Cytoplasmic Expression and Extracellular Deposition of an Antiangiogenic Factor, Pigment Epithelium-Derived Factor, in Human Atherosclerotic Plaques

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Objective—To assess the expression and distribution of a neurotrophic/antiangiogenic factor, pigment epithelium-derived factor (PEDF), related to angiogenesis that is a possibly key event during atherogenesis in human atherosclerotic plaques.

Methods and Results—Twenty fresh aortic samples were used for reverse-transcription polymerase chain reaction (RT-PCR), Western blot, and immunohistochemistry (IHC). In addition, 80 stocked paraffin blocks of coronary arteries from 40 autopsy cases were also used. IHC revealed divergent staining patterns for PEDF in both the aortas and the coronary arteries tested, ie, “cytoplasmic staining” or “extracellular deposition,” were observed, respectively. In the areas showing cytoplasmic staining, double PEDF was expressed in a majority of the foamy macrophages and in some smooth muscle cells, and the PEDF-positive cell frequency was positively correlated with that of microvessels in a cell-rich area in the coronary arteries ($P<0.0001$). Inversely, extracellular deposition of PEDF was seen in acellular areas and was negatively correlated with the number of microvessels ($P=0.0003$).

Conclusions—These results suggest that PEDF may function as an antiangiogenic factor when it is deposited onto the extracellular matrix. Thus, PEDF may play a significant role in determining the balance of angiogenesis/antiangiogenesis during atherogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: angiogenesis, aorta, atherosclerosis, coronary artery, immunohistochemistry, pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF), a neurotrophic/antiangiogenic factor that is a secreted glycoprotein deposited onto the photoreceptor matrix and a member of the serpin superfamily without any activity of serine proteases, was identified in the conditioned medium of cultured fetal retinal pigment epithelial cells. In addition to its neurotrophic activity, PEDF also exerts an alternative function, “antiangiogenesis,” to maintain the avascular condition of the corneal tissue. Despite its known antiangiogenic activity in vivo, there is ultimately little information regarding the pathophysiological role of PEDF in angiogenesis-related diseases and its extracellular expression.

Recent evidence has strongly suggested that atherosclerosis is an angiogenic disease. Neovascularization is frequently seen in human coronary arteries and has been suggested to significantly contribute to plaque progression. Using coronary arteries from autopsy cases, we previously demonstrated that intimal neovascularization was closely associated with the extent of coronary atherosclerosis and histological inflammatory reactions. As a key substance contributing to coronary angiogenesis in human subjects, we and others suggested vascular endothelial growth factor (VEGF)-A; this suggestion was further supported by results indicating the existence of positive correlations among the following factors: the American Hearth Association (AHA) grade of atherosclerosis, the number of intimal neovessels, and the frequency of VEGF-A–positive cells. Our experimental studies have also supported the significant contribution of VEGF-A in this regard, ie, VEGF165 gene transfer into rabbit carotid arteries induced not only the angiomatoid proliferation of endothelial cells forming irregular vascular channels but also intimal hyperplasia. The other group also demonstrated that the administration of VEGF-A protein resulted in enhanced atherosclerosis in rabbits. However, there is only scant information regarding angiogenic inhibitors in cases involving human atherosclerotic lesions.
For this reason, we investigated the expression of PEDF in fresh human aortic tissue obtained from autopsy cases. Furthermore, we also retrospectively assessed the expression and distribution of PEDF with reference to neovascularization in human coronary arteries using immunohistochemical analysis.

Materials and Methods

Fresh Aortic Samples
From May 2002 to June 2003, 2 tissue samples from the descending aorta (1 sample from grossly an atheromatous lesion and 1 sample from grossly nonatherosclerotic tissue) were obtained from each of 20 autopsy cases (age range, 33 to 96 years; male versus female ratio, 14:6) within 10 hours after death. Each sample was sectioned into 3 pieces and subjected to reverse-transcript polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemistry (IHC). On histological examination, all grossly nonatherosclerotic regions were found to exhibit diffuse intimal thickening (DIT). Atheromatous lesions were microscopically classified as being type III, IV, or V, as proposed by the committee of the American Heart Association9 (see Table I, available online at http://atvb.ahajournals.org).

Stock Samples of Human Coronary Arteries
For a retrospective analysis conducted to assess the relationship between PEDF expression and intimal neovessels, 80 stock samples of coronary artery from 40 individuals (age range, 40 to 93; mean, 71.4 years old; male versus female ratio, 24:16) harvested at autopsy from 1994 to 1996 were used for IHC. Histological grading for the AHA classification of 80 tissue samples was performed by 2 independent pathologists (H.B. and Y.Y.) as follows: DIT, I, 10; II, 11; III, 10; IV, 16; V, 8; and VI, 8. No case of death with acute coronary syndrome was involved.

RT-PCR
Nonatherosclerotic aortic segments with DIT (n = 20) and segments containing atheromatous plaques (n = 20) were used for RT-PCR. Segments were selected that were directly adjacent to those used for Western blotting and IHC. The RNAs were isolated using ISOGEN (Nippon Gene, Inc, Tokyo, Japan). cDNA was synthesized from the total RNA, and was subjected to PCR to detect PEDF mRNA (amplicon size, 600 bp; denaturing for 30 seconds at 95°C, annealing for 30 seconds at 65°C, extension for 1 minute at 72°C for 30 cycles). The following primer sequences were used: 5'-CCCATCGAGGATTTCTACTTGG-3' (forward) and 5'-CTTACGTGAGGCCCTGGGGTCCA-3' (reverse). β-Actin was simultaneously amplified as an internal control using primer pairs (amplicon size: 254 bp; forward: 5'-CCCATCGAGGATTTCTACTTGG-3' and reverse: 5'-CTTACGTGAGGCCCTGGGGTCCA-3'). Pseudo-positive amplification caused by the contamination of genomic DNA was ruled out by the inclusion of simultaneous RT-PCR without RT.

Western Blotting
Each tissue sample harvested at autopsy was homogenized, and the supernatant was separated on a 10% SDS-PAGE, and the proteins were transblotted. After blocking using 3.0% nonfat dried milk, the membrane was reacted with a monoclonal mouse antibody against human PEDF (TransGenic Inc, Kumamoto, Japan). Immunoreactivity for PEDF was visualized using the ECL Plus (Amersham Biosciences, Buckinghamshire, UK). For the positive control of glycosylated PEDF, a culture medium of COS7 cells stably transfected with simian immunodeficiency virus-vector based lentiviral vector expressing human PEDF16 was used.

Single Staining Immunohistochemistry
Immunohistochemistry was performed using 4% paraformaldehyde-fixed, paraffin-embedded tissue with the following antibodies, according to the standard streptavidin-biotin complex technique: goat anti-human PEDF antibody (15 μg/mL) (R&D systems, Minneapolis, Minn), anti-CD68 (1:100; DAKO, Glostrup, Denmark), anti-CD34 (1:100; Novocasta, Newcastle, UK), and anti–smooth muscle cell actin (HHF35) (1:100; Enzo Life Science, New York, NY). Anti–single-stranded (ss) DNA (1:300; DAKO, Kyoto, Japan) was used as a reference for detecting apoptotic cells.11 Heat-induced epitope retrieval was performed by immersing sections of tissue in citrate buffer (pH 6.0), except for HHF35 and ss-DNA. Nonimmune IgG corresponding to each isotype was used as a negative control. Antigen absorption for anti-human PEDF antibody using excess recombinant PEDF (molar ratio, 10-fold; CHEMICON International, Inc, Temecula, Calif) was also performed in some cases.

Double Staining Immunohistochemistry
After the first color reaction was developed by using a DAKO LSAB+ System (DAKO), the secondary antibodies for the second antigen were applied in the same manner as that used for the first antigen, and the second color reaction was developed with a 3,3'-diaminobenzidine tetrahydrochloride-peroxidase (brown) or an Alkaline Phosphatase Substrate Kit III (blue) (Vector Laboratories, Inc, Burlingame, Calif). The sections were counterstained with hematoxylin, if necessary.

Statistical Analysis
The total surface area of the intima was classified into 3 different compartments (PEDF-positive cell-rich area, acellular area with or without PEDF deposition; no PEDF-negative cell-rich area was observed in our tissue sections), and the number of microvessels in each area was counted. The correlation between the PEDF-positive cell number and the number of coexisting microvessels was assayed by using Spearman rank correlation test. P < 0.01 was considered to be significant. Wilcoxon signed-rank test was used for evaluating the difference between the number of microvessels in the PEDF-deposited areas and nondeposited areas (excluding DIT). Planimetry was performed for each area by considering the minimum area (6.25×10⁻⁴ mm²) of a 10×10 grid lens (magnification ×40) and taking the sum of the minimum area values obtained.

Results

Expression and Deposition of PEDF in Fresh Aortic Tissue

Detection of PEDF mRNA and Protein
As summarized in Table 1, RT-PCR revealed that the atherosclerotic lesions were positive for PEDF mRNA in 10 of 20 cases (50%), and that nonatherosclerotic regions with DIT were also positive for PEDF mRNA in 8 of 20 cases (40%). However, all samples were positive for PEDF protein at the expected size of 50 kDa, irrespective of lesion type (20/20; 100%). Three of 20 cases (cases 2, 6, 12) of atherosclerotic and nonatherosclerotic lesions are shown as examples (Figure 1).

IHC for PEDF Protein in Aortic Tissue

The Retina as a Positive Control for PEDF
Non diseased region of human retinal tissues from eyes surgically harvested for retinoblastoma were used as positive controls for the IHC of PEDF (0-year-old, male). In this case, visualization by alkaline phosphatase for red was used to distinguish a positive reaction from the brown pigment of RPE cells. The reaction was primarily positive in the retinal pigment epithelial layer and in the inner/outer segments, but was rarely positive in the cytoplasm of other retinal cells (Figure 2a, middle, top and bottom). Antibody absorption using excess recombinant PEDF (Figure 2a, right, top and bottom) and use of nonimmune goat IgG instead of the
primary antibody (Figure 2a, left, top) showed negative reaction.

Nonatherosclerotic Aorta With Diffuse Intimal Thickening

Immunoreactivity of PEDF was diffusely and extracellularly positive in the intima and the media; moreover, the signal tended to be stronger in intima than in the media (Figure 1, available online at http://atvb.ahajournals.org). CD34-positive microvessels were rarely observed in DIT lesions (data not shown). The normal arteries without DIT (3 coronary arteries and 3 aortas from 20- to 25-week-old fetus, 4 splenic arteries from a child, and 3 young adults [3 to 22 years old]) revealed weakly diffuse deposition of PEDF in tunica media (data not shown) similar to that in DIT of aortas.

Atheromatous Plaque

Advanced atheromatous plaques, characterized by a distinct lipid core and fibrous cap, contained macrophages and smooth muscle cells. The cellular cytoplasm, but rarely extracellular area, was PEDF-positive in both the fibrous cap and the shoulder (Figure 2b, right, top and bottom). In contrast, PEDF immunoreactivity was diffusely observed extracellularly in the atheromatous core, irrespective of cell distribution (Figure 2b, left bottom).

Double IHC revealed that a number of PEDF-positive cells in the fibrous cap (Figure 3a, red), which largely corresponded to the distribution of CD68-positive monocytes/macrophages (Figure 3a, brown; doubly positive cells are indicated by arrows). Some HHF35-positive smooth muscle cells were also positive for PEDF (Figure 3b, brown; doubly positive cells were indicated by arrows).

Expression and Deposition of PEDF in Coronary Arteries and Correlation With Intimal Angiogenesis

Because of the relative infrequency of neovessels in the aorta, it is not suitable material for assessing angiogenesis in atherosclerotic plaques; therefore, we further investigated the expression and deposition of PEDF in human coronary arteries from autopsy cases with regard to intimal neovessels.

Similar to the patterns seen in the aortic tissue, 2 independent staining patterns, namely, cytoplasmic expression and extracellular deposition, were also observed in the atherosclerotic intima of human coronary arteries (Figures 4a and 5). The statistical analysis clearly revealed a positive correlation between the severity of the atherosclerotic lesion (AHA classification) and the PEDF-positive cell number in "cell-

Figure 1. Detection of PEDF mRNA and protein in human aortic samples. a, Examples of RT-PCR analysis of PEDF mRNA (600 bp) in atherosclerotic lesions (A) and grossly nonatherosclerotic lesions (histological DIT, N). β-actin (254 bp) was subsequently amplified as an internal control. RT-PCR was simultaneously performed without RT as a negative control (RT–). b, Examples of the Western blot analysis of PEDF protein, corresponding to each of the samples shown in (a). Recombinant PEDF protein secreted into the culture medium of COS7 cells stably transfected a simian lentiviral vector expressing human PEDF (PEDF-CM) used as a positive control.

Figure 2. Immunohistochemical detection of PEDF protein. a, Immunohistochemistry (IHC) for PEDF (red) in the human retina, used as a positive control. The bottom 2 panels are high-powered view of the boxed area shown in the corresponding upper panel, respectively. An intense reaction (red) for PEDF was seen in the inner/outer segments in the acellular zone containing rods and cones (anti-PEDF). No positive reaction was seen in the serial sections reacted with isotype-matched nonimmune antibody (murine IgG1, upper left), as well as with primary antibody absorbed by excess recombinant PEDF (right upper and bottom). Original magnification: upper panels, ×140; lower panels, ×140. INL indicates inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. All sections were counterstained with hematoxylin. b, Immunohistochemical detection of PEDF in an aortic sample with an atherosclerotic lesion (case 20). Three typical lesions, namely fibrous cap (upper right), lipid core (lower left), and shoulder (lower right) lesions, are shown in high-powered views of the respective boxed areas in the upper left panel. A cytoplasmic staining pattern was observed in the fibrous cap and shoulder lesions, whereas a diffuse deposition pattern was observed in the necrotic lipid core. Original magnification: upper left, ×20; others, ×300.
staining was seen in the adventitia; however, the reaction was not significantly lost by antigen absorption (data not shown); therefore, we did not evaluate the adventitial deposition of PEDF.

**Intimal Angiogenesis and Cytoplasmic Expression of PEDF**

As shown in Figure 4a, the cytoplasmic PEDF staining was generally observed in “cell-rich” areas. Such regions were mainly composed of an accumulation of foam cells and showed positive staining of PEDF in the cytoplasm (Figure 4a, top 2 panels, red staining) involving 718 microvessels (56.6%), which were circumvently labeled by CD34 (Figure 4a, top right; brown, arrows). These PEDF-positive cells (red) were also labeled by CD68 (blue) (Figure 4a, middle 2 panels), indicating that the source of PEDF should be macrophages. Double staining for ss–DNA (brown) and CD34 (red) in other sections in these cases revealed that 29.8% (184/618) of CD34-positive microvessels contained at least an endothelial cells positive for ss–DNA in cell-rich area (Figure 4a, bottom 2 panels).

There were 67 cell-rich areas involving PEDF-positive foamy macrophages; as shown in Figure 4b, a positive correlation between the PEDF-positive cell number and CD34-positive microvessels in these areas (Spearman’s $\rho=0.686, P<0.0001$).

**Intimal Angiogenesis and Extracellular Deposition of PEDF**

In contrast, 551 of 1269 microvessels (43.4%) were seen in acellular and fibrous areas in the intima. As shown in Figure 5a, abundant and patchy extracellular deposition of PEDF was seen in these areas (red, upper 2 panels), which was lost by incorporating excess recombinant PEDF (bottom 2 panels). Computer-assisted quantification of the number of mi-
We investigated the localization and cell sources of PEDF, an antiangiogenic factor in human atherosclerotic plaques, and their relationship to intimal angiogenesis of coronary arteries. Key observations were as follows: (1) PEDF protein was detected in all fresh aortic tissue, even though only 50% of the samples expressed PEDF mRNA, suggesting that extracellular deposition in PEDF transcription-negative samples had occurred, a finding supported by IHC study using retinal tissue; (2) double IHC demonstrated that macrophages and some SMCs were major sources of PEDF in both the aorta and in the coronary arteries; (3) 2 divergent staining patterns, namely “cytoplasmic” and “extracellular deposition,” were evident in both the aortic and coronary tissues; and (4) a retrospective study using stored coronary arteries demonstrated a positive correlation among the number of PEDF-positive cells, the number of intimal microvessels, and AHA classification, and inversely, a significantly lower frequency of intimal capillaries was observed in the PEDF-deposited areas than in the PEDF-null areas. To the best of our knowledge, this is the first report investigating the expression and deposition of an angiostatic factor, PEDF, in extraocular lesions, ie, in human atherosclerotic plaques.

We observed 2 different PEDF staining patterns: cytoplasmic staining and extracellular deposition. The latter pattern should be considered as an equivocal finding, because this particular staining pattern is frequently caused by a nonspecific reaction. Therefore, to exclude the influence of such a nonspecific reaction for IHC, we conducted the following series of control experiments: (1) a negative control using isotype-matched nonimmune IgG; (2) a positive control to determine the PEDF staining pattern using human retinal tissue; and (3) a Western blot analysis. The type of extracellular deposition that had been observed in the human aorta and coronary arteries was also seen in the retinal tissue, and 50 kDa of glycosylated PEDF protein was detected in all aortic samples examined, even though the mRNA for PEDF was not consistently observed. We therefore concluded that the results provided definitive evidence of the extracellular deposition of PEDF in human vascular tissue.

An important question may be raised in this context regarding the relationship between PEDF expression and angiogenesis; at a glance, it might appear that paradoxical results were obtained in this study. In other words, a positive correlation was observed between the number of cytoplasmically PEDF-positive cells and the number of microvessels in the intima; inversely, a significantly lower frequency of intimal capillaries was observed in the PEDF-deposited areas than in the PEDF-null areas. Clearly, the latter finding is reasonable in terms of the recent accumulation of evidence suggesting PEDF as an antiangiogenic factor; however, the former is likely to contradict the latter. Furthermore, cytoplasmically PEDF-positive cell species were also found to be positive for not only VEGF-A but also other angiogenic substances including bFGF/FGF-2 and interleukin-8, suggesting the pro-angiogenic potentials of these cells. This paradox can be explained by a recent study: osteoblasts, and possibly also osteoclasts, produce not only PEDF but also an angiogenic factor VEGF-A, as well as its receptors, suggesting a greater range of net stimulatory or inhibitory effects for bone development and angiogenesis. In addition, counterbalance hypothesis between angiogenic stimulators and inhibitors is partly supported by the previous report indicating that VEGF is secreted by differentiated RPE cells, upregulating PEDF via VEGFR-1 in an autocrine manner. Together, there may be the possible mechanism for net angiogenic property through the autocrine-positive feedback loop for the cytoplasmic expression of angiogenic stimulators, ie, VEGF, and inhibitors, ie, PEDF. In contrast, in cases of lesions showing PEDF deposition in acellular area associated with frequent apoptotic neovessels, deposited PEDF may exert its antiangiogenic activity. This hypothesis is reasonable, because PEDF intimately associates with the extracellular matrix and forms a complex with collagen type I.
A limitation related to the current study involves only correlative observations between localization of PEDF and density of microvessels and apoptosis in human coronary arteries, without direct evidence of angiogenic properties of PEDF, which has been uncertain. The observations obtained here, however, strongly support the current hypothetical model regarding the possible mechanism of angiogenic activity of PEDF. Their model is concisely summarized as follows: some integrins, including αβ6, that associate with protein tyrosine phosphatase positively modulate the signaling from angiogenic growth factors including VEGF. Once PEDF binds to integrins directly and/or indirectly via extracellular matrix, dissociated protein tyrosine phosphatase from intracellular domain of integrins cleaves phosphorylation of tyrosine kinase receptors, resulting in silencing the intracellular angiogenic signals including focal adhesion kinase. This model seems to explain well the properties of other angiogenic inhibitors, including endostatin, thrombospondins, and TIMP-2. Further studies, therefore, investigating whether PEDF may exert similar system for antiangiogenesis are called for.

There is one more important question regarding the source of deposited PEDF in the human atherosclerotic plaque. Two possibilities can be raised as follows: (1) PEDF protein may diffuse from plasma onto extracellular matrix in atherosclerotic lesions, a similar pattern to fibrinogen demonstrated in our previous study, because relatively high level of PEDF is circulating in the blood stream and (2) PEDF protein may deposit onto the extracellular matrix after apoptotic or necrotic death of PEDF expressing cells. Further study is still needed to clarify this point.

Recently, PEDF has also been the focus of attention as a possible therapeutic agent because of its potent inhibition of angiogenesis; this is particularly the case in studies attempting to achieve the regression of malignant tumors. It has been emphasized that testing the potential of this agent against a wider range of angiogenic diseases including tumors and a better understanding of its biochemical pathways are needed. The findings presented here may also suggest the potential of PEDF as a plaque stabilizer. In any case, further studies should be performed to clarify whether PEDF treatment may have a favorable effect on plaque stabilization.

In conclusion, we demonstrated here the expression and extracellular deposition of a potent angiostatic factor, PEDF, in human atherosclerotic lesions. The present results suggest that the extracellular deposition of PEDF may be required for this factor to exert its antiangiogenic potential. Thus, we propose that PEDF is an important regulator for maintaining a balance with respect to intimal angiogenesis. Moreover, PEDF appears to play a role in the regulation of the progression of atherosclerosis by controlling angiogenic balance in human subjects.

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References


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