Expression of the Angiogenic Protein, Platelet-Derived Endothelial Cell Growth Factor, in Coronary Atherosclerotic Plaques

In Vivo Correlation of Lesional Microvessel Density and Constrictive Vascular Remodeling


Abstract—Recent information indicates that platelet-derived endothelial cell growth factor (PD-ECGF), a 45-kDa angiogenic protein, is expressed in the endothelium of various tissues and that its level of expression is correlated with the number of microvessels in human tumors. Because the formation of neovessels is also thought to play a role in atherosclerotic vascular remodeling, we analyzed PD-ECGF expression in fresh, coronary plaque tissues obtained by directional coronary atherectomy. Specimens from 31 patients were collected and analyzed by reverse transcription–polymerase chain reaction, histochemical staining, immunohistochemistry, and in situ hybridization with the use of PD-ECGF–specific primers and probes. Lesional vascular remodeling was assessed by intravascular ultrasound. PD-ECGF immunoreactivity and mRNA were found in plaque macrophages, endothelial cells of plaque neovessels, and stellate smooth muscle cells of 20 atherectomy specimens (64.5%). PD-ECGF immunoreactivity was correlated with the number of lesional microvessels and mast cells. Double-staining experiments revealed a close spatial proximity of PD-ECGF–positive cells and mast cells. Furthermore, the numbers of microvessels and mast cells were significantly higher in lesions lacking compensatory enlargement. The data indicate that PD-ECGF is expressed within cells of the atherosclerotic plaque and may be involved in driving angiogenesis in concert with mast cells. (Arterioscler Thromb Vasc Biol. 1999;19:2340-2347.)

Key Words: atherosclerosis ■ angiogenesis ■ endothelium ■ platelet-derived factors ■ growth substances

Atherosclerosis is the principal cause of heart attack, stroke, and peripheral vascular disease and is responsible for 50% of all mortality in the United States, Europe, and Japan.1 The vascular lesions result from an excessive, inflammatory-fibroproliferative response of the endothelium and smooth muscle cells (SMCs) of the arterial wall to various forms of insult.1 The inner parts of the walls of large blood vessels normally do not contain intrinsic vasculature. In conditions such as atherosclerosis or thrombosis, intimal angiogenesis occurs as part of an adaptive change recently addressed as vascular remodeling.2 Histopathologic studies3,4 and intracoronary ultrasound imaging have shown that human coronary arteries enlarge parallel to the formation of atherosclerotic plaque and preserve the lumen area until plaque volume exceeds the compensatory mechanism.5 One current hypothesis of neovascularization in atherosclerosis is that the new, small vessels arise from adventitial vasa vasmorum.6 Several factors that stimulate the proliferation of endothelial cells (ECs) in vitro have been shown to induce angiogenesis in vivo7 and include wide-spectrum multifunctional mitogens (eg, the fibroblast growth factors) as well as factors with distinct specificities for vascular ECs (eg, platelet-derived endothelial cell growth factor [PD-ECGF]). The expression of fibroblast growth factors, PDGFs AA and BB, and their receptors has been investigated in atherectomy tissue and has been correlated with lesion activity.8–10 However, the role of lesional angiogenesis has remained unclear.

PD-ECGF is a 45-kDa nonglycosylated, single-chain polypeptide that stimulates growth and chemotaxis of ECs in vitro and of angiogenesis in vivo11 and has been shown to catalyze the reversible phosphorylation of thymidine to deoxyribose-1-phosphate and thymine, thus reducing thymidine levels that would otherwise be inhibitory to EC growth. Although PD-ECGF has not been detected in the normal aorta12 or myocardium,13 PD-ECGF has been recognized within various human tumors and tumor cell lines,14 as well as in mononuclear cells,15 macrophages, keratinocytes, glial cells, and epithelial cells and within the endothelium of...
the breast, brain, and placenta. Despite the reported lack of PD-ECGF in the cardiovascular system, experiments were designed to examine PD-ECGF expression in coronary atherosclerosis and its relationship to vascular remodeling at the lesion site.

**Methods**

**Patient Characteristics**

Coronary athereectomy specimens were obtained from 31 consecutive patients (the Table; mean ± SD age 58.2 ± 8.8 years; range 41 to 74 years) undergoing directional coronary atherectomy (DCA) at the University of Vienna General Hospital between 1995 and 1997. All specimens were derived from primary, single lesions in the left anterior descending coronary artery (n = 10, 32.2%), circumflex coronary artery (n = 5, 16.1%), or right coronary artery (n = 16, 51.6%). Ischemia in the target vessel–dependent myocardial area was documented by exercise stress test and 201Tl myocardial scintigrams. At the time of the intervention, all patients were in clinically stable Canadian Cardiovascular Society class 0 through 4 (the Table). Conventional coronary angiography and quantitative coronary angiography were performed before and after DCA (mean preprocedural target stenosis by quantitative coronary angiography was 67.6 ± 11.3%). The intravascular ultrasound imaging system consisted of an imaging catheter (Ultracross 2.9F), carrying a 30-MHz transducer, and an intravascular ultrasound imaging console (Clear View Ultra, Boston Scientific Corp). Before DCA, the imaging catheter was introduced over a 0.014-in. coronary guiding catheter, and coaxiality of the imaging catheter in the region of interest was controlled by x-ray fluoroscopy. Imaging was performed by automated pullback at 0.5 mm/s against the direction of blood flow, which is indicated by the arrows in Figure 1. To obtain a numerical measure for vascular remodeling, vessel cross-sectional area in the stenotic segment was subtracted from the average vessel cross-sectional area at the proximal and distal reference segments.

### Summary of Patient Characteristics and Quantitative Tissue Analyses

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CCS indicates Canadian Cardiovascular Society; QCA, percent stenosis at the lesion site of the proximal reference segment by quantitative coronary angiography; Vessel, vessel involved; Remod, category of vascular remodeling as assessed by intravascular ultrasound; PD-EGGF, immunoreactivity status by use of a rabbit antibody and a monoclonal antibody against PD-EGGF; Vessels, number of microvessels per mm²; Mast Cells, number of mast cells per mm² counted at ×200 magnification. Total Thrombus, area of lesional thrombus as a percent of total specimen area; Total Specimen Area, mean total specimen area in mm² calculated from 3 nonparallel sections; Neg, negative; Pos, positive; RCA, right coronary artery; LAD, left anterior descending coronary artery; and CX, circumflex artery.
and divided by the average vessel cross-sectional area at the proximal and distal reference segments. Compensatory enlargement (delineated “Pos” in the Table) was considered to be present when the vessel cross-sectional area at the lesion site was larger than that at the reference site (Figure 1A), ie, when the ratio was $>0$. Inadequate compensatory enlargement (delineated “Neg” in the Table) was considered to be present when the vessel cross-sectional area at the lesion site was smaller than that at the reference site (Figure 1B), ie, when the ratio was $\leq 0$. The study was approved by the investigational review board of the University of Vienna, Austria.

**Tissue Processing**

DCA specimens were flushed from the instrument chamber with saline solution and divided into 2 equivalent parts. The first half was immediately immersed in LN$_2$, and the second half was fixed in 7.5% buffered formalin for 24 hours. Normal transplant-donor coronary arteries and atherosclerotic arteries from heart transplant recipients served as controls. Formalin-fixed tissues were processed with the Miles Scientific Tissue-Tek VIP (Schoeller Pharma) and embedded in paraffin. Serial 3-$\mu$m sections were stained with hematoxylin and eosin to assess general histology. A modified trichrome stain was performed for collagen and fibrin localization. Thrombus was identified by the presence of inflammatory cells, erythrocytes, and aggregated platelets within a fibrin meshwork and strands of proliferating fibroblasts, whereas the smooth, extracellular distribution and red-purple color in the modified trichrome stain identified fibrin. Vessel medial and adventitial tissues were retrieved from 12 and 10 patient samples, respectively (38.7% and 32.2%), which is in good agreement with published reports.

**Immunohistochemistry**

The indirect avidin-biotin–horseradish peroxidase method was used. Bound antibodies were detected with a biotinylated goat anti-mouse or anti-rabbit secondary antibody (Zymed) and aminoethylcarbazole as the substrate reagent. Monospecific IgG1 antibodies and a preimmune rabbit antiserum were used as controls for monoclonal and polyclonal antibodies, respectively. Breast cancer tissue was used for antibody optimization. A protein A–Sepharose–purified rabbit polyclonal antiserum against PD-ECGF (used at a 1:200 dilution in 0.1% normal goat serum) kindly provided by Dr Carl Henrik Heldin, Ludwig Institute for Cancer Research Biomedical Center, Uppsala, Sweden, was used. Because nonspecific background staining was obtained with this antibody, the data were confirmed with a mouse monoclonal anti–PD-ECGF antibody (10 $\mu$g/mL, thymidine phosphorylase/PD-ECGF/gliostatin Ab-1 clone P-GF.44C; Neomarkers, Union City, Calif). Furthermore, mouse monoclonal antibody against CD68 (16 $\mu$g/mL; clone KP1, DAKO, Glostrup, Denmark), mouse monoclonal antibody against SM $\alpha$-actin (clone 1A4, DAKO, used at 10 $\mu$g/mL), mouse monoclonal antibody against von Willebrand factor (clone F8/86, DAKO, used at a 1:150 dilution), and mouse monoclonal antibody against mast cell tryptase (MAB122, Chemicon International Inc, Temecula, Calif, used at 10 $\mu$g/mL) were used.

Immunohistochemical double staining served to colocalize PD-ECGF–expressing cells and mast cells. For this purpose, anti–PD-ECGF was used in the first step with alkaline phosphatase and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt as the color substrate (Zymed). Anti-tryptase was used in the second step with aminoethylcarbazole. Gill’s hematoxylin was used as the counterstain.
Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Tissues frozen in LN_2 were thawed in guanidine thiocyanate followed by total RNA extraction according to the method of Chomczynski and Sacchi. RNA (1 μg) was reverse-transcribed (Boehringer Mannheim Co) with oligo-(dT)_15 primers. The cDNA was amplified by using PD-ECGF–specific primers, forward PD-ECGF primer 5′-gcctgagcgaagcggacatc-3′ and reverse PD-ECGF primer 5′-catctgctctgggctctgga-3′, resulting in a 377-bp product (fragment between nucleotides 143 to 519 of PD-ECGF cDNA) that was analyzed on ethidium bromide–stained agarose gels.

Riboprobe Preparation

A full-length PD-ECGF cDNA (kindly provided by Dr C.H. Heldin, Ludwig Institute for Cancer Research Biomedical Center, Uppsala, Sweden) was cut with EcoRI-I and cloned into pGEM-3Z (Promega, Madison, Wis), yielding a 1363-bp template. Sense and antisense riboprobes were prepared with digoxigenin-labeled UTP (Promega, Madison, Wis), yielding a 1363-bp template. Sense and antisense riboprobes were prepared with digoxigenin-labeled UTP (Promega, Madison, Wis), yielding a 1363-bp template.

In Situ Hybridization

Nonradioactive in situ hybridization was performed on 3- to 5-μm paraffin sections. In brief, sections were mounted onto Superfrost/plus slides (Fisher) and dried at 60°C overnight to improve adhesion. After deparaffinization in xylene and rehydration in graded alcohols, paraffin sections. In brief, sections were mounted onto Superfrost/plus slides (Fisher) and dried at 60°C overnight to improve adhesion. After deparaffinization in xylene and rehydration in graded alcohols, the slides were fixed in 4% paraformaldehyde. The slides were washed in 0.5× SSC (one 10-minute wash; 1× SSC is 150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0) and treated with protease K solution (20 μg/mL for 20 minutes at room temperature). The slides were incubated with prehybridization solution [50% (wt/vol) formamide, 0.3 mol/L NaCl, and 20 mmol/L Tris-HCl (pH 8.0; 5 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficol, 0.02% BSA, 10% (wt/vol) dextran sulfate, and 10 mmol/L DTT] for 1 hour at 42°C in a humidified chamber. Hybridizations were started by adding 0.5 μg/mL digoxigenin-labeled riboprobe in 20 μL of prehybridization buffer containing 2.5 mg/mL tRNA (48°C, overnight). The next day, the slides were incubated with RNase A for 30 minutes and washed for 2 hours in 0.1× SSC and 50% formamide at 48°C, followed by incubation with 10% goat serum and an alkaline phosphatase–labeled anti-digoxigenin Fab fragment (Boehringer Mannheim, 1:1000 in 0.1% goat serum and Tris-buffered saline at room temperature). Signal was developed using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt as the substrate and Gill’s hematoxylin as the counterstain. Parallel sections were analyzed by using a sense probe as the control for nonspecific hybridization.

Computer-Assisted, Quantitative Histological Evaluation of Atherectomy Specimens

All atherectomy specimens were digitized at full size by using a slide scanner (Nikon 6.0 35-mm-film scanner, LS-20, Nikon Corp). Images were processed and the color contrast enhanced with the Adobe Photoshop 3.0 software package (Adobe Systems Inc). Measurement of thrombus area expressed as percent of total area was performed on trichrome-stained specimens by computer-based planimetry (National Institutes of Health Image 1.61/ppc; the Table). Athersclerotic “gruel” in the center of the lesion is indicated by the asterisk (bars represent 10 μm; ×100 magnification).
identities manually counted the number of lesional mast cells, small vessels, and capillaries. The numbers were divided by the respective specimen area in millimeters squared.

**Statistical Evaluation of Data**

ANOVA and correlation analysis were used. A value of $P<0.05$ was considered statistically significant.

**Results**

**Histological Characterization of Lesions**

The primary, single lesions encountered in the present study qualified as complicated lesions according to Stary et al., with moderate inflammation and variable amounts of thrombus, fibrin, macrophages, extracellular lipid deposits, and fibrosis (example shown in Figure 3a). The majority of patient samples (83.8%) contained thrombus. Fibrin was present in 24 specimens (77.4%). Overall there were only 2 specimens devoid of both thrombus and fibrin.

**Identification of PD-ECGF in Coronary Atherosclerotic Plaques**

We used RT-PCR as a sensitive screening assay to determine whether PD-ECGF was expressed in atherosclerotic coronary vessels and coronary atherectomy specimens. By using PD-ECGF–specific primers, a 377-bp band (positive control in Figure 2; lane denoted +) was identified in 13 of 20 atherectomy samples. To verify the specificity of the reaction, bands of the respective size were isolated and the PD-ECGF sequence confirmed by sequence analysis. To ensure the success of the RT reaction, control experiments were performed with primers specific for glyceraldehyde-3-phosphate dehydrogenase (data not shown).

**Identification of PD-ECGF–Expressing Cells in Coronary Atherosclerotic Lesions From Patients With Severe Coronary Heart Disease and in Intimal-Medial Cuts Obtained During DCA**

Light microscopic analysis of sections of whole atherosclerotic coronary arteries revealed PD-ECGF mRNA predominantly within microvessels of the plaque shoulder (Figure 3, panels a through c). As a first step, a more thorough analysis of microvessels within atherectomy specimens (Figure 4a) was performed by probing parallel sections with monoclonal antibodies directed against von Willebrand factor (Figure 4b), antisense PD-ECGF (Figure 4c), monoclonal anti–PD-ECGF (Figure 4d), sense PD-ECGF (Figure 4e), and isotype control antibody (Figure 4f). The data demonstrated PD-ECGF mRNA and immunoreactivity within ECs of intimal neovessels. To examine a PD-ECGF–dependent mechanism of angiogenesis involving tissue mast cells (tryptase stain of a parallel section, Figure 4g), double-staining experiments were performed with the use of antibodies directed against PD-ECGF, followed by anti–tryptase. These experiments confirmed colocalization of PD-ECGF–positive ECs and tryptase-positive cells (Figure 4h).

Second, PD-ECGF signal was detected in plaque macrophages (Figure 3d) compared with the sense control (Figure 3e). A thorough analysis of parallel sections of an atherectomy specimen (shown in Figure 4a) with antisense PD-ECGF (Figure 4i), monoclonal anti–PD-ECGF (Figure 4j), CD68 (Figure 4k), and a monospecific IgG control (Figure 4l) confirmed PD-ECGF–positive cells of the monocyte-macrophage lineage. Parallel analysis of areas with stellate SMCs (Figure 4m) revealed PD-ECGF immunoreactivity (Figure 4o) within SM α-actin–positive cells (Figure 4n), whereas anti–von Willebrand factor staining was negative in these areas (Figure 4p). PD-ECGF mRNA was lacking in medial SMCs of the vessel wall (Figure 3b) but was present in stellate SMCs of neointimal areas (data not shown).

**Statistical Evaluation of PD-ECGF Immunoreactivity Status and Clinical as Well as Morphometric Data**

There was a trend toward greater mass of material retrieved from lesions undergoing compensatory enlargement ($P=0.06$; cf specimen areas in the Table). Staining of 31 consecutive atherectomy specimens demonstrated PD-ECGF immunoreactivity in 21 specimens (the Table). Although all patients were in a stable Canadian Cardiovascular Society class at the time of the intervention in this study, acute coronary syndromes had occurred in 11 patients within 1 and 21 days before DCA (patients identified by bold type in the Table). Statistical analysis of the clinical data in relation to the tissue analysis revealed that there was a significantly more severe angina score in patients from whom specimens with positive PD-ECGF immunoreactivity status had been recovered ($P=0.02$, the Table). Samples with positive PD-ECGF immunoreactivity status had more plaque neovessels ($P=0.03$) than did samples with a negative PD-ECGF immunoreactivity status. Furthermore, mast cell counts were correlated with the number of lesional neovessels ($r=0.78$, $P<0.05$), and mast cell counts per square millimeter of specimen area were higher in specimens with a positive PD-ECGF immunoreactivity status ($P=0.007$, the Table). Mast cells were absent in specimens where PD-ECGF immunoreactivity was absent (the Table). No relationship was found between minimal luminal diameter as assessed by quantitative coronary angiography and PD-ECGF immunoreactivity status. On the basis of data suggesting an interrelationship between vascular thrombus and mast cell recruitment, further analyses were directed at the statistical correlation of thrombus and mast cell recruitment.
correlation of atherectomy thrombus and number of mast cells. Figure 4a represents a typical coronary atherectomy specimen with ≤10% thrombus (4.53 ± 3.49%); mean mast cell counts 11.96 ± 16.6, n = 26. In 5 specimens, relative thrombus area was >10% (34.97 ± 18.57%), and the mean mast cell count was 11.14 ± 8.56. No statistical relationship between mast cell count per square millimeter of area and thrombus area was found.

Analysis of Neovessel Density in Relation to Vascular Remodeling in Atherectomy Specimens

To elucidate the relevance of lesional expression of the EC mitogen PD-ECGF, the numbers of lesional microvessels per square millimeter of specimen area were statistically related to vascular remodeling as assessed by intravascular ultrasound before specimen retrieval (the Table). Numbers of both lesional microvessels and mast cells were statistically higher in vessels demonstrating a lack of compensatory enlargement (P = 0.02 and P = 0.04, respectively).

Discussion

The present study demonstrates expression of the potent EC-specific angiogenesis stimulator PD-ECGF/thymidine phosphorylase in coronary atherosclerosis. The data show that microvessels of the plaque shoulder are predominant sites for PD-ECGF expression. The data further imply that the number of microvessels is a critical determinant of vascular remodeling.

It has been recognized that rupture-related plaque progression due to luminal thrombosis and plaque hemorrhage through fragile, newly formed vessels at the base of advanced plaques are important mechanisms underlying acute coronary syndromes. Recent studies using coronary angiography have indicated that it takes at least 1 month after an ischemic coronary event for target lesions to heal. Therefore, the degree of PD-ECGF and growth factor expression in coronary plaques may be correlated histologically with both the active plaque causing an ischemic coronary syndrome and the repair phase of the individual lesion. As in various tumors, a significant correlation of PD-ECGF immunoreactivity and microvessel density was observed in coronary atherosclerosis. However, the small area that is cut by the DCA catheter may not reflect the biology of the whole vessel segment. Because of intrinsic differences in cut lesions undergoing compensatory enlargement and lesions undergoing shrinkage, there would also not be the same degree of sampling error applicable to both extremes of vascular remodeling. Therefore, the data correlating microvessel numbers and vascular remodeling must be evaluated with caution. Nevertheless, a positive correlation between high numbers of lesional microvessels and PD-ECGF immunoreactivity and between PD-ECGF immunoactivity and angina score suggests a positive relationship between the expression of angiogenic factors, the degree of neovascularization, and lesion severity. These data are in accord with studies correlating clinical scores, neovascularization, and the expression of PDGF-AA, -AB, their receptors, and acidic and basic fibroblast growth factors. In contrast, recent studies of vascular endothelial growth factor expression in SMCs of atherosclerotic coronary arteries have failed to demonstrate a correlation between vascular endothelial growth factor immuno-staining and the extent of vasa vasorum, raising speculations about a possible EC repair effect of this growth factor. Although evidence for this function is still lacking, PD-ECGF has been the subject of similar hypotheses. Like vascular endothelial growth factor, regulation of PD-ECGF expression in atherosclerosis may be mediated by hypoxia.

One further finding was the colocalization of PD-ECGF–positive ECs and mast cells (Figure 4h). Tissue mast cells are generally concentrated around small blood vessels and lymphatics and newly forming microvasculature, and they accumulate at the site of an atheromatous erosion or rupture, eg, in the plaque shoulder. Previous in vitro data have suggested a recruiting function of endothelial PD-ECGF on mast cells, and heparin released from these cells may stimulate capillary EC migration. Increased numbers of mast cells have been found in auricular thrombosis, suggesting attraction of mast cells through components within the thrombus. However, in our study mast cell numbers were independent of thrombus (the Table).

The present study demonstrates that neovascularization is associated with shrinkage of the diseased vessel segment. As an explanation, neovessel fragility could entail intramural bleeding with subsequent scarring. On the other hand, organizing neovascularization could follow thrombosis, secondary to the loss of cross-sectional and luminal vessel areas. Furthermore, mast cell products, eg, serotonin, and platelet products, eg, serotonin and transforming growth factor-β, could mediate vasoconstriction and vascular shrinkage by promoting perivascular fibrosis. Because the lack of compensatory enlargement is an important contributing factor for the loss of luminal diameter in about one-fourth of primary coronary lesions, the data suggest that lesional neovessels promote vascular occlusions. Angiogenic growth factors in atherosclerosis, eg, PD-ECGF, are involved in the process of vascular remodeling and could be targets for therapeutic inhibition to prevent vessel shrinkage.

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References


Expression of the Angiogenic Protein, Platelet-Derived Endothelial Cell Growth Factor, in Coronary Atherosclerotic Plaques: In Vivo Correlation of Lesional Microvessel Density and Constrictive Vascular Remodeling

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