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The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors

(atherosclerosis/wound healing/growth factors/extracellular matrix)

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ABSTRACT Interactions among growth factors, cells, and extracellular matrix are critical to the regulation of directed cell migration and proliferation associated with development, wound healing, and pathologic processes. Here we report the association of PDGF-AB and -BB, but not PDGF-AA, with the extracellular glycoprotein SPARC. Complexes of SPARC and ¹²⁵I-labeled PDGF-BB or -AB were specifically immunoprecipitated by anti-SPARC immunoglobulins. ¹²⁵I-PDGF-BB and -AB also bound specifically to SPARC that was immobilized on microtiter wells or bound to nitrocellulose after transfer from SDS/polyacrylamide gels. The binding of PDGF-BB to SPARC was pH-dependent; significant binding was detectable only above pH 6.6. The interaction of SPARC with specific dimeric forms of PDGF affected the activity of this mitogen. SPARC inhibited the binding of PDGF-BB and PDGF-AB, but not PDGF-AA, to human dermal fibroblasts in a dose-dependent manner. The expression of SPARC and PDGF was minimal in most normal adult tissues but was increased after injury. Enhanced expression of both PDGF-B chain and SPARC was seen in advanced lesions of atherosclerosis. We suggest that the coordinate expression of SPARC and PDGF-B-containing dimers following vascular injury may regulate the activity of specific dimeric forms of PDGF *in vivo*.

Interactions among cells and growth-regulatory molecules are thought to be subject to modulation by specific, locally produced extracellular components. For example, heparan sulfate proteoglycans have been shown to bind the fibroblast growth factors (1–3), granulocyte/macrophage colony-stimulating factor and interleukin 3 (4, 5), and transforming growth factor β (6, 7). Factors bound in this manner might interact directly with cells or remain in the extracellular matrix (ECM), prior to release by enzymes such as heparinase and plasmin (8, 9) to function as soluble, diffusible growth regulators.

A group of extracellular macromolecules with properties distinct from those of collagens, proteoglycans, or adhesive proteins (e.g., fibronectin and laminin) is exemplified by the antiadhesive proteins tenascin, thrombospondin, and SPARC (secreted protein, acidic and rich in cysteine) (10). Although structurally dissimilar, these three secreted glycoproteins share at least one property: modulation of cell shape, in part through interference with molecules that support cell adhesion. SPARC in particular has been shown to promote cell rounding in confluent cultures of endothelial cells, smooth muscle cells, and fibroblasts (11). The prevalence of SPARC in areas of active tissue morphogenesis and remodeling (12, 13) is consistent with a function potentially related to cellular proliferation, migration, and/or differentiation.

Previous work showed that SPARC retarded the cell cycle in bovine aortic endothelial cells by inhibiting progression from G₁ to S phase (14), leading us to speculate that SPARC might be interacting with one or more growth factors to modulate their activity toward cells that would otherwise be responsive to mitogenic stimuli. This hypothesis, coupled with the location of SPARC in platelets (15), prompted our examination of possible interactions between SPARC and the platelet mitogen PDGF.

PDGFs are a family of growth-regulatory molecules capable of inducing directed cell migration, proliferation, and altered cellular metabolism (16, 17). The active forms of PDGF are assembled as disulfide-bonded homo- or heterodimers of two distinct but highly homologous peptide chains (PDGF-A and PDGF-B) that are differentially expressed upon cellular activation. Cells responsive to PDGF express specific surface receptors that are also dimers of two distinct gene products: one, termed the α subunit, binds both the A and the B chain, and the other, the β subunit, binds only the B chain (18–21). The two receptor subunits, which are differentially regulated, are expressed in various amounts and proportions that are characteristic for a given cell type. As a consequence, the capacity of the different dimers of PDGF to induce mitogenesis, as well as several other critical cell functions, depends on both the PDGF dimer present and the relative numbers of receptor subunits on the responding cell (20, 22).

In this report, we examine the association of various dimeric forms of PDGF with SPARC. PDGF dimers containing at least one B chain bound SPARC in a specific and pH-dependent manner. SPARC also inhibited the binding of PDGF to human dermal fibroblasts. Given the colocalization of SPARC and PDGF in platelet α granules and the increased expression of both PDGF-B chain and SPARC in advanced lesions of atherosclerosis, we propose that SPARC might regulate the availability of dimeric forms of PDGF in vascular injury.

MATERIALS AND METHODS

Proteins and Antibodies. Native SPARC was purified from conditioned medium of murine PYS-2 cells (13). Anti-SPARC antibodies were affinity-purified from sera of rabbits immunized with murine SPARC (13) or with a synthetic N-terminal peptide [SPARC-(5–23)] (23).

PDGF-AB was purified from outdated human platelet-rich plasma (24). PDGF-AA_{short} (110-amino acid endothelial form), PDGF-AA_{long} (125-amino acid glial form) and PDGF-BB (109-amino acid form) (17), purified from a re-

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Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; PDGF, platelet-derived growth factor.

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combinant expression system in *Saccharomyces cerevisiae* (25), were kindly provided by Zymogenetics (Seattle). Anti-PDGF antibodies included a goat polyclonal anti-PDGF that recognizes all dimeric forms of PDGF (26), a rabbit polyclonal anti-PDGF-B-chain antibody (24), and a mouse monoclonal antibody specific for the PDGF-B chain, PGF-007 (27), provided by Mochida Pharmaceutical (Tokyo). For some experiments, PDGF-AB and PDGF-AA were iodinated with Iodo-Beads (Pierce). PDGF-BB was modified with the Bolton-Hunter reagent (Pierce) prior to iodination.

Solid-Phase Binding Assays. SPARC (200 ng/ml) or other proteins were adsorbed to microtiter wells (MaxiSorb, Nunc) at 4°C overnight and the plates were blocked with 1% bovine serum albumin (BSA)/0.05% Tween-20 (blocking buffer). Various dimeric forms of PDGF were incubated in blocking buffer for 2 hr at room temperature with coated and blocked plates, followed by incubation with anti-PDGF antibodies for 1 hr. Bound anti-PDGF antibodies were detected with biotinylated second antibody (Vector Laboratories), avidin-biotin-peroxidase (Vector Laboratories), and *o*-phenylenediamine (Sigma).

Immunoprecipitation of PDGF-SPARC Complexes. Purified SPARC (500 ng) was incubated with ¹²⁵I-labeled PDGF-AA, -AB, or -BB (3 or 15 ng) for 16 hr at 4°C followed by incubation with anti-SPARC IgG for 1 hr at room temperature. Immune complexes were removed with protein A-Sepharose and dissociated in SDS/PAGE sample buffer (28) at 95°C for 3 min or 1 M acetic acid at room temperature.

PDGF Binding to SPARC Immobilized on Nitrocellulose. Murine SPARC, BSA, and fibronectin (Telia Pharmaceuticals, La Jolla, CA) were separated in SDS/PAGE 4–20% gradient minigels (Daiichi Pure Chemicals, Tokyo) and were transferred to nitrocellulose. The blots were blocked and were incubated with ¹²⁵I-PDGF-BB (10 ng/ml) for 1 hr at room temperature. Proteins that bound PDGF were visualized by autoradiography.

PDGF Binding to Human Fibroblasts. Cultures of adult human skin fibroblasts were plated in 24-well trays (Costar) and used for both simultaneous and sequential binding studies (29).

Immunohistochemistry. Segments of thoracic aorta from control animals (*Macaca nemestrina*) and animals maintained on a hypercholesterolemic diet (plasma cholesterol, 600–800 mg/dl) were treated with methyl Carnoy fixative (27). Adjacent sections were exposed to antibodies specific for PDGF-B (27) and affinity-purified anti-SPARC antibodies (13). Immunopositive cells were detected with biotinylated secondary antibodies and avidin- or streptavidin-biotin-peroxidase, with diaminobenzidine as the substrate.

RESULTS

PDGF-AB and PDGF-BB, but Not PDGF-AA, Specifically Associate with SPARC. We initially screened a number of extracellular proteins that included collagen types I, III, IV, V, and VIII, as well as SPARC. The binding of platelet PDGF (which contains all dimeric forms of PDGF; ref. 24) with SPARC was dose-dependent and highly significant. Although collagen types III, V, and VIII also demonstrated a dose-dependent binding of PDGF, appreciable levels of the mitogen were also detected in purified preparations of these collagens. Further analysis of the binding of different collagen types to PDGF will therefore require removal of endogenous PDGF. In contrast, preparations of SPARC purified from the murine PYS-2 cell line had low or undetectable levels of PDGF by both immunoassay and radioreceptor assay (data not shown).

To identify the dimeric form of PDGF that bound to SPARC, ¹²⁵I-labeled PDGF-AA, -BB, and -AB were incubated with SPARC, and SPARC-PDGF complexes were detected by

immunoprecipitation with two different anti-SPARC antibodies (Fig. 1A). Both a rabbit polyclonal anti-SPARC IgG (13) and a rabbit antibody to an N-terminal peptide [SPARC-(5–23)] (23) immunoprecipitated complexes of SPARC and ¹²⁵I-PDGF-AB or ¹²⁵I-PDGF-BB, but not ¹²⁵I-PDGF-AA. Immunoprecipitation of these complexes was diminished significantly in the presence of 100-fold and 500-fold excesses of unlabeled PDGF (Fig. 1B).

PDGF-AB and PDGF-BB, but not PDGF-AA, also bound to immobilized SPARC. Unreduced SPARC, BSA, and fibronectin were separated by SDS/PAGE and transferred to nitrocellulose. Incubation of ¹²⁵I-PDGF-BB with the blot demonstrated specific and competitive binding to SPARC, but not to BSA or fibronectin (Fig. 2). In similar experiments PDGF-BB failed to bind to thrombospondin or tenascin (data not shown). Binding was diminished considerably when samples of SPARC were reduced prior to SDS/PAGE. In addition, denaturation of SPARC during purification significantly decreased binding of PDGF. Results similar to those shown in Fig. 2 were obtained with ¹²⁵I-PDGF-AB but not with ¹²⁵I-PDGF-AA_{long} or ¹²⁵I-PDGF-AA_{short} (data not shown).

The Association of PDGF and SPARC Is pH-Dependent. To evaluate the optimal pH range for the association of SPARC and PDGF-BB, SPARC bound to microtiter plates was

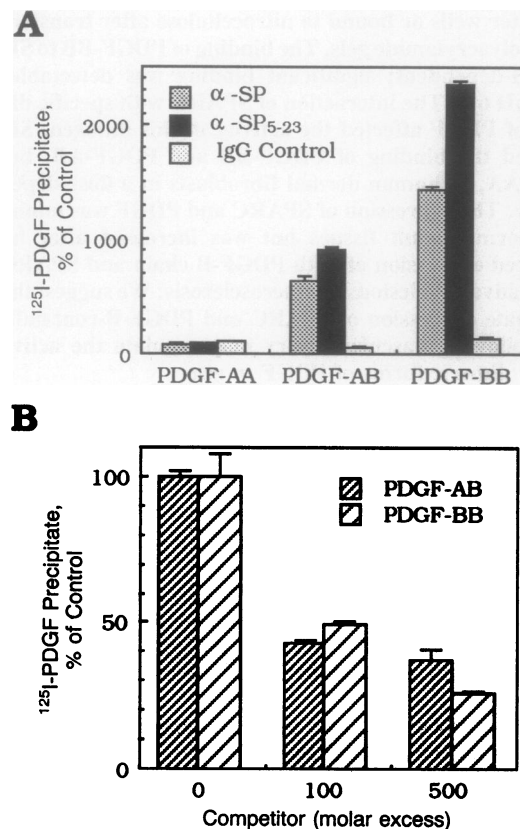


FIG. 1. Anti-SPARC antibodies specifically immunoprecipitate PDGF-BB and PDGF-AB but not PDGF-AA. (A) ¹²⁵I-PDGF-SPARC complexes were precipitated by two independently derived anti-SPARC antibodies (α SP; α SP₅₋₂₃) but not by a preparation of normal rabbit IgG (IgG control). Control binding of ¹²⁵I-PDGF in the absence of SPARC was defined as 100% and was equivalent to 2.06 fmol of PDGF-AA, 0.07 fmol of PDGF-AB, and 0.36 fmol of PDGF-BB. All assays were performed in triplicate, and data were plotted as percentage of control (\pm SEM). (B) Unlabeled PDGF-AB and PDGF-BB inhibit immunoprecipitation of ¹²⁵I-PDGF-SPARC complexes. SPARC was preincubated with 100-fold (300 ng) or 500-fold (1.5 μ g) excess unlabeled PDGF for 4 hr before incubation with labeled PDGF. Binding to SPARC in the absence of nonradiolabeled competitor was defined as 100%.

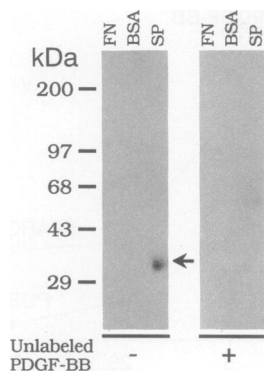


FIG. 2. ^{125}I -PDGF-BB binds to SPARC immobilized on nitrocellulose but not to fibronectin or BSA. SPARC (SP, 500 ng), BSA (2 μg), fibronectin (FN, 2 μg), and molecular size standards were resolved by SDS/PAGE, transferred to nitrocellulose, and incubated with ^{125}I -PDGF-BB (10 ng/ml). PDGF-BB bound only to SPARC (arrow). The binding of iodinated PDGF-BB was eliminated when 100-fold excess unlabeled PDGF was preincubated with the blot prior to the addition of ^{125}I -PDGF-BB.

incubated with PDGF-BB, and binding of PDGF was detected with anti-PDGF antibodies (Fig. 3). At pH 7.5, SPARC bound PDGF-AB and PDGF-BB, but not PDGF-AA, in a dose-dependent manner (data not shown for AB and AA isoforms). Binding of SPARC to immobilized PDGF-BB was also observed at pH 7.5 (data not shown). Below pH 5, no significant binding of PDGF-BB to immobilized SPARC was detected. Binding to SPARC increased rapidly between pH 6.6 and 7.2, with maximal specific binding detectable at pH 7.6. Native conformation of both PDGF-BB and SPARC was required for this association.

SPARC Inhibits the Binding of PDGF-AB and PDGF-BB, but Not PDGF-AA, to Cell Surface Receptors on Human Fibroblasts. To determine whether the binding of PDGF to SPARC affected the ability of PDGF to bind to its receptor, dimeric forms of ^{125}I -PDGF were preincubated with various concentrations of SPARC and were subsequently added to human fibroblasts. A dose-dependent inhibition of the binding of PDGF-AB and PDGF-BB, but not PDGF-AA, to human fibroblasts was observed in the presence of SPARC. Plasma-derived serum, which contains proteins that inhibit

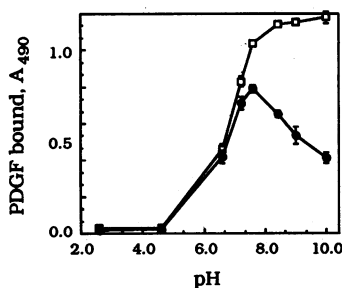


FIG. 3. Binding of PDGF-BB to immobilized SPARC is pH-dependent. Microtiter wells coated with SPARC were blocked and pre-equilibrated for 1 hr in universal buffers of a constant ionic strength (conductance, 15 mS) that were adjusted to pH values between 2.6 and 10.0. PDGF-BB diluted to a final concentration of 50 ng/ml was incubated with SPARC for 2 hr in the same buffers. All wells were re-equilibrated to pH 7.7 and total binding (\square) was determined in triplicate (\pm SEM) at each pH for wells containing SPARC and for control wells containing blocking agent alone with anti-PDGF antibodies. Specific binding (\bullet) represents the total protein bound minus the nonspecific protein bound to control wells. Separate determinations were performed to verify that a constant amount of SPARC remained immobilized at all pH values and that recognition of PDGF by anti-PDGF did not change with treatment (data not shown).

the binding of PDGF to its receptor (29–31), inhibited the binding of all dimeric forms of PDGF to human fibroblasts (Fig. 4). In contrast, type V collagen, a component of vascular ECM, had no effect on the binding of any of the dimeric forms of PDGF to these cells (Fig. 4).

Characterization of the Binding of PDGF-AB and -BB to SPARC. The relative competition of PDGF-AB and -BB was evaluated under conditions identical to those in which SPARC inhibited the binding of PDGF to its receptor (Fig. 5). Increasing concentrations of PDGF-AB and -BB were incubated first with 500 ng of SPARC and then with 15 ng of ^{125}I -PDGF-BB; immune complexes were immunoprecipitated with the rabbit antibody specific for the N-terminal SPARC peptide [SPARC-(5–23)] (23). Twice the concentration of PDGF-AB was required to produce competition comparable to that observed with PDGF-BB. No competition was observed with either the long or the short form of PDGF-AA. Scatchard (32) analysis of the competitive displacement data demonstrated a similar apparent affinity of PDGF-AB and PDGF-BB for SPARC, with a dissociation constant (K_d) of $\approx 10^{-9}$ M.

Expression of SPARC and PDGF-B Chain Is Induced in Lesions of Atherosclerosis. The distribution of PDGF-B chain and SPARC was evaluated by immunostaining of vessels from normal nonhuman primates (*M. nemestrina*) and hypercholesterolemic animals with advanced lesions of atherosclerosis. Normal vessels contained no PDGF-positive cells (data not shown), and SPARC immunoreactivity was confined to areas adjacent to the internal and external elastic laminae (Fig. 6A). However, in advanced lesions of atherosclerosis, cells in the proliferative intimal lesion that have been identified as macrophages (27) stained positively for PDGF-B chain (Fig. 6C). SPARC was clearly increased in atherosclerotic lesions, as compared with normal tissue, and was primarily associated with medial smooth muscle cells and with cells throughout the neointima (Fig. 6B). Many of the same intimal cells that contained PDGF-B chain (Fig. 6C) appeared to contain SPARC (Fig. 6D). However, SPARC was also expressed by additional intimal cells that were identified as smooth muscle cells in adjacent sections.

DISCUSSION

This study suggests a novel role for the secreted glycoprotein SPARC in the regulation of specific dimeric forms of PDGF. Heretofore, major functions for SPARC were proposed in the formation and remodeling of bone (33) and in regions of tissue morphogenesis and repair that required alterations in cell shape (11, 13). Consistent with its role in the modulation of cell shape, SPARC has been shown to effect the dissolution of focal contacts in cultured endothelial cells (34). It was therefore interesting that SPARC inhibited endothelial cell cycle progression and that this effect was independent of apparent changes in cell shape (14). We therefore proposed that SPARC facilitated a temporary withdrawal from the cell cycle that would in turn be requisite for migration or for acquisition of a differentiated phenotype (14). From the data shown here, it appears that, for certain cells, the inhibition of S phase by SPARC could result from the sequestration of PDGF by this protein, a hypothesis that can be tested on cells responsive to both PDGF and SPARC.

Studies of angiogenesis *in vitro* have shown an increase in SPARC mRNA and protein (10, 35). Although the cellular events responsible for the formation of these structures are not completely understood, it is generally agreed that confluent, quiescent endothelial cells disrupt cell–cell and cell–substrate contacts, modulate their secretory phenotype, proliferate, and migrate (36). Since most of these activities require changes in cell shape and/or in proliferative response, SPARC might play an important role in angiogenesis.

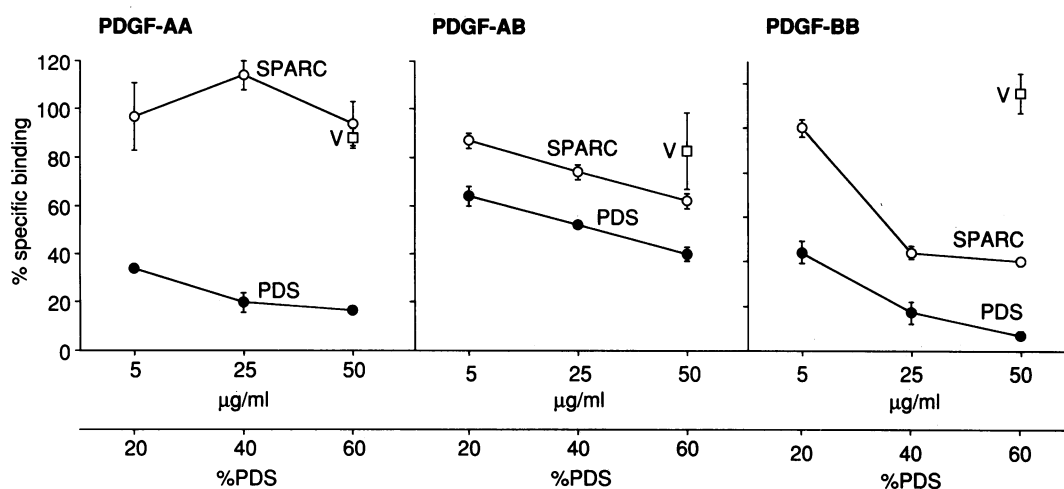


FIG. 4. SPARC inhibits the binding of PDGF-BB and PDGF-AB, but not PDGF-AA, to human dermal fibroblasts. ^{125}I -PDGF dimeric forms were preincubated with SPARC, human plasma-derived serum (PDS), or type V collagen (V), and specific binding to human skin fibroblasts was subsequently measured. Addition of SPARC had no effect on the binding of PDGF-AA to cells (*Left*). However, SPARC inhibited the binding of PDGF-AB (*Center*) and PDGF-BB (*Right*) in a dose-dependent fashion. Type V collagen had no effect on the binding of any dimeric form of PDGF, and plasma-derived serum inhibited the binding of all three dimeric forms. Data represent the mean \pm SEM of triplicate determinations, and the experiment shown is representative of three separate experiments. Samples were checked in a sequential radioreceptor assay and shown to be free of endogenous PDGF (data not shown).

Endothelial cells from microvessels, but not large vessels, respond to PDGF-BB and express the PDGF β receptor (37–39). Therefore, microvascular endothelial cells are capable of responding to PDGF as well as to SPARC and might be primary targets for modulation by a SPARC–PDGF complex.

As demonstrated in this report, the distribution of SPARC and PDGF differs in normal vessels and in the advanced lesions of atherosclerosis. Since both proteins appear to be expressed *de novo* in numerous cells of the proliferative neointima, increased expression of SPARC and PDGF might be indicative of injury and/or repair in certain tissues. The upregulation of specific chains of PDGF and their cognate receptors has been described in all stages of atherosclerotic lesions, in healing vascular grafts, and in cutaneous wounds (27, 40, 41).

The concept of specific ECM components as molecular sinks for morphogenetic and/or growth-regulatory factors is not derived solely from the interaction observed between SPARC and PDGF. Heparan sulfate proteoglycans can bind

a number of growth factors (2–9), and dimeric forms of PDGF containing the basic sequence encoded by exon 6 of the A and the B chain have been shown to bind to heparan sulfate proteoglycans (42). In those studies, the proteoglycans appeared to act as a storage depot for immobilized growth factors. Our observations suggest a more active role for SPARC in blocking the binding of PDGF-B chain to its receptors. The binding of PDGF to its receptor is absolutely required for induction of mitogenesis or chemotaxis. Since SPARC can inhibit cell cycle progression of large-vessel endothelial cells (14), it will be necessary to evaluate whether SPARC can also directly inhibit cell cycle progression in fibroblasts independently of its inhibition of the binding of PDGF to its receptor.

We have shown that the association of PDGF-BB and SPARC requires a pH > 5.0 and that binding is not optimal below pH 7.6. pH values of ≈ 6.5 have been detected in certain pathologic fluids or under conditions of low oxygen tension (43). Several properties of SPARC might affect its association with PDGF: e.g., alteration of tertiary structure by reduction of disulfide bonds or denaturation during purification significantly inhibits the association of PDGF-BB and SPARC. An altered conformation of SPARC could also result from its affinity for several components of the ECM (11, 33, 44), the presence of at least two binding sites for Ca^{2+} (45, 46), and the phosphorylation of serine residue(s) (45, 47).

An interesting feature of the association of PDGF and SPARC is its specificity for the PDGF-B chain. PDGF-BB is the only homodimeric form of PDGF able to bind to the PDGF receptor β subunit with high affinity (20, 21). This subunit is more abundant on most cells than are the α subunits (20, 22). In some cells, such as microvascular endothelial cells, it may be the principal, and perhaps the only, PDGF receptor subunit expressed (38). Increased expression of the β subunits is also associated with inflammatory reactions (48, 49). Thus, in a number of conditions associated with injury, inflammation, and remodeling, coordinate expression of PDGF-B chain, PDGF receptor β subunit, and SPARC might occur. Under these conditions SPARC could limit the availability of dimers containing the PDGF-B chain and consequently control proliferative repair processes.

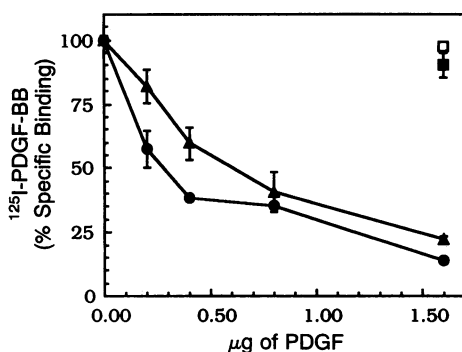


FIG. 5. Competitive inhibition of the binding of ^{125}I -PDGF-AB and -BB to SPARC. SPARC (500 ng) was incubated 4 hr with the indicated amounts of PDGF and then with 15 ng of ^{125}I -PDGF-BB. ^{125}I -PDGF–SPARC complexes were precipitated with antibody to SPARC-(5–23) peptide. Control binding of ^{125}I -PDGF-BB to normal rabbit IgG (<12% of PDGF–SPARC binding) was used to evaluate nonspecific binding. All points were determined in triplicate and plotted as percent specific binding (\pm SEM). In this experiment, 3 ng of ^{125}I -PDGF-BB was bound to SPARC in the absence of competitive inhibitor. \blacktriangle , PDGF-AB; \bullet , PDGF-BB; \square , PDGF-AA_{short}; \blacksquare , PDGF-AA_{long}.

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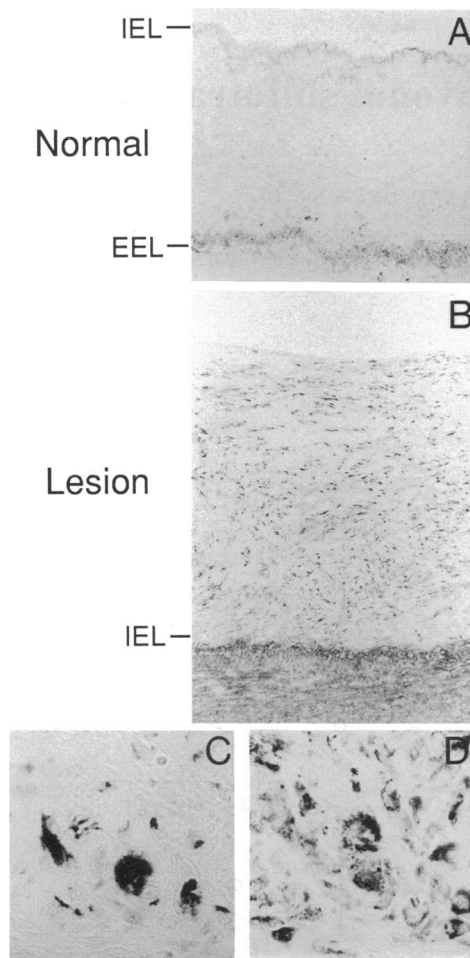


FIG. 6. Expression of PDGF-B chain and SPARC is induced in lesions of atherosclerosis. Sections of vessels from normal (A) or hypercholesterolemic (B–D) monkeys were incubated with antibodies to PDGF-B chain (C) or to SPARC (A, B, and D). Reaction product (dark stain) was visualized by an avidin–biotin–peroxidase technique. Normal vessels contained no PDGF-positive cells (data not shown), and immunoreactivity for SPARC was limited and confined to areas adjacent to the internal (IEL) and external (EEL) elastic laminae. Expression of SPARC was increased in advanced atherosclerotic lesions and was observed in most medial smooth muscle cells and in scattered cells of the neointima (B). Examination of adjacent sections of the advanced lesion at higher power demonstrated localized expression of PDGF-B chain in intimal cells (C), previously identified as macrophages, and expression of SPARC by apparently the same cells and in additional neointimal cells (D), identified in adjacent sections as smooth muscle cells (data not shown). ($\times 36$ in A and B; $\times 225$ in C and D.)

PDGF-AA and -BB and antibodies to PDGF, and Mochida Pharmaceutical for monoclonal antibody PGF-007. We thank Brenda Wood for assistance with the manuscript, and Kris Carroll and Mary Lucas Bohidar for assistance with preparation of the figures. This work was funded in part by National Institutes of Health Grants HL18645, GM40711, and RR-00166 to the Primate Center. T.F.L. is a predoctoral fellow supported by National Institutes of Health Training Grant HL07312.

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