7.9, 10 m*M* $MgCl_2$, 50 m*M* KCl, 7 m*M* β-mercaptoethanol, 20% (v/v) glycerol, 0.2 pmol of the 130-bp T7A1 promoter DNA fragment, and 1 pmol of RNA polymerase. The final volume of each reaction was 20 μl, and the final concentration of challenging nucleotides were 0.25 m*M* each of ATP, GTP, CTP, and UTP. Gel electrophoresis and autoradiography were according to (*14*).

24. The transcription reactions were performed in a 15-μl volume containing 0.1 pmol of the 130-bp T7A1 promoter DNA fragment and 0.5 pmol of RNA polymerase in 40 m*M* tris-HCl, *p*H 7.9, 10 m*M* $MgCl_2$, 7 m*M* β-mercaptoethanol, 100 m*M* KCl, 5% (v/v) glycerol, 0.1 m*M* each of [α-^{32}P]UTP (5 to 10 Ci/mmol), CTP, and GTP. The ATP concentration was 0.25 m*M* in lanes 1 and 2 and 0.1 m*M* in lanes 3 and 4. The concentration of CpA in lanes 3 and 4 was 0.25 m*M*. Incubation was for 15 min at 37°C. The reactions were stopped and the samples were directly applied to a polyacrylamide–8*M* urea gel (20% acrylamide and 3% *N*,*N*′-methylene-bisacrylamide), which were run and autoradiographed as described [S. Malik, M. Dimitrov, A. Goldfarb, *J. Mol. Biol.* **185**, 83 (1985)]. The oligonucleotide products were identified by using different nucleoside triphosphates as the source of α-^{32}P label.

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Localization of PDGF-B Protein in Macrophages in All Phases of Atherogenesis

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Lesions of atherosclerosis occur in the innermost layer of the artery wall and consist primarily of proliferated smooth muscle cells surrounded by large amounts of connective tissue, numerous lipid-laden macrophages, and varying numbers of lymphocytes. Growth-regulatory molecules may be involved in intimal accumulation and proliferation of smooth muscle cells responsible for the occlusive lesions of atherosclerosis. Platelet-derived growth factor (PDGF) B-chain protein was found within macrophages in all stages of lesion development in both human and nonhuman primate atherosclerosis. Thus macrophages may play a critical role in the disease by providing PDGF, a potent chemotactic and growth-stimulatory molecule, to the intimal smooth muscle cells.

Atherosclerosis is a disease of large- and medium-sized arteries and is characterized by focal thickening of the inner portion of the artery wall in association with fatty deposits (*1*). This results in the clinical sequelae of myocardial infarction, cerebral infarction, gangrene of the extremities, and loss of function. Fatty deposits, although common in Western society, may be missing in populations where other risk factors such as hypertension, cigarette smoking, and diabetes are associated with increased incidence of the disease (*2–6*). The common underlying events responsible for the formation of lesions are the intimal proliferation of smooth muscle cells, formation of new connective tissue matrix by these cells, and the possible accumulation of lipid. Smooth muscle cells appear in the lesions of experimentally induced atherosclerosis concomitant with, or following the appearance of, monocyte-derived macrophages and T cells (*7–10*). The smooth muscle cells may be derived from existing intimal cells or from cells that migrate in from the underlying media. As the smooth muscle cells proliferate within the intima, the lesion increases in size and forms a fibrous cap that overlies a deeper region of macrophages, T cells, and debris, culminating in a large connective tissue–rich lesion that contains varying amounts of lipid. Platelet-derived growth factor (PDGF) (a homodimer of PDGF-A or PDGF-B chains or an AB heterodimer) may be one of the principal growth-regulatory molecules responsible for the migration of medial smooth muscle cells into the intima and for the proliferation of existing collections of intimal smooth muscle cells. The source of PDGF is not well defined; PDGF may be secreted by platelets, monocyte-derived macrophages, the overlying endothelial cells, or the smooth muscle cells themselves (*6, 11, 12*).

By Northern (RNA) analysis, we examined the expression of growth-regulatory molecules and their receptor genes in advanced lesions of atherosclerosis induced in nonhuman primates that had been made hypercholesterolemic by ingesting a diet rich in cholesterol for 1 year. In comparison with the dissected normal aortas from control animals, transcripts for several growth factors and their receptors were increased in the advanced lesions, whereas others remained unchanged (Fig. 1): PDGF-B, c-*fms* (the colony-stimulating factor type 1 receptor), the β subunit of the PDGF receptor, interleukin-1 (IL-1), and transforming growth factor β1 (TGF-β1) were increased, whereas PDGF-A and β-actin were not. These observations are consistent with the Northern analysis of human carotid endarterectomy specimens in which increased lev-

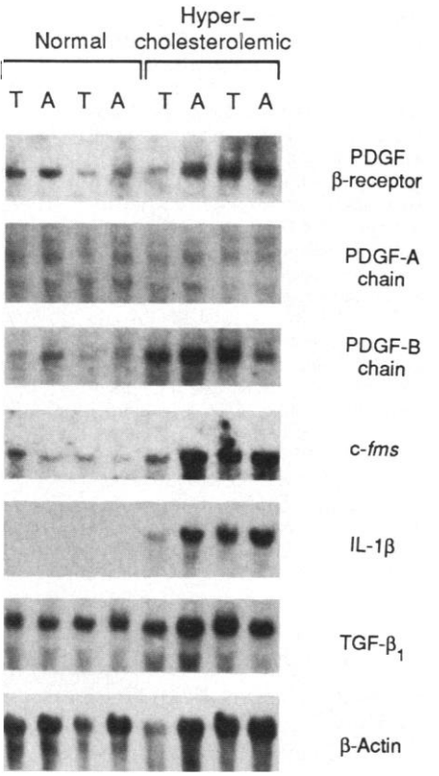

Fig. 1. Comparison by Northern (RNA) analysis of arterial segments of nonhuman primates maintained on a hypercholesterolemic diet (600 to 800 mg/dl) for 1 year with control normocholesterolemic animals. Two pigtail monkeys (*Macaca nemestrina*) between 3 and 5 years of age were maintained on a hypercholesterolemic diet as described (*8*) and killed after 1 year on the diet. An additional two monkeys received normal monkey diet and served as controls. Segments (1 cm) of thoracic (T) or abdominal (A) aorta were removed from each animal and dissected as described for preparation of tissues for immunocytochemistry (*10*) and snap frozen for isolation and analysis of total RNA (*27*). The same blot was sequentially rehybridized with the indicated cDNA probes or cRNA probe. β-actin hybridization was used as a reference for relative mRNA load per lane. Message sizes were 5.7 kb for the PDGF receptor (β subunit); 2.8, 2.3, and 1.9 kb for the PDGF-A chain; 3.7 kb for the PDGF-B chain; 4.3 kb for c-*fms*; 1.8 kb for IL-1β; 2.5 kb for TGF-β1; and 2.1 kb for β-actin.

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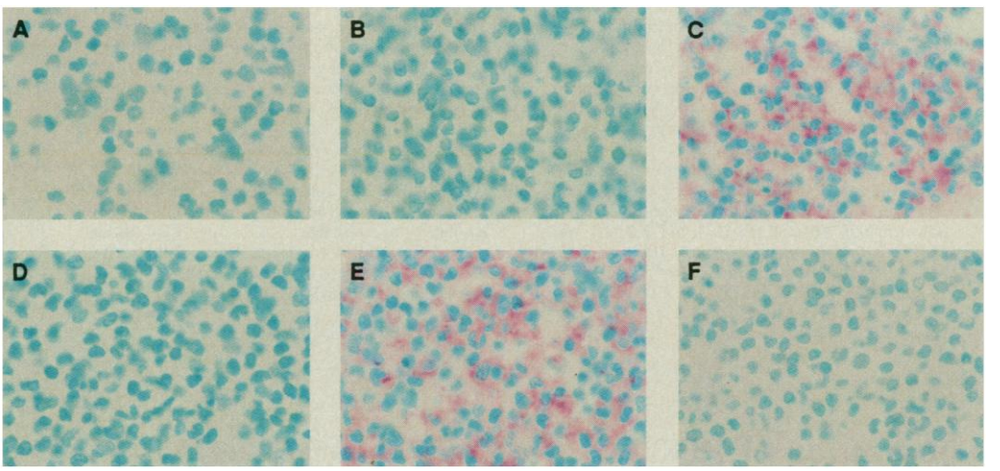

Fig. 2. Specificity of anti–PDGF-B immunostaining of activated human monocytes. Human monocytes were isolated from human leukocyte fractions obtained from the blood bank. The cells were either immediately pelleted and fixed in methyl Carnoy's fixative (**A**), or cultured in the presence of 10% plasma-derived serum and phytohemagglutinin (10 μg/ml) for 24 hours and then pelleted and fixed (**B** to **F**). Deparaffinized sections of the pellets were stained with MAb PGF-007 (1.25 μg/ml, 8.3×10^{-9} M) (A and C); an equivalent concentration of an irrelevant monoclonal (also IgG1a) (B); PGF-007 (1.25 μg/ml) preincubated for 1 hour at 37°C with immunizing peptide at 1.7×10^{-7} M (D); PGF-007 (1.25 μg/ml) incubated for 1 hour at 37°C with an analogous peptide from the PDGF-A chain, residues 67 to 92 (E); or PGF-007 (1.25 μg/ml) preincubated for 1 hour at 37°C with 6.6×10^{-8} M platelet PDGF (F). Incubations with the primary antibodies were done at 4°C overnight, after which incubations were done at room temperature with biotinylated second antibody and avidin-biotin immunoalkaline phosphatase. All sections were counterstained with methyl green. Original magnification, ×1000.

els of PDGF-B mRNA were correlated with increased expression of c-*fms* (macrophage-specific) and to a lesser extent (~10%) with von Willebrand factor (endothelial cell–specific) (*13*). Similarly, in situ hybridization for PDGF mRNA in a series of fibrotic lesions of human atherosclerosis suggested that smooth muscle is the predominant source of PDGF-A, whereas PDGF-B hybridized with "mesenchymal-appearing" intimal cells and with what was interpreted to be endothelium of vasa vasorum (*14*). Regions rich in foam cells were negative. The authors concluded from their studies that macrophages were not a major source of transcripts for either chain of PDGF. The lesions they studied appeared to be very advanced and were relatively acellular, with large regions of necrosis.

To determine the source of PDGF-B protein in different stages of atherosclerosis, we have used a monoclonal antibody (MAb), PGF-007, that was generated to a 25–amino acid peptide located near the COOH-terminus of the B chain of PDGF (residues 73 to 97 of the mature B chain) (*15*). On immunoblots, PGF-007 recognizes PDGF-AB and PDGF-BB, but not PDGF-AA, in contrast to a goat polyclonal antibody to PDGF, developed in our laboratory to a mixture of PDGF molecules, that recognizes all three dimeric forms of this molecule (*16*). PGF-007 does not cross-react with human epidermal growth factor, fibroblast growth factor, or platelet factor–4 (*15*). The specificity of PGF-007 is demonstrated in Fig. 2. Freshly isolated monocytes express and secrete low or undetectable levels of PDGF, whereas activated monocytes inducibly express and secrete PDGF (*17*). We prepared pellets of freshly isolated human monocytes and monocytes activated for 24 hours in culture, and we analyzed them with PGF-007. Although PGF-007 showed only occasional cell staining in preparations of freshly isolated monocytes (Fig. 2A), it showed intense staining of monocytes activated in culture for 24 hours (Fig. 2C). The immunostaining of PGF-007 was completely blocked by a 20-fold molar excess of the peptide used for immunization (Fig. 2D) or an 8-fold molar excess of platelet PDGF (Fig. 2F), but was unaffected by a 20-fold molar excess of a PDGF-A peptide (residues 67 to 92) analogous to the peptide used for immunization (Fig. 2E). An irrelevant monoclonal antibody of the same subtype showed no staining of freshly isolated monocytes (*16*) or activated monocytes (Fig. 2B). The amounts of peptide and PDGF required for competition in immunostaining were the same as those required to compete for PGF-007 binding to a purified PDGF-coated well in an enzyme-linked immunosorbent assay (ELISA).

We studied atherosclerotic lesions from human arteries obtained at surgery (Fig. 3) and from nonhuman primates fed a high-fat, high-cholesterol diet (Fig. 4). The PGF-007 staining was specifically associated with cells located in the core of the lesion. To identify the cell type in which the PDGF-B chain was localized, these lesions were also immunostained with monocyte lineage–specific or smooth muscle–specific MAbs, either in parallel sections or in the same section, by the use of a double immunostaining technique that combines immunoalkaline phosphatase and sequential silver-enhanced immunogold (IGSS) (*18*). A human fibrous plaque, removed by carotid endarterectomy, contained numerous smooth muscle cells surrounded by connective tissue matrix with intermingled macrophages and T lymphocytes. Double immunostaining of sections at the base of this lesion, and examination of adjacent sections stained individually, showed that many of the macrophages in each of these lesions contained the PDGF-B chain (in the form of PDGF-BB or PDGF-AB) (Fig. 3). The majority of the positive macrophages appeared to have either small amounts or no visible lipid within their cytoplasm. The majority of the apparently lipid-rich foam cells were negative for PDGF. In all of the cases examined, the smooth muscle cells in these lesions did not react with the antibody to the B chain (anti-PDGF-B).

The fatty streaks of the nonhuman primate lesions contained some positive macrophages that stained somewhat less intensely with PGF-007 than those observed in the fibrofatty lesions or the more advanced fibrous plaques (Fig. 4) (*19*). Closer examination at higher magnification of these macrophages (Fig. 4B) suggested that PDGF is located in a perinuclear or Golgi-associated region. In each of these cases, the smooth muscle cells were negative for PDGF-B, whereas 20 to 30% of the identifiable macrophages were positive for PDGF-B (Fig. 4). Our data identify the PDGF-B protein within many of the macrophages of the atherosclerotic lesions, which suggests that the increased expression of B-chain transcript observed in the nonhuman primate lesions (Fig. 1) and in human carotid endarterectomy specimens (*13*) originated from the macrophages that had not yet become foam cells.

The presence of PDGF-B in the non–foam cell macrophages, and its absence in

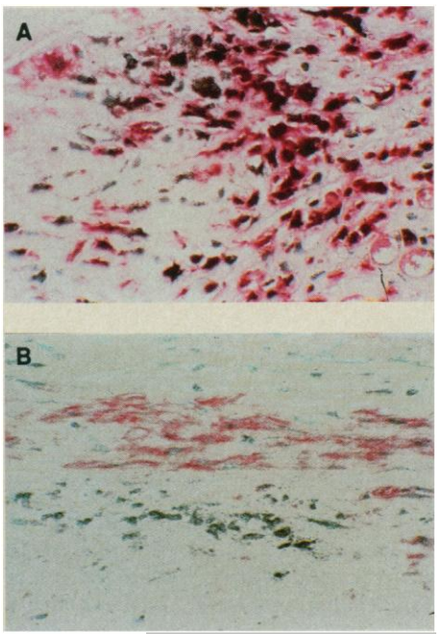

Fig. 3. Double-immunostained preparations with PGF-007 and cell type–specific monoclonal antibodies. The procedure used is IGSS for localization of PDGF-B chain, and avidin-biotin immunoalkaline phosphatase conjugated to cell-specific MAbs for simultaneous cell type analysis. (**A**) An advanced human carotid artery plaque is sequentially immunostained with PGF-007 (black, granular reaction product) and HAM56, a macrophage-specific MAb (*28*) (red reaction product). (**B**) The identical lesion is sequentially immunostained with PGF-007 (black, granular reaction product) and HHF35, a muscle actin–specific antibody, which in this context identifies only smooth muscle cells (red reaction product) (*28*). All sections were counterstained with methyl green. Original magnifications, ×250.

apparently lipid-rich foam cells, suggests that the entry of monocytes into the artery and their differentiation into macrophages may stimulate PDGF-B gene expression and protein synthesis. This may be followed by lipid accumulation and decrease in PDGF-B gene expression, similar to observations in lipid-loaded endothelial cells (*20*). Preliminary data suggest that lipid loading of human peripheral blood monocyte-derived macrophages leads to decreased PDGF gene expression (*21*). Alternatively, activation of B-chain gene expression may change with the state of macrophage differentiation during lesion formation and progression. Examination of experimentally induced lesions suggests that monocyte entry and macrophage exit from the lesions is a continuous process (*8, 10*).

Since PDGF exerts its mitogenic effects by means of high-affinity membrane receptors (*22*), the nature of the receptor populations available to bind PDGF also needs to be evaluated. There are two separate forms of the PDGF receptor, termed α and β subunits (*23*), each of which has specific affinities for the different dimeric forms of PDGF. Each chain of the PDGF dimer binds to one receptor subunit to form a receptor dimer, and then induces mitogenesis through a series of intracellular signals. Both of these receptor subunits are present on arterial smooth muscle in culture (*24*). PDGF β-receptors, able to bind only PDGF-B chain, are found in lesions of atherosclerosis on HLA-DR$^+$ smooth muscle cells by immunocytochemical techniques associated with HLA-DR$^+$ T cells and macrophages (*25*). In contrast, PDGF β-receptors were not detected in smooth muscle cells in the media of the arteries or in regions of the intima unaffected by atherosclerosis. Thus, the interactions among T cells, macrophages, and smooth muscle may stimulate the smooth muscle cells in this microenvironment to increase the number of PDGF β-receptors, making them susceptible to the mitogenic action of the PDGF-B chain–containing protein observed in the macrophages within the lesions of atherosclerosis.

The "response-to-injury hypothesis of atherosclerosis" was formulated to test the proposal that endothelial injury and platelet interactions were responsible for initiating the proliferative smooth muscle lesions of atherosclerosis. Testing of this hypothesis has led to modification on the basis of data confirming the fact that although endothelial changes and platelet interactions are observed at branches and bifurcations of the arteries, they are not always present. Monocyte-derived macrophages are ubiquitous in the lesions of human and experimental atherosclerosis (*26*). Within the microenvironment of the lesions, interactions between monocytes, T cells, and endothelium may lead to the formation and release of growth-regulatory and other bioactive molecules, which may induce further chemotaxis of leukocytes as well as smooth muscle cells from the media into the intima. Continued proliferation of the smooth muscle cells and their migration toward the lumen can eventually lead to the formation of a mature advanced lesion, or fibrous plaque. The observations presented in this report demonstrate the presence of PDGF-B chain–containing protein in macrophages during all stages of both human and nonhuman primate atherogenesis. Such localized production of this potent smooth muscle mitogen and chemoattractant by macrophages is consistent with the chronic inflammatory and focal nature of the lesions of atherosclerosis (*3, 8–10*).

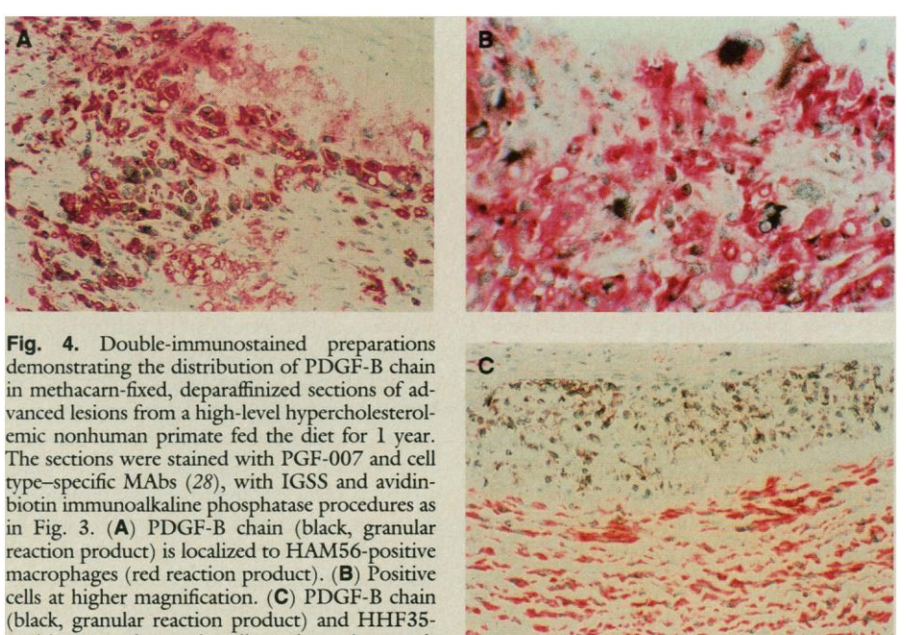

Fig. 4. Double-immunostained preparations demonstrating the distribution of PDGF-B chain in methacarn-fixed, deparaffinized sections of advanced lesions from a high-level hypercholesterolemic nonhuman primate fed the diet for 1 year. The sections were stained with PGF-007 and cell type–specific MAbs (*28*), with IGSS and avidin-biotin immunoalkaline phosphatase procedures as in Fig. 3. (**A**) PDGF-B chain (black, granular reaction product) is localized to HAM56-positive macrophages (red reaction product). (**B**) Positive cells at higher magnification. (**C**) PDGF-B chain (black, granular reaction product) and HHF35-positive smooth muscle cells (red reaction product) are identified in nonoverlapping cell populations. All sections were counterstained with methyl green. (A and C) Original magnifications, ×250; (B) original magnification, ×400.

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A Novel Nucleoprotein Complex at a Replication Origin

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The viral protein p6, required for the protein-primed initiation of replication of *Bacillus subtilis* phage φ29, forms a nucleoprotein complex at the viral replication origins that shows novel features. Deoxyribonuclease I and hydroxyl radical footprinting data, as well as the induction of positive supercoiling, support a model in which a DNA right-handed superhelix tightly wraps around a multimeric p6 core. The interaction occurs through the DNA minor groove. The activity of p6 not only requires the formation of the complex but also its correct positioning, indicating that the other proteins involved in the initiation of replication recognize, at a precise position, either the p6 core or the DNA conformational change induced by p6.

THE INITIATION OF REPLICATION OF circular (or topologically constrained) double-stranded DNA genomes is associated with the formation of a nucleoprotein complex at the replication origin that severely alters the DNA conformation and allows or stimulates the assembly of the replication machinery (*1*). Such complexes may also occur in circular genomes that replicate by a rolling-circle mechanism (*2*). The initiation of replication of linear double-stranded DNA genomes that have a terminal protein covalently linked at both 5′ ends has been studied mainly in adenovirus (*3*) and bacteriophage φ29 of *Bacillus subtilis* (*4*). In both systems there is evidence for the formation of nucleoprotein complexes at replication origins (*5–7*).

Adenovirus and φ29 initiate replication at both genome ends through the formation of a covalent linkage between the first deoxynucleoside monophosphate (dNMP) and a free molecule of the terminal protein, which is used as primer; the linkage is catalyzed by the viral DNA polymerase. Once the initiation reaction occurs, elongation proceeds, displacing the nontemplate strand until a genome-length strand is synthesized. In the case of φ29, in addition to the terminal protein and the DNA polymerase, the viral protein p6 is required for the phage DNA replication in vivo (*8*). Protein p6 stimulates both the initiation reaction and the transition to the elongation process in a φ29 DNA in vitro replication system (*9*).

Protein p6, which is very abundant in infected cells, interacts with double-stranded DNA (*7*) presumably as a dimer of 24 kD (*10*). By deoxyribonuclease I (DNase I) footprinting it was shown that protein p6 cooperatively forms a periodic DNA–multimeric protein complex at the φ29 replication origins (*7*) that extends along the terminal 200 to 300 bp. The major determinant of p6 binding seems to be the ability of these sequences to assume a well-defined conformation; this has been related to the ability of the DNA to bend (*11*). Binding of p6 to nonterminal φ29 DNA fragments has been observed, but it does not show the periodicity or salt-resistance of binding to terminal fragments (*7*). The formation of the nucleoprotein complex at the replication origins is required for the activity of protein p6 in the initiation of replication (*11, 12*).

The DNase I footprint pattern of p6 bound to the φ29 replication origins consists of strong hypersensitive bands periodically spaced every ~24 bp, the remaining positions being protected, except an unprotected or moderately hypersensitive site in the middle (*7*) (see also Fig. 1). Hydroxyl radical footprinting (*13*) was used to further study the interaction of protein p6 with the φ29 replication origins, since it allows one to determine the positions of the DNA backbone contacted by the protein with a

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