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Identification of Platelet-Derived Growth Factor A and B Chains in Human Renal Vascular Rejection

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Platelet-derived growth factor (PDGF) exists as a dimer composed of two homologous but distinct peptides termed PDGF-A and -B chains, and may exist as AA, AB, and BB isoforms. The PDGF-B chain has been implicated as a mediator of renal vascular rejection by virtue of up-regulated expression of its receptor, PDGF β-receptor, in affected arteries. A role for PDGF-A chain in mediating intimal proliferation has been suggested in human atherosclerosis (Rekhter MD, Gordon D: Does platelet-derived growth factor-A chain stimulate proliferation of arterial mesenchymal cells in human atherosclerotic plaques? Circ Res 1994, 75:410), but no studies of this molecule in human renal allograft injury have been reported to date. We used two polyclonal antisera to detect expression of PDGF-A chain and one monoclonal antibody to detect PDGF-B chain by immunohistochemistry in fixed, paraffin-embedded tissue from 1) normal adult kidneys, 2) a series of renal transplant biopsies chosen to emphasize features of vascular rejection, and 3) allograft nephrectomies. Immunohistochemistry was correlated with in situ hybridization on adjacent, formalin fixed tissue sections from nephrectomies utilizing riboprobes made from PDGF-A and -B chain cDNA. PDGF-A chain is widely expressed by medial smooth muscle cells of normal and rejecting renal arterial vessels of all sizes by immunohistochemistry and in situ hybridization. PDGF-A chain is also expressed by a population of smooth muscle cells (shown by double immunolabeling with an antibody to α-smooth muscle actin) comprising the intima in chronic vascular rejection. In arteries demonstrating acute rejection, up-regulated expression of PDGF-A chain by endothelial cells was detected by both immunohistochemistry and in situ hybridization. In contrast, PDGF-B chain was identified principally in infiltrating monocytes within the rejecting arteries, similar to its localization in infiltrating monocytes in human atherosclerosis. Although less prominent than the case for PDGF-A chain, PDGF-B chain also was present in medial and intimal smooth muscle cells in both rejecting and nonrejecting renal arteries. PDGF-A and -B chains have now been localized at both the mRNA and protein levels to the intimal proliferative lesions of vascular rejection. These peptides, which are known stimuli for smooth muscle cell migration and proliferation in experimental vascular injury, may have similar stimulatory effects on smooth muscle cells in an autocrine and/or paracrine manner to promote further intimal expansion and lesion progression in this form of human vasculopathy. (Am J Pathol 1996, 148:439–451)

Intimal proliferation in muscular arteries is a hallmark of vascular rejection in renal allografts.[1–3] In the chronic injury phases of this process, occurring after the earliest stages of endothelial injury and subendothelial inflammatory cell infiltration, the intimal expansion is characterized by prominent accumulations of smooth muscle cells and an extracellular matrix that is presumably synthesized by these cells.[4] However, little is known of the factors that

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mediate this recruitment and proliferation of smooth muscle cells within these vascular lesions.

There are a number of reasons to consider members of the platelet-derived growth factor (PDGF) system as likely contributors to the development of chronic vascular rejection lesions. In humans, upregulated expression of the PDGF β-receptor, which binds the PDGF-B chain, has been demonstrated in chronic renal vascular rejection.[5] In animal models of angioplasty injury, which result in intimal proliferative lesions having considerable resemblance to those of chronic vascular rejection, prominent participation of PDGF-A and -B chains has been demonstrated.[6–8] Finally, it has been noted that there are a number of similarities between the neointimal proliferative lesions of human atherosclerosis and chronic vascular rejection of solid organ allografts; in cardiac allografts, this process has even been termed accelerated or transplant atherosclerosis/arteriosclerosis by some investigators.[9–11] In atherosclerosis, PDGF-A chain has been demonstrated in the vascular smooth muscle cells of both medial and intimal components of these lesions, whereas production of PDGF-B chain is a prominent feature of macrophages that are a common infiltrative component of these lesions.[12–14]

In this study, we build upon this foundation of observations to demonstrate in lesions of both acute and chronic human renal vascular rejection the presence of both PDGF-A and -B chains. We suggest that these molecules, in conjunction with other molecules that share similar qualities of being mitogenic and chemotactic for smooth muscle cells, have potentially important roles to play in the development and progression of this particular vasculopathy.

Materials and Methods

Normal human kidney (n = 24) was obtained fresh from uninvolved portions of kidneys surgically resected for localized renal cell carcinoma or from cadaver donor kidneys unable to be used for transplantation. Portions of these tissues were fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid), and other portions were fixed in 10% neutral buffered formalin and processed and embedded in paraffin according to conventional techniques.

A total of 31 renal allograft biopsies were also used in this study. Allograft biopsies were obtained as core needle biopsies and comprised the following categories: 1) biopsies of donor kidneys before transplantation (n = 2), 2) cases of acute cyclosporine nephrotoxicity as determined by subsequent clinical course and compatible biopsy findings (n = 5), 3) cases of mild interstitial inflammatory infiltration of uncertain significance not clearly related to rejection (n = 5), 4) cases of acute cellular rejection of at least moderate severity and demonstrating features of tubulitis (n = 4), 5) cases of acute vascular rejection, as previously illustrated and defined[4] (n = 13), and 6) cases of chronic rejection (n = 2). The diagnosis of acute vascular rejection (also termed allograft arteritis or endothelialitis by some) required the subendothelial infiltration of leukocytes into the arterial intima; endothelial swelling and lifting often accompanied this process but was not considered sufficient to establish the diagnosis. Features of inflammation or necrosis of the vessel wall media was not required for this diagnosis. Allograft kidneys (n = 14) excised for irreversible rejection were also used. These allograft nephrectomies invariably had features of cellular (interstitial) and vascular rejection. The infiltrates of cellular rejection were usually of moderate to severe intensity but focally distributed within the renal parenchyma so that portions of tissue showed only minimal inflammatory infiltration. Arterial vessels in these specimens showed a range of rejection processes ranging from absent to subendothelial inflammation only (acute vascular rejection) to diffuse intimal inflammation and intimal accumulation of smooth muscle cells and matrix (acute and chronic vascular rejection) to minimal or absent intimal inflammation with prominent accumulation of smooth muscle cells and matrix (chronic vascular rejection), as previously illustrated.[4] All of the allograft nephrectomy tissue sections had arteries exhibiting changes of chronic rejection; all but one case also had at least some arteries with features of additional superimposed acute rejection.

All biopsies other than pretransplant donor kidneys were obtained from patients under conditions of routine immunosuppression protocols employing cyclosporine and prednisone. The biopsies were obtained before more specific or intensified therapies such as administration of OKT_3. The nephrectomy specimens in general were exposed to multiple courses of routine and intensified immunosuppression before excision and represent a heterogeneous sample from a clinical standpoint.

Antibodies

PDGF-A chain

Anti-PDGF-A (Santa Cruz Biotechnology, Santa Cruz, CA) is an affinity-purified rabbit polyclonal an-

tibody raised against a 30-amino-acid peptide corresponding to the amino terminus of the human PDGF-A chain. The antibody specifically recognizes human PDGF-A chain under reducing and nonreducing conditions and is nonreactive with either reduced or unreduced PDGF-B chain or unreduced human PDGF-AB. The second antisera used is a rabbit polyclonal antibody raised against purified anti-human PDGF-AA (Upstate Biotechnology, Lake Placid, NY), which specifically recognizes PDGF-AA homodimer and PDGF-AB heterodimer. The immunogen for this antisera was purified human recombinant PDGF-AA homodimer, and this antisera neutralizes PDGF-AA and PDGF-AB in a PDGF stimulation of 3T3 cells bioassay. Absorption studies and Western blots demonstrating the specificity of both of these reagents in normal and developing human kidneys have been published previously.[15]

PDGF-B chain

Murine monoclonal antibody PG7–007 (provided by Mochida Pharmaceutical, Tokyo, Japan) has been previously characterized,[16] and its ability to specifically recognize PDGF-B chain in methacarn-fixed tissue has been demonstrated.[14,17,18] The specificity of the immunohistochemical activity of this antibody has been demonstrated in cell culture in which its activity has been abolished by preincubation with immunizing PDGF peptide[14] and by studies by our group that tightly link increased expression of the PDGF peptide as recognized by this antibody in tissue sections with increased synthesis of mRNA encoding PDGF as detected by Northern analysis and *in situ* hybridization in rat glomeruli.[19,20] Western blotting experiments to further define the specificity of this reagent are detailed below.

Smooth Muscle Cell Markers

Murine monoclonal antibody 1A4 (Dako Corp., Carpinteria, CA) has been characterized by tissue immunohistochemistry and Western blotting[21] and has been previously demonstrated to recognize smooth muscle α-actin in methyl Carnoy's-fixed tissues.[18,22]

Monocyte/Macrophage Markers

A murine monoclonal antibody PG-M1 (Dako) directed against the CD68 epitope of human monocytes and macrophages[23] was used in methyl Carnoy's-fixed tissues. This antibody has been shown to recognize cells of monocyte/macrophage lineage in fixed tissues with no loss of sensitivity compared with frozen tissue specimens.[23] The specificity of this antibody has been established by studies of transfected and untransfected cell lines and by immunocytochemical surveys of numerous cell lines and examples of neoplastic and non-neoplastic hematopoietic tissues, as previously described.[23] It does not react with normal granulocytes or lymphoid cells but has been shown to react with leukemic cells of M_4 and M_5 myeloid leukemias, mast cells in systemic mastocytosis (but not normal mast cells), and neoplastic cells of some nonhematopoietic solid tumors.[23]

Endothelial Cell Markers

Monoclonal mouse anti-human CD31, clone JC70 (Dako) is reactive with a formalin-resistant epitope of CD31 in vascular endothelial cells. It has been previously characterized by Western immunoblotting of membrane fractions and studies with transfected cells.[24]

Immunohistochemistry

Immunohistochemistry was performed on methyl Carnoy's-fixed, paraffin-embedded tissues following a standard avidin-biotin complex (ABC) method, as previously described.[18,25]

Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide, and nonspecific binding was blocked by incubation in 10% normal goat serum. The sections were then incubated overnight with the anti-PDGF-A or anti-PDGF-AA antisera or the PGF-007 monoclonal antibody in a humid chamber at 4°C. After washes in phosphate-buffered saline (PBS), the sections were sequentially incubated with biotinylated secondary antibody (goat anti-rabbit antisera or horse anti-mouse antisera; (Vector Laboratories, Burlingame, CA), the ABC-Elite avidin reagent (Vector Laboratories), and finally 3,3′-diaminobenzidine (with nickel chloride enhancement) as the chromogen. The sections were counterstained with methyl green, dehydrated, and coverslipped.

For all samples, negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with both irrelevant murine monoclonal antibodies or nonimmune rabbit sera and PBS. Positive controls included concurrent staining of fixed human fetal kidney, a tissue with

detectable constitutive expression of PDGF-B chain by mesangial cells,[18] as previously described, and PDGF-A chain by visceral epithelial cells, collecting ducts, urothelium, and vascular smooth muscle cells, as previously described.[15]

Double Labeling Immunocytochemistry

Methyl Carnoy's-fixed, paraffin-embedded tissues were sectioned and mounted on aminopropylmethoxysilane-coated slides. After deparaffinization and rehydration, the slides were sequentially incubated with the anti-α-smooth muscle actin antibody and goat anti-mouse IgG-gold (Amersham, Arlington Heights, IL) diluted in PBS plus 1% bovine serum albumin (BSA) and 0.1% gelatin for 1 hour at room temperature. Sections were washed, and the gold was visualized with an IntenSE M silver enhancement kit (Amersham). The sections were then incubated sequentially with 1) either rabbit anti-PDGF-A chain or rabbit anti-PDGF-AA dimer diluted in PBS plus 1% BSA overnight at 4°C, 2) biotinylated goat anti-rabbit IgG (Vector Laboratories), and 3) avidin-biotin-alkaline phosphatase complex (Vector Laboratories). The alkaline phosphatase was developed with a red substrate kit (Vector Laboratories), and the slides were counterstained with methyl green.

For double immunolabeling with the rabbit polyclonal anti-PDGF-A antisera and the murine monoclonal antibodies anti-actin, anti-CD68, anti-CD31, and anti-PDGF-B, the tissue sections were first immunolabeled with the first primary antibody at standard concentration using a conventional three-step ABC-horseradish peroxidase method, in which the horseradish peroxidase was visualized with 3,3′-diaminobenzidine to give a brown reaction product. For the second immunolabeling, the sections were then incubated sequentially with the second primary antibody, diluted approximately 10 times further than the standard concentration, and horseradish peroxidase-labeled anti-mouse. The sections were then reacted with True Blue peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After washing and counterstaining with nuclear fast red, the sections were dehydrated, coverslipped, and viewed. Controls for the double labels were done by substitution of the first or second primary antibody with PBS.

Antibody Absorption

Microtiter enzyme-linked immunosorbent assay plates were coated with PDGF-BB control peptide (from UBI, Lake Placid, NY) diluted in 50 mmol/L carbonate buffer, pH 9.0, at a concentration of 20 μg/ml. After overnight incubation at 4°C, the antigen solution was removed and the plates were blocked with PBS containing 1% BSA and 0.02% sodium azide for 2 hours at room temperature. The plates were then washed with PBS and allowed to air dry. The murine antibody PGF 007 diluted in PBS plus 2% BSA was added, and the plates were incubated overnight at 4°C. The supernatant was removed from the wells and used as the absorbed primary antibody in a standard ABC immunohistochemistry procedure. Positive controls (ie, repetition of the absorption procedure without antigen-specific absorption of the antisera) were done by using carbonate buffer only and PDGF-AA peptide (UBI) to coat the microtiter wells. This control antisera was used in procedures identical to those of the primary unabsorbed and absorbed antisera and gave results similar to that of the original unmanipulated antisera (data not shown). Similar absorption studies for the PDGF-A antisera have been previously described.[15]

Western Blotting

Homogenates of normal and transplanted adult and developing human fetal kidneys were electrophoresed on a 15% sodium dodecyl sulfate polyacrylamide gel and then blotted onto polyvinylidene difluoride membranes. The blots were blocked with 5% BSA in PBS for 1 hour at 37°C and then incubated with PGF 007 diluted in 10 mmol/L PBS containing 0.1% BSA and 10 mmol/L sodium azide (PBS-BSA) for 2 hours at room temperature. After washing, the blots were incubated sequentially with biotinylated horse anti-mouse antisera (Vector Laboratories) and ABC-alkaline phosphatase (Vector Laboratories) for 2 hours. The blots were then visualized with 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium. In control experiments, reduced and unreduced recombinant human PDGF-BB peptide (kindly provided by Drs. Ron Seifert, University of Washington, and Charles Hart, Zymogenetics, Seattle, WA) were used to demonstrate specificity of the antisera. As a negative control, the primary antibody was replaced by normal mouse IgG at an equivalent dilution.

Molecular Probe

A 1280-bp human PDGF-A chain cDNA[26] was subcloned into SP64 vector. This probe was a kind gift of Dr. J. N. Wilcox, Emory University. The construct was linearized and transcribed into an antisense riboprobe using reagents obtained from Promega Biotec (Madison, WI), except [^{35}S]UTP, which was obtained

from New England Nuclear (Boston, MA). The transcription reaction mixture contained 1 μg of PDGF-A cDNA (either sense or antisense), 250 μCi of [^{35}S]UTP (1100 to 1300 Ci/mmol), 500 μmol/L each of ATP, CTP, and GTP, 40 U of RNAsin, 10 mmol/L dithiothreitol, 40 mmol/L Tris, and 10 U of either SP6 or T7 polymerase. After 75 minutes at 37°C, the DNA was digested by adding 1 U of DNAse (Promega) and incubation at 37°C for an additional 15 minutes. Free nucleotides were then separated with a Sephadex G-50 column. Specific activity of the probes ranged from 5 to 30 × 10^7 cpm/mg. Probes were used immediately.

The probe for PDGF-B chain was derived from a 278-bp segment of PDGF-B chain cDNA[27] subcloned into a PGEM3-Z vector (gift of Dr. Tom Barrett and Kathy Gordon, University of Washington). Additional preparation of the sense and antisense probes used in the *in situ* hybridization procedures were as described for the PDGF-A chain probes above.

In Situ Hybridization

Adult kidney tissue that had been fixed in 10% formalin and embedded in paraffin was deparaffinized following standard protocol. The sections were washed with 0.5X standard saline citrate (SSC; 1X SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) and digested with proteinase K (1 μg/mL; Sigma Chemical Co., St. Louis, MO) in RNAse A (Promega) buffer for 40 minutes at 37°C. Several 0.5X SSC washes were followed by prehybridization for 2 hours in 50 μl of prehybridization buffer (0.3 mol/L NaCl, 20 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 1X Denhardt's solution, 10% dextran sulfate, 10 mmol/L dithiothreitol). The hybridizations were started by adding 5 × 10^5 cpm of ^{35}S-labeled riboprobe in 50 μl of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, sections were washed with 0.5X SSC, treated with RNAse A (20 μg/ml for 30 minutes at room temperature), washed in 2X SSC (twice for 2 minutes each), followed by three high stringency washes in 0.1X SSC/Tween 20 (Sigma) at 37°C, and several 2X SSC washes. After the tissue was dehydrated and air dried, it was dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 4 weeks. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, mounted, and viewed. Positive cellular labeling was defined as five or more silver grains concentrated over a single cell. Examples of positive and negative hybridization using the antisense and sense probes, respectively, are illustrated in Figure 1.

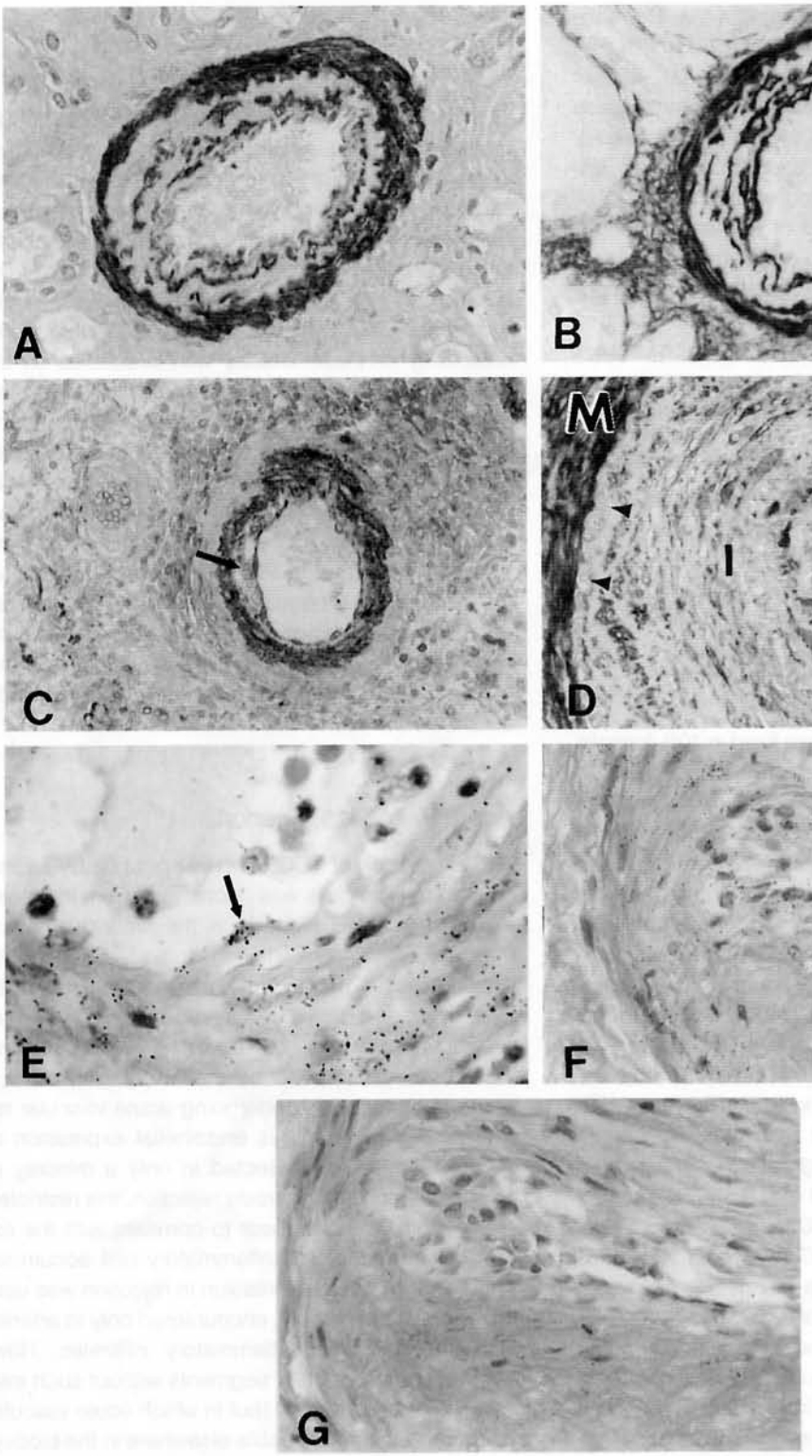
Results

Normal Arteries

The localization of PDGF-A chain mRNA by *in situ* hybridization techniques and PDGF-A chain peptide by immunocytochemistry previously has been demonstrated in vascular smooth muscle cells of the media of renal arteries as well as in smooth muscle cells constituting the arterial intima in renal arteriosclerosis.[15] For comparison with rejecting arteries, illustrations of this finding are given in Figure 1, A and B. Double immunolabeling of these arteries with antibodies to the smooth muscle marker α-actin has confirmed that this expression of PDGF-A chain is by smooth muscle cells.[15] PDGF-B chain peptide also could be identified as a constitutively expressed peptide by some medial smooth muscle cells of normal or arteriosclerotic renal arteries in this study. Correlative Western blotting studies also demonstrated PDGF-B chain peptide within the parenchyma of normal adult kidneys. Immunolocalization studies detected such expression within tubular epithelial cells, parietal epithelial cells, and glomerular mesangial cells in addition to vascular smooth muscle cells.

Acute Vascular Rejection

The expression of PDGF-A chain peptide by arterial smooth muscle cells was unchanged from that seen in untransplanted kidneys in the setting of acute vascular rejection. A striking finding, not normally encountered in normal, aging kidneys or in arteries from allograft kidneys uninvolved by acute vascular rejection, was the up-regulated expression of PDGF-A chain peptide by the endothelial cells in some of the arteries undergoing acute vascular rejection (Figure 1C). This endothelial expression of PDGF-A chain was detected in only a minority of arteries demonstrating acute rejection; this restricted expression did not appear to correlate with the extent of subendothelial inflammatory cell accumulation. PDGF-A chain expression in rejection was usually, but not exclusively, encountered only in arteries with subendothelial inflammatory infiltrates. However, at least two artery segments without such infiltrates in a single biopsy (but in which acute vascular rejection was demonstrable elsewhere in the biopsy) demonstrated endothelial expression of PDGF-A chain. In a second case, arterial endothelial cell expression of PDGF-A chain was encountered in a protocol biopsy in a patient without concurrent morphological or clinical evidence of active rejection. However, 6 days after this biopsy was obtained, the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patient experienced an acute onset of renal insufficiency (doubling of serum creatinine from baseline) that responded to a combined therapeutic intervention of pulse steroid administration and lowering of cyclosporine dosage. The overall clinical impression was a probable rejection episode and possible concurrent cyclosporine nephrotoxicity; however, no biopsy was obtained at this late time or subsequently to evaluate the possibility of vascular rejection.

The endothelial expression of PDGF-A chain persisted in arteries showing features of both active (subendothelial inflammatory cell infiltration) and chronic (prominent accumulations of neointimal smooth muscle cells and matrix) rejection (Figure 1D). Double immunolabeling studies that identified endothelial cells by virtue of their expression of the CD31 antigen confirmed this endothelial expression of PDGF-A chain (see Figure 3D). Correlative *in situ* hybridization studies demonstrated production of PDGF-A chain mRNA by both endothelial cells and underlying intimal smooth muscle cells in this setting (Figure 1E).

PDGF-B chain localization in acute vascular rejection differed significantly from that of PDGF-A chain. In this setting, PDGF-B chain was prominently expressed by infiltrating mononuclear leukocytes within the subendothelial region of the arterial intima (Figure 2). Sequential immunolabeling of replicate tissue sections and double immunolabeling on single tissue sections identified the great majority, if not all, of such PDGF-B-chain-producing leukocytes as $CD68^+$ monocytes (Figure 2, C and D). Smooth muscle cell components of the vessel wall had persistent expression of PDGF-B chain in this setting. The percentage of smooth muscle cells expressing PDGF-B chain varied from case to case and even among arteries within a single tissue section. Smooth muscle cells with PDGF-B chain expression were present within both the intima and vessel media (Figure 2, A and G).

Chronic Vascular Rejection

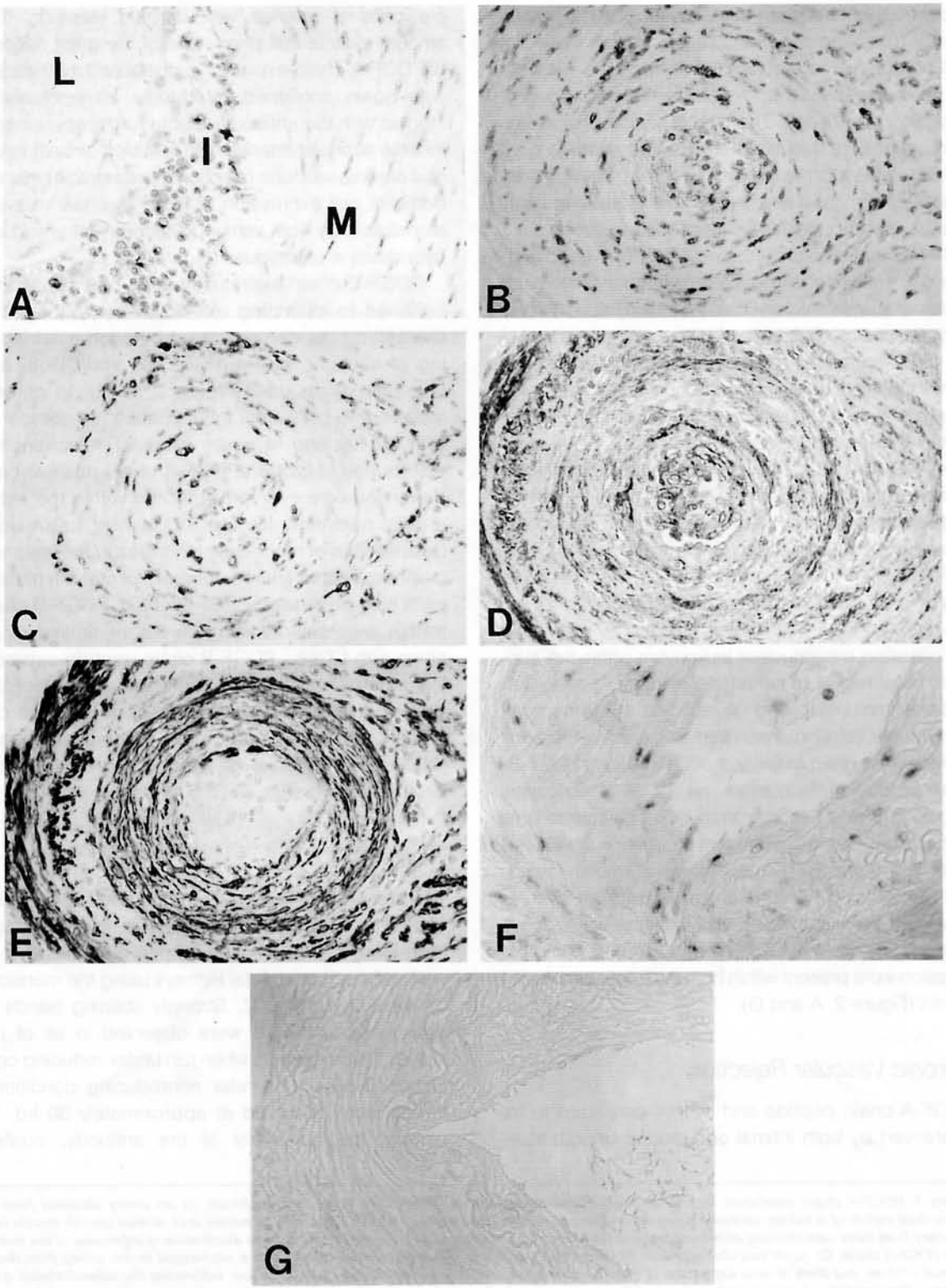
PDGF-A chain peptide and mRNA continued to be expressed by both intimal and medial smooth muscle cells in arteries with chronic rejection. The smooth muscle cell phenotype of the great majority of PDGF-A-chain-expressing intimal and medial cells was again confirmed by double immunolabeling studies with the antibody directed against α-smooth muscle actin. In arteries with features of both acute and chronic vascular rejection, persistent luminal endothelial cell expression of PDGF-A chain was demonstrable by both immunocytochemical and *in situ* hybridization techniques (Figure 1E).

PDGF-B chain expression most often appeared confined to infiltrating monocyte/macrophages in this setting, as demonstrated by sequential labeling of sections by the PGF 007, anti-CD68, and anti-actin antibodies (Figure 2, D–G). In chronic lesions with persistent inflammation, as compared with the lesions of acute vascular rejection, the distribution of $CD68^+$ PDGF-B-chain-positive cells generally were evenly distributed within the entire arterial neointima (Figure 3A), similar to previous descriptions of monocytic infiltrates in the lesions.[4] It appeared that limited numbers of smooth muscle cells also expressed PDGF-B chain. PDGF-B chain mRNA was demonstrable in the neointima of lesions with $CD68^+$ PDGF-B chain peptide-expressing monocytes, but confident assignment of the detected PDGF-B chain message to specific cell types could not be made due to technical reasons relating to dispersal of isotopic grains in highly cellular lesions (Figure 3, B and C).

Western Blotting

The presence of PDGF-B in human kidney was confirmed by Western blot analysis of tissue extracts from normal and rejecting human adult kidneys as well as from human fetal kidneys using the monoclonal antibody PGF-007. Strongly staining bands at approximately 17 kd were observed in all of the kidney tissue extracts when run under reducing conditions (Figure 4). Under nonreducing conditions, bands were observed at approximately 30 kd. To confirm the specificity of the antibody, purified

Figure 1. *PDGF-A chain expression in untransplanted and rejecting renal arteries.* A: *Renal arteriosclerosis, in an artery obtained from an uninvolved region of a kidney excised for neoplasia. There is widespread expression of PDGF-A chain by medial and intimal smooth muscle cells.* B: *Artery from same case showing distribution of* α*-actin-expressing smooth muscle cells, showing congruent distribution of expression of this marker with PDGF-A chain.* C: *Acute vascular rejection. PDGF-A chain expression by medial smooth muscle cells is unchanged in this setting from that of normal arteries, but there is now expression of PDGF-A chain by luminal endothelial cells. Leukocytes are infiltrating the subendothelial space* (arrow), *characteristic of acute rejection. Some of the mononuclear leukocytes infiltrating the periarterial connective tissue also express this peptide.* D: *Chronic vascular rejection, with some persistent inflammatory infiltration. There is widespread expression of PDGF-A chain by medial* (*M*) *and neointimal* (*I*) *smooth muscle cells, and endothelial expression of PDGF-A chain persists* (arrow). *Some of the neointimal cells expressing PDGF-A chain may also be infiltrating mononuclear leukocytes.* Arrowheads *delineate the internal elastic membrane separating the media from the neointima.* E: In situ *hybridization shows that arteries demonstrating chronic and active rejection as in* D *show production of PDGF-A chain mRNA by both endothelial cells* (arrow) *and many of the cells of the neointima, which are likely to be smooth muscle cells based on immunostaining of alternate tissue sections.* F: *Another artery with acute and chronic vascular rejection demonstrating prominent PDGF-A chain mRNA as indicated by silver grain label and using an antisense probe.* G: *Same artery as in* F *reacted with sense probe for PDGF-A chain mRNA. There is no detectable hybridization, demonstrating the specificity of the procedure.*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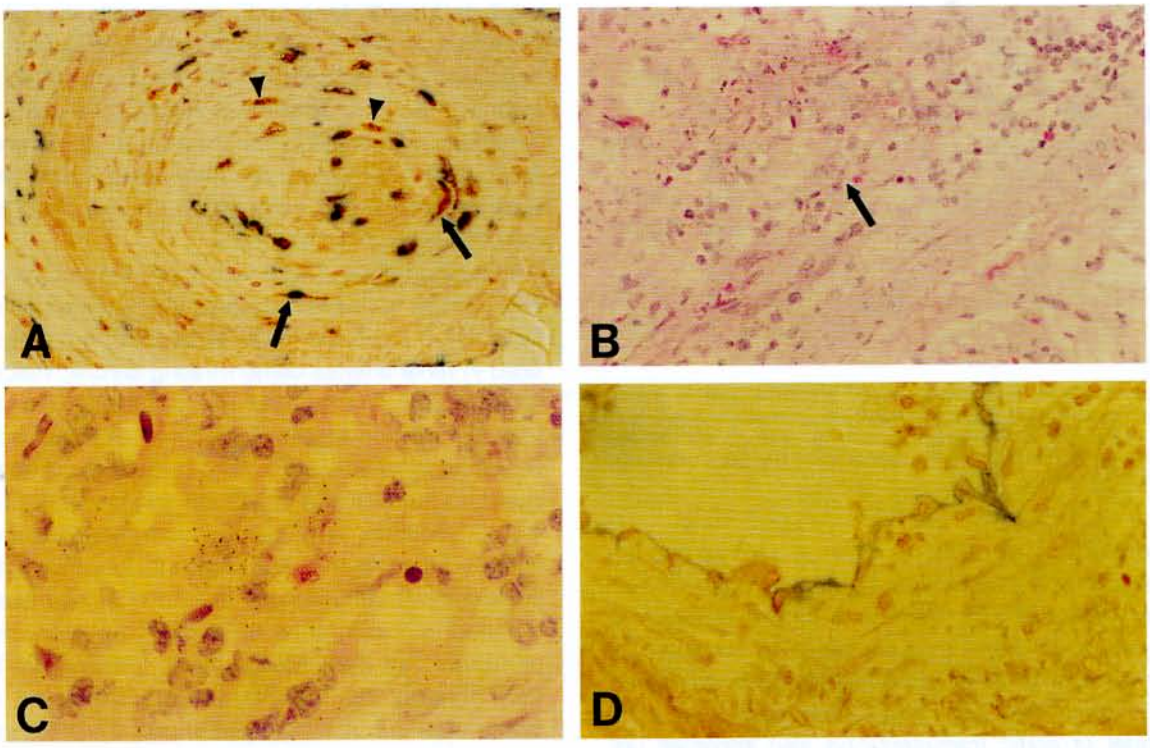

Figure 3. A: *Chronic and acute rejection. Double immunolabeling for PDGF-B chain in blue/black and CD68 in brown demonstrates that at least some CD68$^+$ monocyte/macrophages express the PDGF-B chain identified in these lesions* (arrows), *whereas* arrowheads *denote some CD68$^+$ cells without detectable PDGF-B chain.* B: *Low power view of* in situ *hybridization study of an artery with chronic active rejection, showing PDGF-B chain mRNA production by some cells in the expanded, inflamed neointima* (arrow). C: *Higher power view of artery in* B, *showing PDGF-B chain mRNA localization to cell types having a morphological appearance of monocyte/macrophages.* D: *Acute vascular rejection. The arterial endothelium is labeled both for PDGF-A chain in brown and CD31 antigen expression in blue.*

PDGF-BB, PDGF-AB, and PDGF-AA were also run under both reducing and nonreducing conditions. Both PDGF-BB and PDGF-AB showed bands at approximately 15 and 30 kd, whereas PDGF-AA was negative. These results are consistent with the reported multiple molecular weight forms of PDGF (27 to 31 kd unreduced and 14.4 to 17.5 kd reduced).[28]

Immunohistochemical Controls

No specific cellular staining was seen with substitution of control non-immune rabbit sera or irrelevant murine monoclonal antibodies or PBS for the primary antibody. Replication of the immunohistochemical procedures with absorbed antisera removed the specific staining identified with the anti-PDGF-A antisera and with the PGF-007 antibody (Figure 2, F and G).

Discussion

In this study, we have used well characterized antisera and probes for mRNA to localize both production and expression of PDGF-A and -B chains in the intimal proliferative lesions of acute and chronic renal vascular rejection. Previously, each of these growth factors has been shown to be localized to intimal proliferative lesions in experimental vascular

Figure 2. *PDGF-B chain in rejecting arteries.* A: *Acute vascular rejection. Artery with subendothelial intimal inflammatory infiltrate characteristic of acute rejection (I). A subpopulation of infiltrating cells (approximately one-fourth to one-third of the infiltrating leukocytes) expresses PDGF-B chain, as labeled in black. None of the infiltrating cells were found to express PDGF-A chain when an adjacent histological section was examined. L, lumen; M, media.* B: *Small muscular artery with chronic active rejection demonstrates a population of cells scattered throughout the vessel wall that express PDGF-B chain, labeled in black.* C: *Serial section of same artery as* B. *Most of the PDGF-B chain-expressing cells seen in* B *are likely to be CD68-expressing monocyte/macrophages, which have an identical distribution within the artery as identified by immunohistochemistry in this section.* D: *Serial section of same artery in* B *and* C *showing expression of PDGF-A chain. PDGF-A chain expression is widely divergent from PDGF-B chain, and appears to be expressed principally by endothelial cells and smooth muscle cells in both the intima and media, and perhaps some monocyte/macrophages.* E: *Serial section of same artery in* B, C, *and* D *showing expression of α-smooth muscle actin, demonstrating that some of the PDGF-A chain-expressing cells in* D *are vascular smooth muscle cells.* F: *Demonstration of specificity of the immunohistochemistry procedure. Artery with chronic rejection shows scattered intimal cells labeled for PDGF-B chain expression.* G:. *Serial section of same artery in* F *in which the antisera to PDGF-B chain has been pre-absorbed with PDGF-B chain peptide. All immunolabeling has been abolished.*

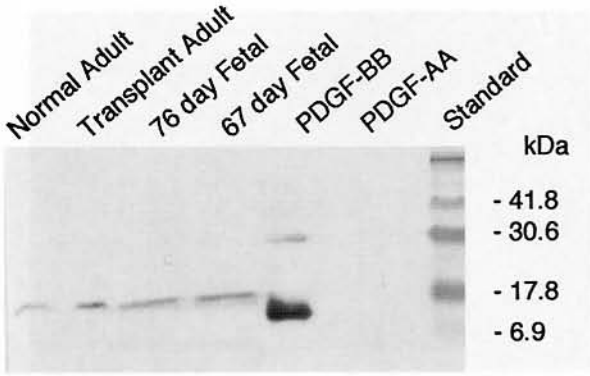

Figure 4. *Western blot showing presence of PDGF-B chain in normal, rejecting, and fetal kidneys, with bands identified at approximately 17 kd. The antibody recognizes monomeric and dimeric purified PDGF-BB peptide run under reducing conditions but is unreactive with PDGF-AA.*

injury; a role for PDGF-B chain in particular in the genesis of the experimental lesions appears well established.[7,8,29,30] There is a limited literature in experimental heart transplantation to suggest up-regulated expression of PDGF β-receptor is involved in chronic vascular injury,[31] and studies in a rat model of aortic transplantation also implicate up-regulated expression of PDGF-B chain and PDGF β-receptor in the chronic vascular rejection observed in that model.[2] This study is the first to demonstrate that at both the mRNA and protein product levels, both PDGF-A and -B chains can be localized to analogous intimal proliferative lesions that characterize chronic renal vascular rejection in humans.

Extensive studies of the activities of PDGF-B chain in the balloon injury model of neointimal development in the rat and in other species have indicated that this factor functions as a weak smooth muscle cell mitogen *in vivo* but that it is an important agent for promoting the smooth muscle cell migration from the arterial media that forms the cellular substrate of the neointima created as a response to injury.[7,8,29] Complementary studies of mesenchymal and smooth muscle cells *in vitro* also point to important chemotactic activity mediated by PDGF-B chain and its receptor.[32–35] PDGF-B chain has also been identified in lesions of human atherosclerosis,[14,36] where it is most prominently expressed within macrophages.[14] It has been speculated, although not proven, that the PDGF-B chain present in human atherosclerosis plays a role similar to that observed in experimental balloon injury. There is also some evidence that PDGF-B chain production by vascular smooth muscle cells may also occur in the course of intimal lesion development, as demonstrated in the neointima created by placement of synthetic vascular grafts in the baboon, and that the PDGF-B chain produced locally by smooth muscle cells may also contribute to lesion development by both autocrine and paracrine pathways.[30] On the basis of such evidence, we have speculated that PDGF-B chain is important in mediating the processes of smooth muscle cell accumulation that characterize the neointima of human solid organ chronic vascular rejection.

In support of this hypothesis, previous studies by Fellström, et al., have demonstrated that a receptor for PDGF-B chain, the PDGF β-receptor, is up-regulated in renal arteries demonstrating chronic vascular rejection.[5] This receptor is ordinarily not detectable by immunocytochemical techniques in the walls of human renal arteries.[5,37] The present study demonstrates that PDGF-B chain, the only known ligand for this receptor, is present in both the early and actively inflamed chronic rejection lesions, primarily in monocyte/macrophages, akin to what has been reported in human atherosclerosis.[14] By *in situ* hybridization, we demonstrate that it is likely that at least some of the PDGF-B chain accumulating in these lesions is actively synthesized by infiltrating macrophages. We also show that, whereas PDGF-B chain in these lesions is expressed primarily by cells of monocyte/macrophage origin, some of the smooth muscle cells present, both in the arterial media and neointima, also produce PDGF-B chain similar to what has previously been reported in the baboon synthetic graft model of vascular injury.[30] In demonstrating the presence of both PDGF-B chain and a relevant receptor in the human lesion, the first step in establishing the hypothesis that PDGF-B chain mediates the neointimal accumulation of smooth muscle cells characteristic of chronic vascular rejection has been achieved.

This study also established that PDGF-A chain is widely present in renal vascular rejection, albeit in patterns distinct from that observed for PDGF-B chain. It has previously been noted in developing human renal arteries, in human renal arteriosclerosis unassociated with transplantation, and in human atherosclerosis that PDGF-A chain is widely expressed by the vascular smooth muscle cells of both the intima and media of human arteries.[12,13,15] This pattern is maintained in rejecting arteries. Double immunolabeling confirmed that α-actin-expressing smooth muscle cells are the predominant cell type expressing PDGF-A chain in these lesions. Although expression by other cell types, in particular macrophages, cannot be excluded, it is clear from these double immunolabeling studies that such cells could account for only a small minority of the PDGF-A-

chain-producing cells within the wall of rejecting arteries.

However, a striking finding of this study was that PDGF-A chain expression by vascular endothelial cells could be identified in arteries undergoing acute rejection. This finding, not previously observed in other human vasculopathies, was also not identified in other forms of transplant pathology, including cyclosporine toxicity and interstitial cellular rejection without a vascular component. The single exception to this pattern, in which arterial endothelial expression of PDGF-A chain was observed in a biopsy obtained only to conform to a clinical protocol, preceded a clinically diagnosed episode of rejection occurring within 6 days of this biopsy. Although requiring more extensive clinical study before it can be considered established, this finding suggests endothelial expression of PDGF-A chain might be a useful diagnostic or prognostic marker for rejection in the management of allograft recipients, and it clearly establishes PDGF-A chain as a marker of injured or activated endothelial cells in this setting. We are unable to determine whether this up-regulated expression of PDGF-A chain by endothelial cells is critical to initiation, progression, or resolution of this form of acute vascular injury. In particular, our understanding of the significance of PDGF-A chain expression in any of the vasculopathies in which it has been observed, eg, arteriosclerosis and atherosclerosis, is hindered by our current inability to localize the PDGF α-receptor, the only receptor known to bind PDGF-A chain in human morphological lesions *in situ*. Consequently, we remain unable to determine the exact site of activity of the observed PDGF-A chain expression occurring in rejection. Activities of PDGF-A chain as determined by studies *in vitro* that could be important in the progression of vascular rejection include promotion of angiogenesis,[38] mitogenesis,[12] or some kind of nonmitogenic trophic function as implied by the widespread constitutive expression of this molecule by smooth muscle cells in human arteries.[13,15] PDGF-A chain may also be an intermediate that modulates some of the effects on smooth muscle cells produced indirectly by other cytokines, such as angiotensin II,[39] transforming growht factor-β,[40] and interleukin-1.[41] However, there is no body of evidence equivalent to that available for PDGF-B chain that would currently allow us to imply a specific functional role for PDGF-A chain in the pathogenesis of chronic vascular rejection.

In one model of denuding endothelial injury in the rat carotid artery and aorta, PDGF-A and PDGF-B chains have been shown to be expressed by endothelium.[42] In this study, PDGF-A and -B protein and mRNA were found to be expressed only in injured endothelium of the large vessels. Although this model is not ideal for the study of acute vascular rejection, this particular study provides an additional demonstration that injured endothelial cells are capable of PDGF-A and -B chain synthesis *in vivo*.

Little is known about other growth factor mediators of chronic vascular rejection involving solid organ allografts. There is preliminary evidence that basic fibroblast growth factor, probably arising primarily from infiltrating leukocytes, may also participate in the evolution of vascular intimal proliferative lesions, particularly those of vascular rejection.[43] Other factors, which largely remain unidentified, are also likely to play a significant role in the evolution of this form of injury. Based on the complexity of growth factor and cytokine interactions observed in human atherosclerosis and other examples of arterial intimal proliferative injury,[44] it is highly unlikely that chronic vascular rejection will be explainable by the activity of only a few such molecules. Nonetheless, the identification of PDGF-A and -B chains in human lesions provides substantial evidence that these molecules are likely to play a significant pathophysiological role in the evolution of vascular rejection, which in the case of PDGF-B chain is most likely the result of its activity in promoting smooth muscle cell migration into the intima. Although other specific functions of these factors in this site in human lesions remain unknown, we believe that identification of the participants in this form of injury will lead to an understanding of progressive vascular injury in solid organ transplants.

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