Platelet-derived Growth Factor mRNA Detection in Human Atherosclerotic Plaques by In Situ Hybridization

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Abstract

Platelet-derived growth factor (PDGF) mRNA, and mRNA for its receptor, have been localized to specific cell types within the human atherosclerotic plaque, using in situ hybridization. The predominant cell types found to express PDGF A and B chain mRNA are mesenchymal-appearing intimal cells and endothelial cells, respectively, with little or no expression detected in macrophages. The distribution of PDGF receptor mRNA containing cells was also examined and found to be localized predominantly in the plaque intima.

Introduction

Platelet-derived growth factor (PDGF)[1] is a dimer composed of two homologous polypeptide chains, A and B (1, 2). The genes encoding these polypeptide chains have been mapped to different chromosomes (3, 4) and their expression is independently regulated (3). The gene encoding PDGF B chain mRNA is c-sis which is the cellular counterpart to the v-sis gene of simian sarcoma virus (5–7). PDGF has been hypothesized to be responsible for intimal proliferation in atherosclerosis. This hypothesis originated with the observations that platelets contain a mitogen for smooth muscle and that platelets accumulate and release their granules at sites of endothelial denudation (8, 9). The source of PDGF was therefore presumed to be external to the vessel wall, in the form of blood borne platelets. A requirement for platelets, however, could not explain certain experimental observations. With hypercholesterolemia, smooth muscle proliferation can precede the loss of endothelium (10). In hypertension, smooth muscle replication occurs without recognizable endothelial denudation (11–14). Finally, in the balloon-injured rat carotid artery, smooth muscle proliferation continues long after most of the platelet interaction with the vessel wall has ceased (15–17).

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1. *Abbreviations used in this paper:* PDGF, platelet-derived growth factor; TGF-α, transforming growth factor-alpha.

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Subsequent studies in PDGF biology revealed that many cell types, including macrophages (18–21), endothelial cells (22–24) and even arterial smooth muscle cells (25–27) can produce PDGF, at least in vitro. Since these same cell types are present in human atherosclerotic plaques (28, 29), the PDGF hypothesis of atherogenesis has been modified to include the possible production of PDGF from within the developing human intima (30). Indeed, local synthesis of PDGF in the atheroma is supported by recent studies showing that PDGF B chain mRNA can be detected by Northern blots of human carotid plaques removed at surgery (31).

In the present series of experiments we report the first successful detection of PDGF gene expression within the human atherosclerotic plaque by in situ hybridization.

Methods

In situ hybridizations. In situ hybridizations were carried out as described previously (32–35). Human carotid endarterectomy specimens were obtained from surgery and within 15 min of removal fixed in 4% paraformaldehyde 0.1 M sodium phosphate buffer for 3–18 h at 4°C followed by immersion in sterile 15% sucrose PBS overnight at 4°C. The tissue was then frozen in O.C.T. (Miles Scientific, Elkhart, IN) and 7 μm frozen sections thaw-mounted onto polylysine-coated slides and stored at −70°C with desiccant. Before hybridization the sections were pretreated with paraformaldehyde (10 min) and proteinase K (1 μg/ml) (10 min), prehybridized for 1–2 h in 50 μl of prehybridization buffer (0.3 M NaCl, 20 mM Tris pH8.0, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM DTT). The hybridizations were started by adding 300,000 cpm of the ^{35}S-riboprobe in a small amount of prehybridization buffer. After hybridization the sections were washed with 2× SSC (2 × 10 min) (1× SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), treated with RNase (20 μg/ml, 30 min room temperature), washed in 2× SSC (2 × 10 min), followed by a high stringency wash in 0.1× SSC at 52°C for 2 h. All SSC solutions up to this point of the procedure contained 10 mM β-mercaptoethanol and 1 mM EDTA to help prevent nonspecific binding of the probe. The tissue was then washed in 0.5× SSC without β-mercaptoethanol (2 × 10 min) and dehydrated by immersion in graded alcohols containing 0.3 M NH_4Ac. The sections were dried and coated with NTB2 nuclear emulsion (Kodak Co.) and exposed in the dark at 4°C for 4 to 8 wk. After developing the sections were counterstained with hematoxylin and eosin.

Probes. Probes specific to PDGF A chain (3), PDGF B chain (5), PDGF receptor (36) or transforming growth factor alpha (TGF-α) (37) were labeled by transcription (38) using ^{35}S-labeled UTP (sp act 1,200 Ci/mmol; Amersham Corp., North Chicago, IL). The final specific activity of these probes was 300 Ci/mmol.

To control for the possibility that some tissues might show a variable hybridization response dependent on postoperative handling, each tissue was probed first with synthetic oligomers to human von

Willebrand's factor (vWf) mRNA (complementary to bases 3430-3481 [reference 39]) and to smooth muscle alpha-actin mRNA (60 bases long complementary to the most 3′ end of the coding region [40]); both labeled by terminal transferase (41) using [^{35}S]CTP (New England Nuclear, Boston, MA; sp act 1139 Ci/mmol). These probes specifically hybridized to endothelial cells or medial smooth muscle cells, respectively. Only 1 of 8 carotid plaque samples failed to show hybridization to these probes and was discarded from our analysis.

Identification of cell types. In an attempt to identify cell types expressing PDGF in the plaque the in situ autoradiographed slides were counterstained with hematoxylin and eosin and examined by light microscopy at higher magnification. In some cases serial sections were processed for immunocytochemistry using markers specific for endothelial cells (anti-vWf; Vector Laboratories, Burlingame, CA; Ulex Europaeus lectin binding [42, 43]), smooth muscle cells (HHF-35; 44), T cells (anti-Leu4; Becton Dickinson Co., Oxnard, CA), or macrophages (HAM56; 28). Immunocytochemistry was performed according to the manufacturer's direction using the Vectastain ABC alkaline phosphatase system (Vector Laboratories). The final reaction product was stained with the alkaline phosphatase substrate kit I to give a final stain that appeared red.

One technical problem encountered was that the HAM56 monoclonal antibody recognizes capillary endothelium as well as monocyte/macrophage appearing cells (28). However, on comparing serial sections immunostained for HAM56 and either anti-vWf or Ulex Europaeus lectin, it became apparent that endothelial cells were arranged either in a linear monolayer on the plaque surface, or as two to five cells arranged as vascular profiles within the plaque. Individual cells or cell clusters located elsewhere in the plaque, did not react with either anti-vWf or Ulex Europaeus, but did react with the HAM56 antibody, and were therefore interpreted as monocyte/macrophages. In agreement with previous studies (45) almost all cells with foamy cytoplasm at the light microscope level (foam cells), reacted with the HAM56 antibody but not with markers for endothelial cells or smooth muscle cells.

Results

Expression of PDGF and PDGF receptor mRNA by plaque regions. Serial 10-μm sections of eight human carotid atherosclerotic plaques removed at surgery were hybridized to ^{35}S-labeled PDGF A- or B-chain-specific cRNA probes. The most immediately impressive result was the recognition at low magnification that the mRNAs for both chains of PDGF were distributed nonrandomly within the plaque, and involved a subset of cells. Based on cell counts performed on random sections of four carotid plaques 7.2% of all plaque cells counted (308/4306 cells) showed hybridization for B-chain and 15.2% (669/4405 cells) revealed PDGF A-chain hybridization. Similarly 6.5% of all cells (251/3888 cells) revealed PDGF receptor hybridization.

Cells containing mRNA encoding both PDGF A and B chains could be detected in similar locations in the plaque (Fig. 1, *A* and *B*). Cells containing the mRNA for the B-chain of PDGF were most prominent at the lumenal edges of vascular structures and in plaque regions rich in small, penetrating blood vessels (Fig. 1 *B*). Cells containing the mRNA for the A chain of PDGF were found over similar vascularized plaque regions (Fig. 1 *A*) but also over sparsely distributed mesenchymal-appearing intimal cells distributed in the fibrous portions of the plaque (Fig. 3). Areas with large numbers of foam cells, most of which appeared to be macrophages did not show PDGF production. No message was detected over areas of necrosis and only occasionally in cells in the underlying tunica media. PDGF receptor mRNA could be identified in cells found primarily in the plaque intima co-localized in plaque regions synthesizing A and B chain mRNA with a few positive cells detected in the tunica media.

We used multiple probes to confirm the specificity of the hybridization reaction and to control for the possibility that hybridizations at tissue edges could represent a form of edge artefact. Serial sections from every experiment were hybridized with ^{35}S-labeled cRNAs to: PDGF A, PDGF B, PDGF receptor, or to transforming growth factor-alpha (TGF-α). These probes were all labeled to the same specific activity and equivalent amounts of each radioactive probe were added to the hybridization reactions. Each probe gave distinct, different patterns of hybridization. Positive autoradiography was not seen at the cut edges of the media or plaque using the TGF-α or PDGF receptor probes. In no case was positive TGF-α hybridization seen on any vascular section examined, and so this probe served as a negative control for every hybridization. The TGF-α probe was however effective in labeling epidermal cells in normal human skin using the same methodology (46).

As already noted, we observed a strong correlation between those plaque regions making PDGF B-chain mRNA and the intraplaque capillaries. Examination of this tissue at higher magnification suggested that endothelial cells were a major site of PDGF B-chain synthesis since positive cells were found at the luminal surfaces (Fig. 2). Serial sections stained with anti-vWf or Ulex Europaeus lectin confirmed that endothelial cells were present at the luminal surfaces of these vascular profiles.

Serial sections hybridized with A-chain specific probes showed less intense labeling of endothelial cells. However, some vascular profiles appeared to express both A and B chains (Fig. 2). More often, A-chain hybridization was found in cells adjacent to the endothelium in the capillary wall (Fig. 2 *D*) or in mesenchymal-appearing intimal cells (Fig. 3). Only occasional endothelial-appearing cells displayed A-chain hybridization, whereas most of such cells displayed B-chain hybridization.

Not all plaque capillary profiles expressed PDGF. More plaque capillaries could be identified by reactivity to anti-vWf antibody or with the Ulex Europaeus lectin than were positive by in situ hybridization for either PDGF A- or B-chain mRNA. To control for the possibility that not all endothelial cells had mRNA available for hybridization, some serial sections were hybridized with the oligomer directed against vWf mRNA tailed with [^{35}S]CTP. A comparison of this in situ vWf labeling to that of PDGF B chain confirmed that there were more capillary endothelial-appearing cells containing vWf mRNA than PDGF B-chain mRNA supporting our hypothesis that there is a subset of endothelial cells making PDGF B chain mRNA.

PDGF B chain mRNA was also detected in endothelial cells of normal internal mammary artery (Fig. 2 *E*). In these same internal mammary artery specimens, little hybridization was seen with the PDGF A-chain probe (Fig. 2 *F*) or the PDGF receptor probe, and none was seen with the TGF-α probe (results not shown). The background was very low for all of these probes, confirming the specificity of our result to the B chain of PDGF. Few or no macrophages were detected in these tissues by immunocytochemistry on serial sections.

Macrophages, identified as either foam cells or hemosiderin containing cells, did not appear to be a major site of PDGF mRNA biosynthesis in the plaque. Few if any macro-

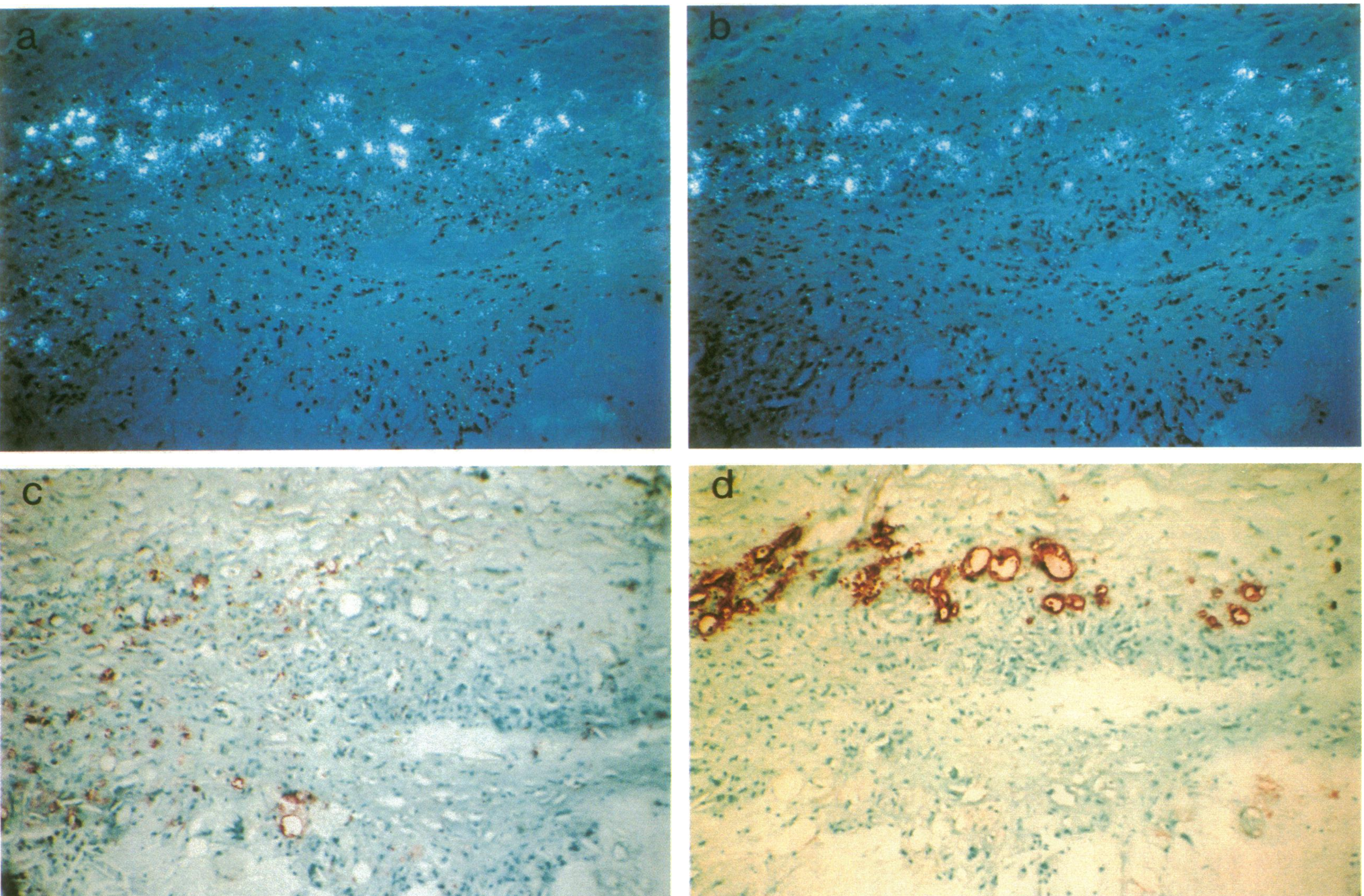


Figure 1. Overview of PDGF gene expression in the human atherosclerotic plaque. Human endarterectomy specimens were processed by in situ hybridization using probes specific for PDGF A (*A*) or B (*B*) chain mRNA. A comparison of serial sections stained for human macrophages (*C*) or endothelial cells (*D*) by immunocytochemistry suggested that PDGF mRNA was found primarily in regions rich in endothelial cells and capillaries (*A* and *B* vs. *D*) but not macrophages (*A* and *B* vs. *C*). Photographs were taken using polarized light epiluminescence or bright field illumination. ×125.

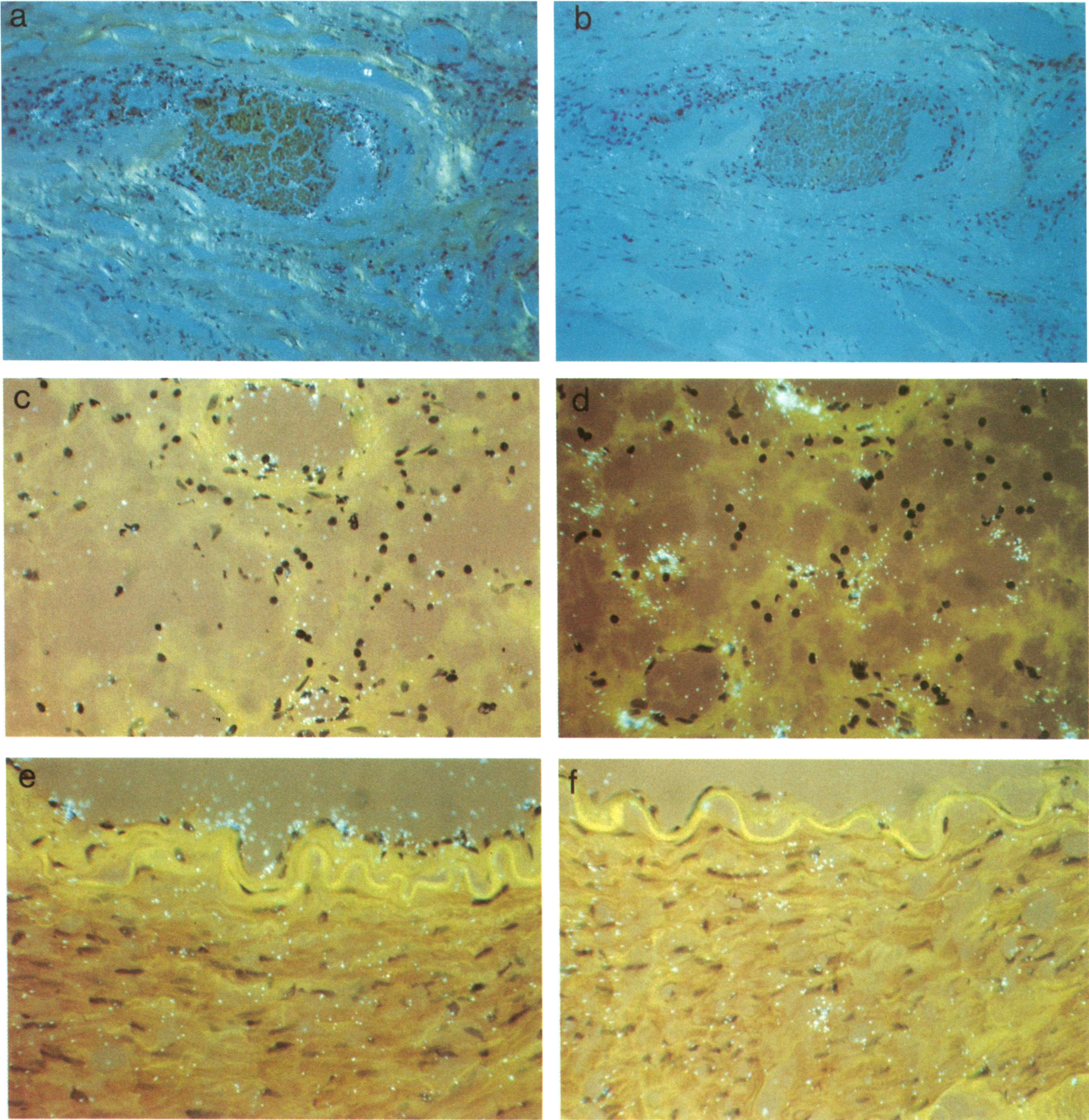


Figure 2. Localization of PDGF B chain mRNA in endothelial cells of capillaries within the human atherosclerotic plaque (*A* and *C*) or the normal internal mammary artery (*E*). Serial sections hybridized with PDGF A chain specific probes showed little labeling of endothelial cells with much more intense hybridization to cells adjacent to the endothelial layer (*B* and *D* arrows) or to mesenchymal-appearing intimal cells (*D*). Serial sections from the internal mammary artery specimen hybridized with PDGF A (*F*), PDGF receptor, or TGF-α probes were negative. Specimens were photographed using a combination of polarized light epiluminescence and bright field illumination. (*A* and *B*) ×125; (*C–F*) ×310.

phages were considered positive for either A- or B-chain expression (Table I; Fig. 3 *A*). Serial sections stained for macrophage antigens by the Ham56 antibody suggested a very poor correlation of PDGF expression with the localization of macrophages, in contrast to the correlation seen with the endothelial cell markers (Fig. 1). Preliminary studies combining in situ hybridization for PDGF A-chain mRNA localization and Ham56 immunocytochemistry on the same tissue section confirm our impression that the macrophage contributes little (if any) to local PDGF production in the atherosclerotic plaque (Fig. 4). Similarly, no positive hybridizations to either PDGF chain were seen among lymphocyte-appearing cells (Fig. 3 *A*, Table I).

Although by autoradiographic grain intensity, B-chain hybridization was most intense among the endothelial-appearing cells of plaque capillaries, the largest number cells positively hybridized for B- and A-chain were noncapillary, mesenchymal-appearing intimal cells (Table I). Some of these cells had

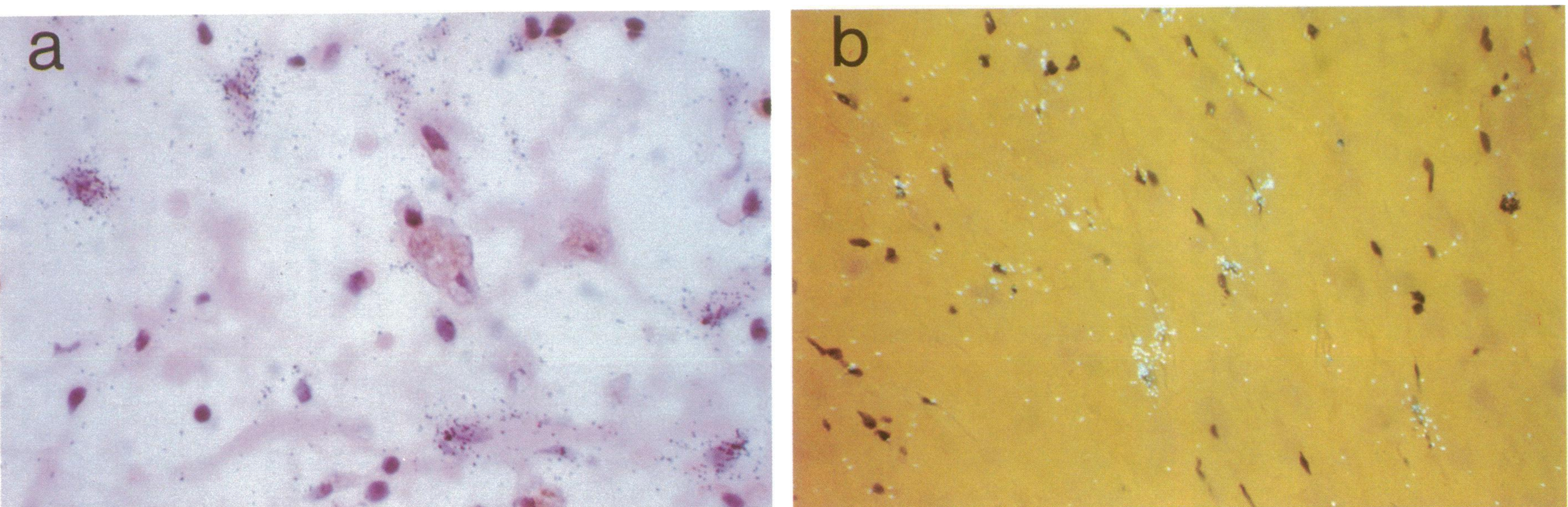


Figure 3. Cell types expressing PDGF A chain mRNA in the human atherosclerotic plaque. In a region in the plaque intima rich in inflammatory cells positive hybridization was found to a stellate shaped cell we have referred to as a mesenchymal-like intimal cell (*A*). PDGF A chain mRNA was also detected in cells with a spindle shaped nucleus and elongated cytoplasm consistent with smooth muscle cell morphology in the fibrous cap of the lesion (*B*). Serial sections of A stained by immunocytochemistry using either the HAM56 or anti-Leu4 antibodies indicated that the surrounding mononuclear cells were macrophages and T cells. A clear example of a macrophage identified by the presence of phagocytosed hemosidrin is shown in the center of the picture and does not contain PDGF A chain mRNA. The positive A chain expressing mesenchymal-like intimal cells did not stain using any of the antibodies tested (HHF35, HAM56, Ulex Lectin, VWF). (*A*) ×500, (*B*) ×310.

Table I. Percentage of All Positive Hybridizations for PDGF A-chain, B-chain, and PDGF Receptor mRNA Broken Down by Cell Type

	A-chain	B-chain	Receptor
Hemosiderin/Foam	6.3%	6.2%	4.4%
Capillary associated*	4.3%	15.9%	0.0%
Lymphocytes	0.1%	0.3%	0.4%
MICs‡	85.1%	75.0%	83.7%
Medial cells	3.9%	0.9%	11.2%

Cell counts were performed on four carotid endarterectomy specimens screened for PDGF A and B chain, and PDGF receptor gene expression by in situ cRNA:mRNA hybridization. Random fields were examined by light microscopy (at 400 magnification) to identify cell type. Hybridization results and cell counts were pooled for all four specimens for each probe. Results are expressed as the percentage of all positively hybridizing cells that fell into a particular cell type category based on hematoxylin and eosin stain appearance. One slide per probe for each specimen was used for counting, all with 6–7 wk autoradiography exposures. Cells with five or greater associated autoradiographic grains were considered positive. Similar qualitative results were evident on the remaining three carotid specimens studied.
* Capillary-associated cells include both endothelial cells and smooth muscle cells.
‡ MICs, mesenchymal-appearing intimal cells.

the light microscopic appearance of smooth muscle cells, i.e., spindle-shaped cells with long, slender nuclei (Fig. 3 *B*) (47). Many more cells had a stellate shape and displayed variable amounts of cytoplasm (Fig. 3 *A*). However, most of the stellate cells did not stain well with any of the cell type-specific antibodies used. Thus the identity of these cells remains somewhat unclear.

Localization of PDGF receptor to the arterial intima. All of the samples so far discussed were hybridized in parallel with PDGF receptor probe as well. Many cells in the intima of the plaque make mRNA for PDGF receptor (Fig. 5 *B*). Again, almost all such cells had the same mesenchymal morphology as described above for the majority of A- and B-chain positive cells. No lymphocyte-appearing cells, no endothelial-appearing cells, and almost no foam cells or hemosiderin-containing cells were positive for PDGF receptor. Similarly, by serial section immunocytochemistry analysis, PDGF receptor positive cells did not appear to react with monocyte/macrophage or lymphocyte antibodies, nor did they show Ulex Europaeus binding. PDGF receptor positive cells were distributed in regions previously seen to be A- or B-chain positive. In one sample of coronary artery taken from a diseased heart removed during transplant surgery we found a diffuse intimal thickening without atherosclerotic change, and with no evidence of macrophages or T cells. This sample was hybridized with the PDGF receptor probe and displayed prominent labeling of the intimal cells (Fig. 5 *A*). Few PDGF receptor positive cells were identified in the media of both this coronary artery specimen and carotid plaques.

Discussion

Our data using in situ hybridization corroborate existing data in demonstrating that PDGF mRNA is expressed in athero-

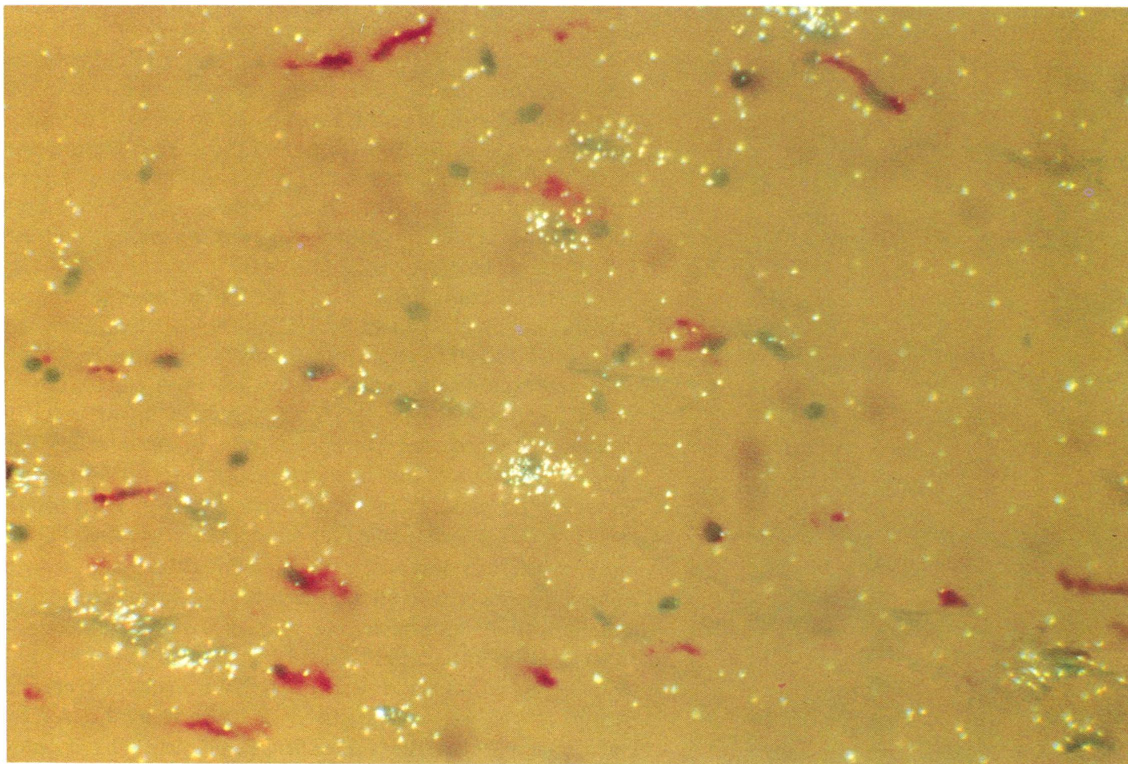
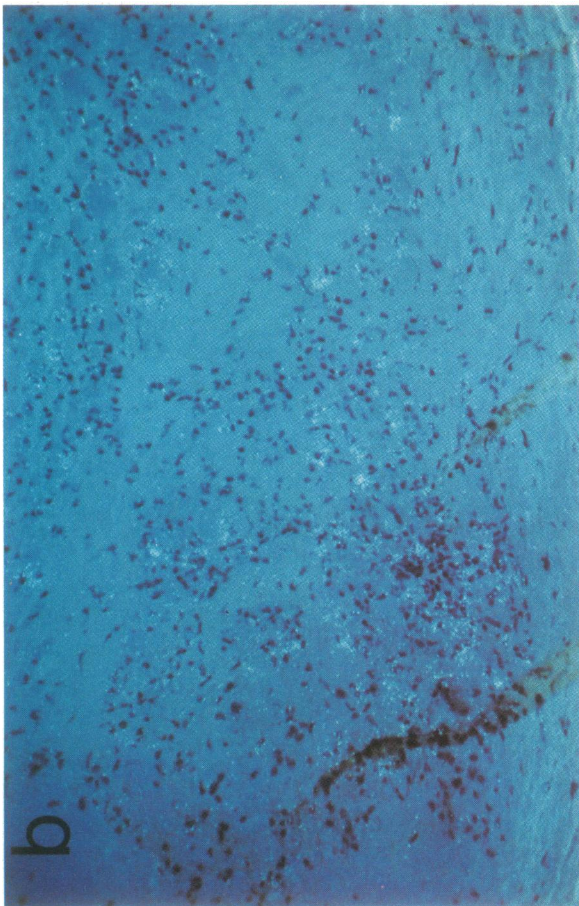


Figure 4. Demonstration that macrophages do not make PDGF A chain mRNA in the human atherosclerotic plaque by simultaneous in situ hybridization and immunocytochemistry. Frozen sections were hybridized as described using the ^{35}S-labeled PDGF A specific riboprobe. After the posthybridization washes the tissue was stained by immunocytochemistry using the HAM56 antibody as described followed by dehydration and coating with emulsion. In preliminary studies it was found that the prior processing of the tissue by the in situ procedure intensified subsequent immunostaining. ×310.

sclerotic plaques. Expression of PDGF receptor, and its selective expression by the intimal mesenchymal cell, and not medial smooth muscle cells, establishes the presence of a target for this agonist in the part of the vessel known to undergo selective proliferation in atherosclerosis. The absence of medial cells hybridizing with PDGF A- or B-chain or receptor probes suggests that either a fundamental change occurs in the smooth muscle cells hypothesized to arrive in the intima from the media (30), that some intimal mesenchymal cells may be in a different stage of differentiation than the cells of the media (48), or that intimal cells have in some way become transformed (49, 50). These are consistent with the finding that the vessel wall contains different types of nonendothelial mesenchymal cells distinguished by their content of desmin and smooth muscle-specific actin (51–54).

The idea that plaque growth is controlled by the production of growth factors by vessel wall cells themselves is intriguing for several reasons. Several forms of smooth muscle replication occur without platelet or leukocyte involvement, which could act as a source of growth factors exogenous to the vessel wall. This includes the response to hypertension (11–14) and normal development. Endothelial cells are capable of producing PDGF in vitro but this ability depends on whether the cells are proliferating as monolayer sheets or are stimulated to form tubes (22); only the former form expresses significant levels of PDGF. Perhaps controls exist in vivo, allowing the endothelium to respond to injury by producing PDGF, which then initiates smooth muscle replication. Smooth muscle cells taken from adult rat aorta do not produce PDGF except to a small extent in primary culture (27). Cells taken from the same vessel of newborn rats constitutively produce PDGF in culture (25), a phenomenon also reported by Walker et al. (26) for cells taken from the balloon injured carotid artery of adult rats. These data imply that the ability to produce PDGF may be under developmental control and may become remanifest when smooth muscle cells are stimulated to respond to injury. Alternatively, the local environment could regulate the production of PDGF by endothelial cells. Such factors as thrombin or transforming growth factor beta, or cAMP have been shown to affect PDGF synthesis in capillary endothelial cells (55) and may be produced at focal sites in the lesion.

Several caveats should be expressed. First, measurement of mRNA contents by in situ hybridization is relative. Thus the lack of hybridization seen among macrophage-appearing cells does not exclude the possibility of low level expression of PDGF by this cell type, but does suggest a higher level of expression among nonmacrophage cells in the plaque. The lack of PDGF production by macrophages was surprising since these cells do make PDGF under appropriate in vitro conditions (18, 21). It is possible that macrophages produce PDGF in atherosclerosis only when they first enter the lesion or with some appropriate activation signal. It is important to realize that the carotid atherosclerotic plaques we studied were advanced, complicated, and clinically significant lesions in individuals over 50 years old. Recent observations by Barrett and Benditt (56) suggested that PDGF B chain is synthesized by macrophages in human atherosclerotic plaques. This conclusion was based on their finding of a correlation between expression of PDGF B chain and *fms* by Northern blot analysis of portions of carotid endarterectomy specimens. These data are not necessarily in conflict with our results but may only reflect strong PDGF expression in endothelial and intimal cells



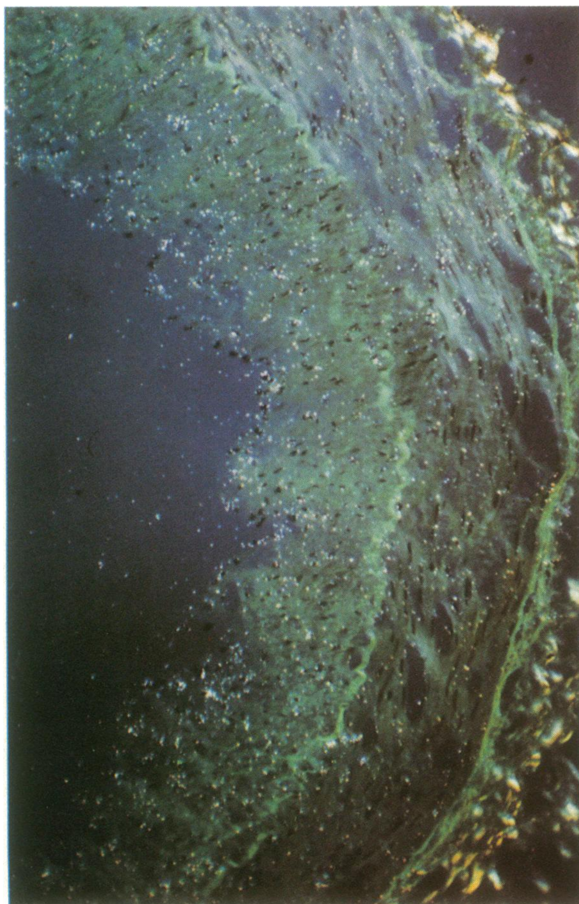


Figure 5. Localization of PDGF receptor mRNA containing cells in the human atherosclerotic plaque (*B*) and a coronary artery removed from a patient with idiopathic conjestive cardiomyopathy (*A*). ×125.

in regions of the plaque rich in macrophages. The coronary specimen with diffuse intimal thickening was interesting because it suggested that PDGF receptor expression may be a feature of early intimal thickening or early atherosclerosis. This finding must, however, be verified by studying earlier lesions in younger individuals more extensively.

We have no direct evidence that the PDGF produced in the plaque is stimulating replication. There are almost no data on proliferation occurring in human arterial tissue. The studies that have been performed using either in vitro thymidine labeling or flow cytometry have revealed labeling indices of $< 0.1\%$ (57–59). Aside from being a mitogen, PDGF has other properties, including inducing chemotaxis and contraction of smooth muscle cells (60–61). Therefore, if PDGF protein translation and secretion are occurring in the atherosclerotic plaque, proliferation need not be the result. The detection of PDGF mRNA and the identification of cells synthesizing it is a first step in elucidating the possible sequence of events leading to the development of the atherosclerotic plaque.

The nature of the mesenchymal-appearing intimal cell type is not clear. By light microscopy (Fig. 3) these cells were of the type sometimes called intimal smooth muscle cell (62), stellate cells (63), or synthetic state smooth muscle cells (64). Our failure to find a positive immunocytochemical marker for these cells is not surprising. In previous immunocytochemical and ultrastructural studies of the human atherosclerotic plaque a significant number of intimal cells have remained unidentified (28, 29, 62). The usual conclusion has been that these cells are altered smooth muscle cells, since this cell type is believed to be the sole connective tissue cell comprising the inner tunica media from which the intimal cells are hypothesized to arise (30). Whatever its cell lineage, this cell comprises a large portion of the human atherosclerotic intima and appears to be responsible for most of the local PDGF A chain production.

One of the most striking features of atherosclerosis has been the localization of proliferating smooth muscle cells to the intima. Martin (65) proposed that this depends on loss of contact dependent control of growth by sparsely scattered smooth muscle cells in the intima. Benditt proposed that atherosclerosis represents a monoclonal, neoplastic growth at sites of high mutagen accumulation due to lipid deposition (49, 50). Ross and others have proposed that localization of proliferating smooth muscle cells depends on accumulation of toxic or stimulatory products along a permeability gradient (30). None of these ideas suggest the special properties that make intimal smooth muscle cells proliferate. The focal nature of expression of A chain mRNA by the scattered mesenchymal-appearing intimal cells and the localization of receptor expression to the intima may be important clues to identifying the special properties of intimal smooth muscle cells that may contribute to the development of the atherosclerotic plaque.

Acknowledgments

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