening.

PDGF α-Receptor. Transcript abundance for PDGF α-receptor in carotid neointima appeared greatly reduced compared with that of PDGF β-receptor both by RNA blot analysis and in situ hybridization (Fig. 4 *F*). Cells positive for PDGF α-receptor mRNA were only detected occasionally and were scattered throughout the neointima. Among four different experiments, PDGF α-receptor-positive SMC were more variable in number (generally very few) and location within the neointima than were PDGF-A and PDGF β-receptor-positive cells. A striking luminal localization was not seen in any of the sections examined.

Immunohistochemistry

The distribution of PDGF-A-containing SMC within carotid neointima was examined using an antibody that recognizes PDGF-AA and PDGF-AB, but not PDGF-BB (Hart et al., 1990). Positive intracellular immunostaining was consistently found in neointimal SMC, particularly among those located at or near the luminal surface (Fig. 5). This staining pattern is quite similar to the distribution of PDGF-A mRNA in neointimal SMC as detected by in situ hybridization (above). Perinuclear staining (possibly Golgi complex) was frequently observed. Normal uninjured carotid SMC exhibited faint, perinuclear staining with no luminal concentration. Substitution of anti-PDGF-AA with normal rabbit IgG (Fig. 5), phosphate buffered saline or an irrelevant antibody (anti-transforming growth factor-α) (not shown) produced no staining of carotid neointimal sections.

Discussion

We and others have suggested that SMC proliferation at sites of arterial injury might depend on growth factors produced locally by vessel wall cells themselves (Reidy, 1985; Schwartz et al., 1985; Ross, 1986; Walker et. al., 1986; Libby et al., 1989; Majack et al., 1990). This model requires that SMC also express functional growth factor receptors during tissue repair. We explored these possibilities in this study by examining expression of PDGF-A and B (ligand) and PDGF receptor (α and β subunits) genes during repair of extensive injury to rat carotid artery. For purposes of discussion the results are divided into four stages based on our earlier studies of the kinetics of SMC proliferation in this model (Clowes et al., 1983; Clowes and Schwartz, 1985; Schwartz et al., 1985).

Stage 1: PDGF Gene Expression in Normal Artery

Quiescent, uninjured carotid artery contained transcripts for PDGF-A, PDGF-B, and both PDGF receptor subunits indicating that normal vessel wall has the potential to synthesize and respond to PDGF isoforms. Since DNA synthesis is a rare event in adult rat carotid (Clowes et al., 1983), this

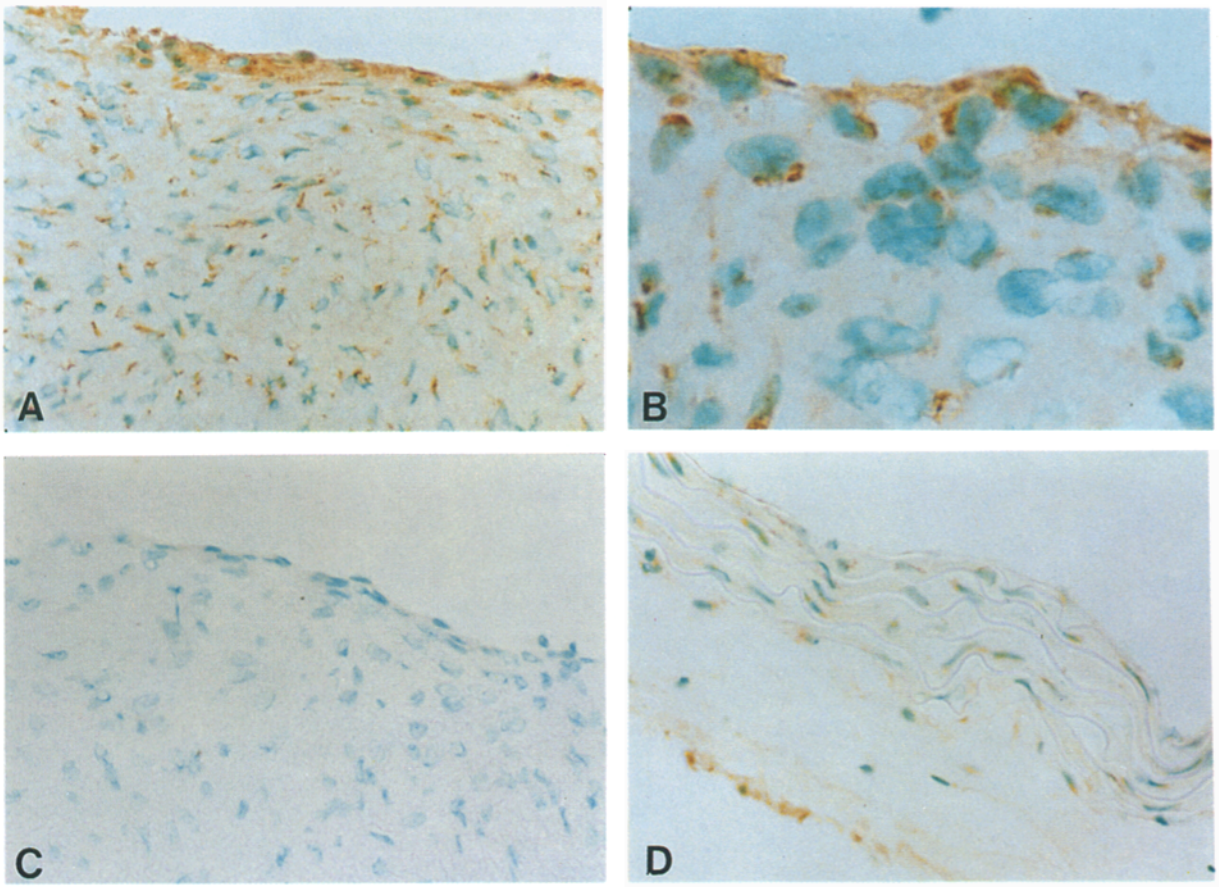

Figure 5. Immunocytochemical localization with anti-PDGF-AA antibody in rat carotid neointima. Frozen sections of rat carotid were stained with a polyclonal rabbit anti-PDGF-AA IgG (1:100) (Hart et al., 1990). (*A*) Carotid neointima 2 weeks after injury. Note the strong positive staining along the luminal surface and more scattered staining in the rest of the neointima. (×100). (*B*) A luminal portion of carotid neointima 2 wk after injury (×400). (*C*) Carotid neointima 2 wk after injury stained with control rabbit IgG (1:100) (×100). (*D*) Normal, uninjured rat carotid stained with anti-PDGF-AA (1:100). Note scattered, low level of positive staining in the media and adventitia with a lack of luminal distribution (×100).

might suggest that PDGFs produced locally have some nonmitogenic role in artery wall function (Berk et al., 1986; Barrett et al., 1988; Majesky et al., 1990). The finding of PDGF α and β-receptor transcripts in normal artery is consistent with reports that each of the PDGF isoforms (AA, AB, BB) can evoke contractions of freshly isolated strips of rat aorta (Berk et al., 1986; Block et al., 1989). Indeed, control of smooth muscle contractile tone may be one function for locally produced PDGFs in normal artery wall (Berk et al., 1986; Block et al., 1989; Majesky et al., 1990).

Stage 2: PDGF Gene Expression in the First 48 h

Balloon catheter injury stimulates carotid SMC to rapidly exit the quiescent (G_0) state and synchronously progress through the prereplicative (G_1) and S phases during the first 48 h after wounding (Majesky et al., 1987). We show here that a large and transient increase in PDGF-A mRNA precedes SMC S phase entry. The kinetics of stimulated PDGF-A gene expression after carotid injury are similar to transient increases in PDGF-A mRNA in cultured fibroblasts exposed to PDGF or epidermal growth factor (Paulsson et al., 1987). The presence of PDGF α-receptor transcripts in injured carotids suggests that SMC could respond to PDGF-A-containing isoforms that they produce. SMC-derived PDGFs might function as part of an intercellular cytokine signaling network (Raines et al., 1989) coordinating changes in gene expression and anabolic metabolism needed for cellular growth prior to DNA synthesis and cell division (Cochran et al., 1981; Pegg, 1986).

Acutely injured carotids also contain PDGF β-receptor transcripts. Because PDGF released from rat platelets is mostly PDGF-BB (Bowen-Pope et al., 1989), SMC appear able to respond to rapid appearance of PDGF at sites of vascular injury resulting from platelet degranulation. The rapid decrease in PDGF β-receptor mRNA levels seen in the first 4 h after injury implies that signals generated by the injury event itself are important regulators of PDGF β-receptor gene expression in vivo (Gronwald et al., 1989).

Stage 3: PDGF Gene Expression at 2 and 7 d after Injury

Days 2 and 7 after carotid injury are characterized by continued SMC proliferation, directed migration, and appearance of a fibrocellular neointima (Clowes et al., 1983).

Whereas cell replication is very active at these times (Clowes et al., 1983), the low levels of PDGF-A and B gene expression detected by RNA blot analysis do not provide obvious clues as to a local stimulus for this proliferation. Increases in PDGF β-receptor mRNA detected between 2 and 7 d after wounding may be due to selectively increased amounts of this transcript in neointimal SMC. Rats made deficient in circulating platelets by an antiplatelet antibody had a delayed appearance of SMC in the intima of wounded carotids (Fingerle et al., 1989). Since neointimal SMC contain abundant PDGF β-receptor mRNA, this delay might suggest that PDGF-BB released from platelets plays an important role in neointima formation. Stimulation of SMC chemotaxis and extracellular matrix production are two known properties of PDGF in vitro (Ross et al., 1986) that seem particularly relevant in this regard.

Stage 4: PDGF Gene Expression in Intima and Media at 14 d

Between 7 and 14 d after injury two cellular compartments become established in regenerating carotid arteries (Clowes et al., 1983). Neointimal thickening proceeds by SMC proliferation and extracellular matrix accumulation. In contrast, SMC in underlying media are restored to original numbers and cell replication returns to basal rates. When strippings of neointimal versus underlying medial tissues were compared, RNA blot analysis showed that PDGF-A, PDGF-B, and PDGF α-receptor transcript levels were very low in both layers, whereas PDGF β-receptor mRNA was three- to fivefold higher in abundance in neointima than in media. The later results are similar to findings of Rubin et al. (1988) that greater amounts of PDGF β-receptor protein are found in SMC of human carotid atherosclerotic intima than normal artery.

In situ hybridization analysis identified a subpopulation of neointimal SMC localized along the luminal surface with a different pattern of gene expression than underlying SMC. Luminal SMC were strongly positive for PDGF-A and PDGF β-receptor transcripts. SMC positive for PDGF-B mRNA were not seen and PDGF α-receptor mRNA was found only occasionally in cells scattered throughout the neointima. Ongoing cell proliferation at 14 d after injury was previously found to be limited to a luminal subset of neointimal SMC (Clowes et al., 1983). However, luminal SMC express a PDGF ligand and receptor pair (PDGF-A and PDGF β-receptor) that interact poorly, if at all, with each other (Hart et al., 1988; Heldin et al., 1988; Seifert et al., 1989). Since PDGF-B mRNA was barely detectable in carotid neointima and was not found accumulated within luminal SMC, exogenous sources of PDGF-BB such as platelets (Bowen-Pope et al., 1989) or monocytes (Shimokado et al., 1985) would seem probable candidates for stimulating luminal SMC proliferation. However, platelets and monocytes are found infrequently in rat carotid neointima (Reidy, 1985; Jonasson et al., 1988). Thus, the local stimulus for neointimal SMC replication remains unclear, although the possibility that local PDGF-A concentrations might be high enough to activate at least some PDGF β-receptors cannot be ruled out.

It is also possible that coordinate expression of PDGF-A and PDGF β-receptor suggests a role for PDGFs in communication between different cell types during carotid repair. This scheme would allow one cell type to produce a form of PDGF that signals a neighboring cell type bearing the appropriate PDGF receptor without the consequence of autocrine stimulation. For example, endothelial cells might secrete PDGF-BB (DiCorleto et al., 1983; Collins et al., 1985; Davies et al., 1988; Zerwes et al., 1987) during regeneration as a signal to neointimal SMC bearing PDGF β-receptors that an endothelial lining has been restored. Large vessel endothelial cells themselves do not contain PDGF β-receptors (Ross et al., 1986). A similar role for PDGF-AA has recently been suggested in cell-to-cell communication between type-1 astrocytes and glial progenitor cells in the developing rat optic nerve (Raff, 1989).

Specialization of "Pseudoendothelial" SMC

Neointimal SMC at the luminal surface had a markedly different pattern of PDGF ligand and receptor gene expression than underlying carotid SMC. Luminal SMC are morphologically specialized to provide a nonthrombogenic pseudoendothelium in regions of incomplete endothelial regeneration (Schwartz et al., 1975). Together with our previous findings that cultured neointimal SMC have stable differences in morphology and gene expression compared to medial SMC (Walker et al., 1986), these data raise the intriguing possibility that structural specialization of luminal SMC described earlier (Schwartz et al., 1975) extends to functional and growth control levels. This may have important implications for mechanisms of intimal SMC proliferation in vascular disease.

We thank Stella Chao, Colleen Irvin, and Nels Eric Olson for animal surgery; Adam Evans for assistance with RNA blot analyses; Thomas McDonald for immunohistochemical assays; Kathleen Smith and Judith Hasko for help with in situ hybridizations; Christer Betsholtz and Carl-Henrik Heldin (University Hospital and Ludwig Institute for Cancer Research, Uppsala, Sweden); Mark Mercola, Charles Stiles, Kyu-Ho Lee (Johns Hopkins University) and Lewis Williams (University of California, San Francisco) for cloned DNA probes; and Virginia Wejak for preparation of this manuscript. We acknowledge valuable discussions during the course of these studies with our colleagues Earl Benditt, Alexander Clowes, Thomas Barrett, Ronald Seifert, Elaine Raines, and Russell Ross.

This work was done during the tenure of Established Investigatorships of the American Heart Association (M. A. Reidy, D. Bowen-Pope) with funds from the Washington Affiliate. National Institutes of Health Grants HL-03174, HL-30203, GM-35501, HL-42270 and HL-07312 provided support.

Received for publication 17 July 1989 and in revised form 1 June 1990.

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