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Placental Growth Factor Promotes Atherosclerotic Intimal Thickening and Macrophage Accumulation

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Background—Placental growth factor (PlGF) has been implicated in the pathophysiological angiogenesis and monocyte recruitment that underlie chronic inflammatory disease, but its role in atherosclerosis has not been examined. We investigated the effects of exogenous PlGF, delivered by adenoviral gene transfer, on atherogenic intimal thickening and macrophage accumulation induced by collar placement around the rabbit carotid artery and examined the effects of PlGF deficiency on atherosclerosis in apolipoprotein E–deficient (apoE$^{-/-}$) mice.

Methods and Results—Periadventitial transfer of PlGF2-encoding adenoviruses significantly increased intimal thickening, macrophage accumulation, endothelial vascular cell adhesion molecule-1 expression, and adventitial neovascularization in the collared arteries of hypercholesterolemic rabbits and increased the intima-to-media ratio in rabbits fed a normal diet. Neointimal macrophages were associated with increased expression of the PlGF receptor Flt-1. The size and macrophage content of early atherosclerotic lesions were reduced in mice deficient in both apoE and PlGF compared with apoE-deficient mice.

Conclusions—Local adenoviral PlGF2 delivery promotes atherogenic neointima formation in hypercholesterolemic rabbits, and PlGF is required for macrophage infiltration in early atherosclerotic lesions in apoE$^{-/-}$ mice. These findings support a novel role for PlGF in the pathogenesis of atherosclerotic disease. (***Circulation***. 2005;111:2828-2836.)

Key Words: angiogenesis ■ atherosclerosis ■ cell adhesion molecules ■ endothelium ■ monocytes

Vascular endothelial growth factor (VEGF or VEGF-A) is essential for endothelial cell (EC) differentiation and angiogenesis during embryogenesis and plays a major role in neovascularization in diverse human diseases.[1,2] Two protein tyrosine kinase receptors for VEGF, VEGFR-2/KDR/Flk-1 and VEGFR-1/Flt-1, are essential for embryonic vascular development, but signal transduction and biological responses in endothelia are mediated primarily via KDR.[1,3] Flt-1 is believed to function during embryogenesis as a negative regulator of KDR-mediated actions, in part by acting as a "decoy" receptor and in part through direct modulation of KDR activity.[1,4] However, a growing body of evidence indicates that the VEGF homologue, placental growth factor (PlGF), stimulates pathophysiological angiogenesis and other biological responses in the postembryonic state by signaling through its receptor Flt-1 on ECs.[5] Loss of PlGF impairs collateral artery growth in mouse limbs and neovascularization in tumors and ischemic retinas, whereas exogenous PlGF delivery stimulates angiogenesis and collateral growth in ischemic hearts and limbs.[5–7] Inhibition of Flt-1 suppresses pathophysiological angiogenesis[8]; in addition, Flt-1 mediates PlGF-induced monocyte chemotaxis,[9] and rescue of impaired ischemia-driven arteriogenesis in the rabbit hind limb or in PlGF$^{-/-}$ mice is effected by infusion of exogenous PlGF and prevented by ablation of monocytes,[10] which indicates that some biological effects of PlGF in vivo are mediated by acting on Flt-1$^+$ monocytes. Flt-1 has been implicated in atherogenesis because anti-Flt-1 blocking antibody reduced atherosclerotic plaque growth in apolipoprotein (apo) E$^{-/-}$ mice,[6] but the involvement and mechanisms of action of its ligand PlGF in atherosclerosis or associated cellular processes remains to be determined.

In the present study, the role of PlGF in atherogenic lesion formation was investigated by 2 distinct approaches. First, we examined the effects of local periadventitial transfer of PlGF-encoding adenoviruses on intimal thickening and macrophage accumulation in the rabbit collar model. In this model, neointimal lesions are induced by periadventitial placement of an inert silicone collar around the carotid artery in rabbits maintained on a normal or cholesterol-rich diet without major damage to the endothelium or medial vascular smooth muscle cells (VSMCs), with the collar device also

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providing the means of local gene delivery.[11–16] Second, we evaluated the effects of loss of the PlGF gene on the growth and macrophage content of atherosclerotic lesions in apoE-deficient mice. The results show that adenoviral PlGF expression increases intimal thickening, neointimal macrophage accumulation, and adventitial neovascularization in hypercholesterolemic rabbits and demonstrate a requirement of PlGF for macrophage accumulation and plaque growth in early atherosclerotic lesions in apoE$^{-/-}$ mice. These findings support the conclusion that PlGF promotes atherogenic intimal thickening and macrophage infiltration and identify a novel role for PlGF in the pathogenesis of early atherosclerosis.

Methods

Adenoviruses

Adenoviral constructs (E1-, E3-deleted) encoding either LacZ (Ad.LacZ) or mouse PlGF2 (Ad.PlGF2) were produced as described previously.[6,12] Adenoviruses were desalted with G50 Sephadex columns (Boehringer Mannheim) immediately before their use in animal studies.

Collar Placement and Gene Transfer

All experiments were conducted in accordance with the animal care guidelines and Ethics Committee of University College London and the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Twenty-six New Zealand White male rabbits (weight 2.5 to 3.2 kg) were fed a normal diet supplemented with 1.5% cholesterol for 1 week before collar placement and throughout the experiment. In parallel, 22 rabbits were maintained on normal chow without added cholesterol. Placement of a biologically inert silastic collar (Ark Therapeutics Ltd) around the right carotid artery was performed in anesthetized rabbits as described previously.[12] The contralateral carotid artery was sham operated by surgical dissection from surrounding tissues and exposed to a similar degree of stretch. The wounds were sutured and the animals allowed to regain consciousness. Five days later, the collared arteries were exposed, 100 μL of each adenoviral vector solution containing 5×10^9 pfu was placed within the space between collar and artery with a pipette, and the wound was sutured. Serum samples were taken for assay of total cholesterol, LDL cholesterol, and HDL cholesterol (Boehringer-Mannheim) 7 days before initiation of the high-cholesterol diet, 1 day after initiation of the diet, and 9 days after gene transfer. Nine days after gene transfer, animals were killed, and collared and contralateral control arteries were excised. Arteries were flushed with ice-cold saline and divided into 2 segments. The proximal part was immersion-fixed in 1% paraformaldehyde/7.5% sucrose in PBS, pH 7.4, for 6 hours, rinsed in 70% ethanol, then transferred to 70% ethanol and embedded in paraffin. The distal part was either immersion-fixed in 4% paraformaldehyde/7.5% sucrose/PBS, pH 7.4, for 30 minutes, rinsed in PBS for 15 minutes, embedded in OCT compound (Miles), and stored at −80°C or snap-frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from 30 to 50 mg of frozen tissue (pooled from 2 carotids or nontargeted organs) by an RNEasy spin-column method, adapted to optimize RNA yields from fibrous tissue by incorporating an additional proteinase K digestion step (RNeasy fibrous tissue mini kit, Qiagen). The tissue was homogenized with a rotor-stator (Polytron 3100) and treated with RNase-free DNase-1. Total RNA (500 ng) was reverse transcribed with superscript III RT (Invitrogen) and random hexamers according to the manufacturer's instructions. For PlGF2 and β-actin, polymerase chain reaction (PCR) was performed with Platinum Taq DNA Polymerase (Invitrogen) and transgene-specific primers (5′ primers selected from the

cytomegalovirus promoter and 3′ primers from the coding region) with the following PCR cycle parameters: 1 minute at 94°C, followed by 34 cycles comprising 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, and the last extension step was continued for 7 minutes at 72°C. Forward and reverse primers for PlGF2 and β-actin were, respectively, 5′GAT CTT GAA GAT TCC CCC CAA TCG G3′ and 5′ATA GAG GGT AGG TAC CAG CAG GGA GGG3′, and 5′CTC ATG AAG ATC CCT CAC GGA GC3′ and 5′GCA CAG CTT CTC CTT GAT GTC C3′. A 270-bp amplicon for PlGF2 was generated. Reaction products were run on a 2% agarose gel (Invitrogen). PlGF2 cDNA derived from Ad.PLGF2-transfected human umbilical vein ECs served as a positive control. Products of the reverse-transcription reaction, in which the reverse-transcriptase (RT) enzyme had been omitted, served as negative controls.

LacZ Staining

Detection of β-galactosidase activity was performed by overnight incubation of whole arterial segments or 6-μm sections in the dark at 37°C in β-gal staining solution as described previously.[12]

Immunohistochemistry

The following antibodies were used: mouse IgG1 to rabbit vascular cell adhesion molecule-1 (VCAM-1; Rb1/9; gift of MI Cybulsky) at a 1:100 dilution in frozen sections; macrophage-specific RAM-11 (1:50, Dako); mouse anti-human CD31 (1:500; Dako); VSMC-specific α-actin antibody (1:150; Dako); rat anti-mouse PlGF monoclonal antibody (Pharmingen Inc; 10 μg/mL); and Flt-1 anti-mouse monoclonal antibody (Santa Cruz Inc; 1:50). Primary antibodies were diluted in Tris-buffered saline, pH 7.2. Staining was performed on deparaffinized or frozen sections as described previously and visualized with a Vectastain Elite ABC Kit (Vector Laboratories).[12] For PlGF staining only, a tyramide amplification step (Perkin Elmer Inc) was performed before secondary antibody incubation. The 5-μm frozen sections were fixed for 10 minutes in acetone at −20°C and then air dried. Negative controls for all immunostainings were performed by omission of the primary antibody.

Morphometry and Image Analysis

Images of sections at ×5 and ×40 were acquired with a high-resolution color camera (Zeiss microscope, Jenoptik Camera) and analyzed with automated image-analysis software (Image J, National Institutes of Health). Intimas were defined as the regions between luminal endothelium and the internal elastic lamina. The media was defined as the area between the internal and external elastic laminas and analyzed blindly. Ratios of intimal to media areas were determined in serial sections cut at 500-μm intervals and stained with hematoxylin and eosin; intima-media values were averaged and expressed as mean±SE. Neovascularization was quantified by counting the numbers of CD31-positive vessels. CD31 staining was regarded as positive if a single cell or a vessel with a lumen was present, and numbers of CD31-positive cells and vessels were expressed per square millimeter of total adventitial area. Total RAM-11–positive macrophages were counted in the intima and expressed per square millimeter. VCAM-1 immunostaining was quantified on high-resolution (1300×1030 pixel) images with Open-Lab 3.14 software (Improvision Ltd) and expressed as the number of pixels representing endothelial VCAM-1 immunostaining as a percentage of the total endothelial pixel count.

apoE$^{-/-}$ and apoE$^{-/-}$:PlGF$^{-/-}$ Mice

apoE$^{-/-}$ mice were intercrossed with previously generated PlGF$^{-/-}$ mice,[8] which yielded apoE$^{+/-}$:PlGF$^{+/-}$ offspring. Breeding of these compound heterozygous mice generated apoE$^{-/-}$:PlGF$^{-/-}$ mice and their apoE$^{-/-}$:PlGF$^{+/+}$ littermates. Mice (all female) were maintained on regular chow for 5 weeks, after which they were fed a cholesterol-rich diet for an additional 10 or 25 weeks.[6] Mice were anesthetized (60 mg/kg pentobarbital; Nembutal, Abbott Laboratories) and perfused with saline followed by 1% phosphate-buffered paraformaldehyde. Hearts were harvested and postfixed in the same fixative
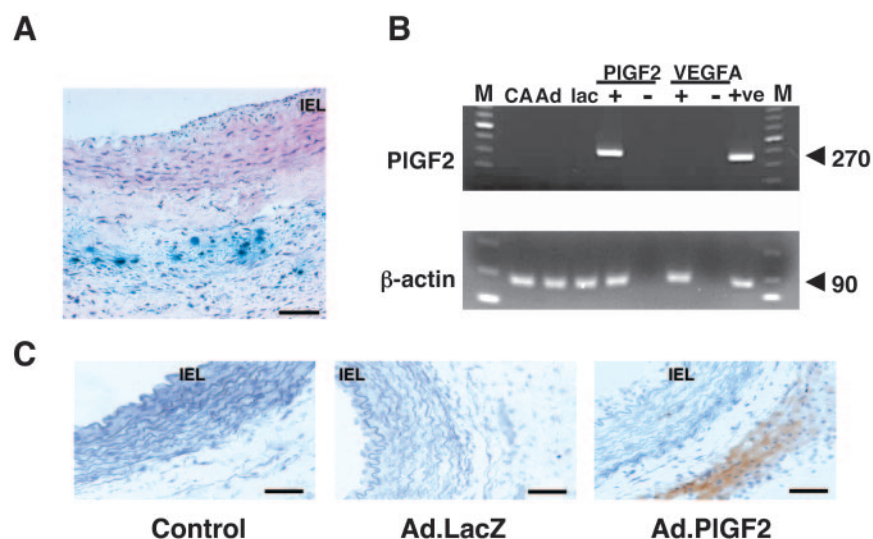
Figure 1. Expression of Ad.PlGF2 in collared rabbit carotid arteries. A, Periadventitial Ad.LacZ delivery to collared carotid arteries resulted in efficient β-galactosidase expression predominantly in adventitia. Bar=40 μm. B, RNA was extracted from collared transfected carotid arteries in cholesterol-fed rabbits, and PlGF2 transgene expression was determined by RT-PCR in presence (+) or absence (−) of reverse transcriptase with β-actin as reference gene. Predicted PCR product of 270 bp corresponding to PlGF2 was detected only in arteries transduced with appropriate adenovirus and was absent from Ad.lacZ-transduced arteries (lac), adenoviral backbone (Ad), or uninfected control contralateral arteries from same rabbit (CA). Product of 90 bp corresponding to β-actin was present at similar level in all samples. RT-PCR of PlGF2 adenoviruses was used as positive control (+ve). C, Arterial PlGF2 expression was detected by immunostaining with specific antibody for mouse PlGF2. Ad.lacZ-infected and -uninfected sham-operated (control) arteries stained with PlGF antibody showed little detectable expression. PlGF2 immunostaining in Ad.PlGF2-transduced arteries was detected mainly in adventitia, with some neointimal staining. Position of internal elastic lamina (IEL) is indicated. Bar=40 μm.

for another 3 hours, dehydrated, and embedded in paraffin. Seven-micrometer cross sections of the aortic origin and the descending aortas (thoracic and abdominal region) were cut, and cross-sectional plaque area measurements were performed as described previously.[6,17] Macrophage content was analyzed on sections stained with a rat anti-mouse macrophage-specific Mac-3 antibody (Pharmingen). Morphometric analyses on Mac-3–stained sections were performed with a Zeiss Axioplan2 microscope (Zeiss), a 3CCD video camera (DXC-93OP; Sony), and KS300 software. Plaque macrophage content is expressed as the ratio of the area positive for Mac3 staining to total plaque area. Plaque microvessels were analyzed after staining of cross sections of the aortic root for endothelium-specific markers, von Willebrand factor (Dako) and CD31 (Pharmingen), as described previously.[6] T lymphocytes were stained with a rabbit anti-CD3 antibody (Laboratory Vision Corporation).

Statistical Analysis
Differences in serum cholesterol levels and morphometric differences between different treatment groups in rabbits were evaluated by ANOVA and Bonferroni correction (SPSS). Data obtained from mouse knockout models, expressed as mean±SE, were analyzed with the unpaired Student t test and the Mann-Whitney U test, which yielded similar results. Data were considered statistically significant at $P<0.05$.

Results

Periadventitial PlGF2 Adenoviral Gene Delivery in Collared Carotid Arteries
Collar placement around the carotid arteries of rabbits fed a normal diet for 14 days induces the formation of neointimal lesions composed of VSMCs with no detectable involvement of macrophages.[11,12] In contrast, collaring of the carotid artery in rabbits fed a 1.5% cholesterol diet generates neointimas comprising VSMC with the involvement of Ram-11–stained macrophages (results not shown), very similar to previous findings.[12] Blood LDL cholesterol and total cholesterol increased to 10 and 14 mmol/L, respectively, after 7 days on the high-cholesterol diet and 20 and 26 mmol/L after 21 days. The contralateral sham-operated carotid arteries in the cholesterol-fed rabbits displayed no evidence of either intimal

thickening or increased Ram-11 immunostaining (results not shown). Immunostaining of ECs with CD31 antibody indicated that neither collaring nor hypercholesterolemia caused significant discontinuities in the endothelium, which remained essentially intact (results not shown). Staining for β-galactosidase in arteries transduced with a control LacZ gene revealed abundant strongly stained cells in the adventitia consistent with a high efficiency of gene transfer (≈5% to 10%) and in agreement with previous findings (Figure 1A).[12,18]

Expression of the PlGF2 transgene after periadventitial delivery of Ad.PlGF2 to carotid arteries was examined by RT-PCR and immunohistochemistry. An amplicon of predicted size, 270 bp, corresponding to PlGF2 was detected only in RNA prepared from collared arteries transduced with Ad.PlGF2 (Figure 1B). Expression of β-actin was very similar in RNA prepared from all transduced arteries. No PlGF2 transgene expression was detected in Ad.LacZ-transduced arteries, segments of the transduced carotid arteries distal to the collared arterial region, contralateral noncollared control arteries, or other nontargeted tissues, including lung, liver, kidney, and testis (results not shown). These results indicate that the perivascular collar was effective in targeting transgene expression specifically to the collared region of the artery. Immunostaining of sections of transduced arteries with a specific antibody to mouse PlGF2 showed strong expression of PlGF in the adventitia and additional expression in the neointima and endothelium (Figure 1C), which confirmed the RNA analysis.

Ad.PlGF-2 Increases Macrophage Accumulation and Flt-1 Expression
Ad.PlGF2 delivery to collared carotid arteries in cholesterol-fed rabbits significantly increased intimal thickening (Table 1; Figure 2A), and it significantly enhanced neointima formation in the collared carotid arteries of rabbits fed a normal low-cholesterol diet (Figure 2B). Immunostaining with Ram-11 showed that PlGF2 increased neointimal macro-

TABLE 1.   Effects of Ad.PlGF2 Gene Delivery on Lesion Formation in Collared Rabbit Carotid Arteries

	1.5 % Cholesterol Diet		Normal Diet	
	Ad.LacZ (n=10)	Ad.PlGF2 (n=10)	Ad.LacZ (n=8)	Ad.PlGF2 (n=8)
Intima/media	0.15±0.02	0.28±0.01‡	0.097±0.006	0.16±0.02†
Macrophages/mm² neointima	246±30	470±66*	ND	ND
Macrophages/mm² adventitia	13.5±1.5	22.5±3.6*	24.4±3.3	47.1±4.1‡
CD31/mm² adventitia	6.7±2.3	28.7±2.3§	9.3±2.7	22.4±4.4*
% Endothelial VCAM1	24.0±1.6	47.8±4.4*	ND	ND

Values represent mean±SE for carotid arteries infected with Ad.LacZ or Ad.PlGF2 in rabbits fed either 1.5% cholesterol or normal diet. Neointimal macrophages and endothelial VCAM1 staining were not detected (ND) in rabbits on a normal diet. Other experimental details are provided in Methods and figure legends.

*$P<0.05$, †$P<0.01$, ‡$P<0.001$, and §$P<0.0001$ for Ad.PlGF2 vs Ad.LacZ.

phage accumulation in the collared arteries of hypercholesterolemic rabbits (Figure 3A). Compared with either Ad.LacZ-transduced arteries or nontransfected collared arteries, periadventitial Ad.PlGF2 delivery significantly increased the neointimal density of Ram-11–positive cells (Figure 3B). Ad.PlGF2 also increased the number of adventitial macrophages in collared hypercholesterolemic rabbits from 13.5±1.5/mm² to 22.5±3.6/mm² (Table 1). Although macrophages did not accumulate in the neointimas of collared arteries in rabbits fed a normal diet, consistent with previous findings,[12] a significant increase in adventitial macrophage staining was detected in Ad.PlGF2-transduced arteries compared with Ad.LacZ (Table 1).

Expression of the PlGF receptor Flt-1 was also examined in collared arteries. In Ad.PlGF2-transduced arteries of hypercholesterolemic rabbits, Flt-1 expression was readily detected in neointimal cells and in areas of the endothelium and adventitia, and it was increased in Ad.PlGF2-transduced vessels compared with Ad.LacZ-expressing arteries (Figure 3C). Ram-11 staining of serial sections showed close associ-

ation between some Flt-1–positive regions and Ram-11–positive staining in the neointima, which suggests that lesion macrophages expressed Flt-1.

Ad.PlGF-2 Induces Neovascularization and Endothelial Activation

Recent findings have discovered a role for PlGF in pathophysiological angiogenesis in vivo.[6–8] It was therefore examined whether local Ad.PlGF2 delivery increased neovascularization in collared carotid arteries. Immunostaining of CD31 revealed the presence of new adventitial vessels in the Ad.PlGF2-transduced arteries of rabbits fed a high-cholesterol diet (Figure 4A) or normal diet (results not shown). Quantification of the adventitial density of CD31-positive vessels indicated that neovascularization was increased in the Ad.PlGF2-transduced arteries of rabbits regardless of dietary regimen (Figure 4B). In both experimental groups, CD31-positive cells and vessels were not detected in the media, and apart from luminal staining of the endothelium, they were also absent from neointimal lesions.
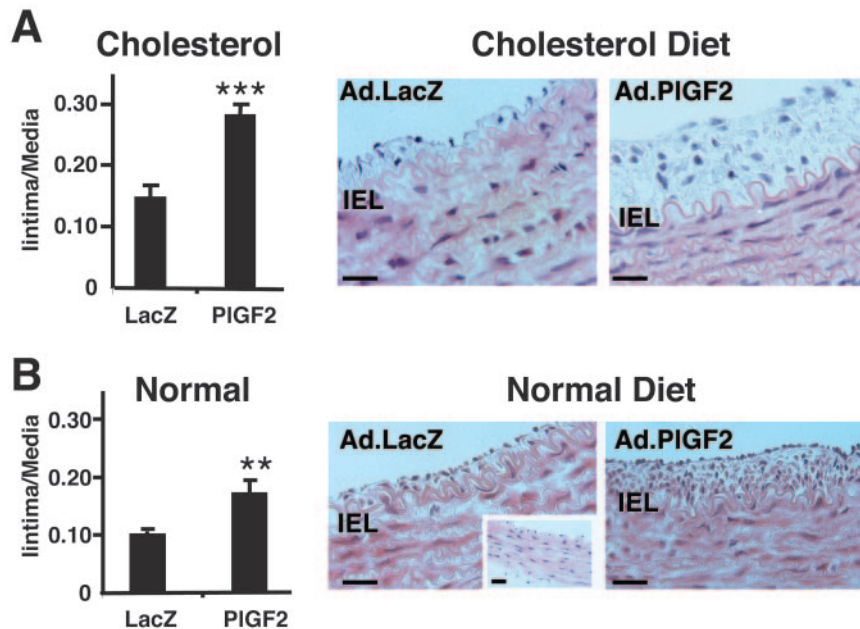

Figure 2. Ad.PlGF2 enhances collar-induced intimal thickening. A, Collar placement and gene transfer were performed in cholesterol-fed rabbits and intima/media ratios were determined in collared carotid arteries infected with Ad.LacZ (n=10) or Ad.PlGF2 (n=10) as described in Methods. Intimal thickening was significantly increased in Ad.PlGF2-transduced arteries: ***$P<0.001$ for PlGF2 vs lacZ. Representative hematoxylin-and-eosin–stained sections indicating position of internal elastic lamina (IEL) are shown. Bar=20 μm. B, Ad.PlGF2 delivery to collared carotid arteries significantly increased intimal thickening in rabbits fed normal low-cholesterol diet. **$P<0.01$ for PlGF2 (n=8) vs lacZ (n=8). Representative hematoxylin-and-eosin–stained sections indicating position of IEL are shown. Inset, Sham-operated collateral carotid artery shows no intimal thickening. Bar=20 μm.
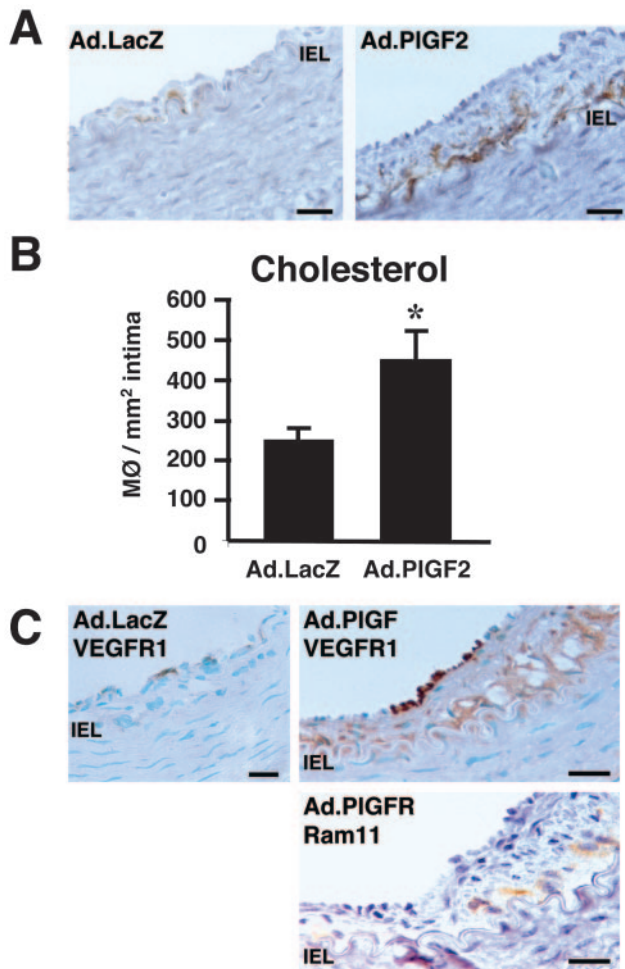
Figure 3. Effect of Ad.PlGF2 on intimal macrophage accumulation in hypercholesterolemic rabbits. A, Collared carotid arteries in rabbits on high-cholesterol diet were transfected with lacZ or Ad.PlGF2, and macrophage content was determined by immunostaining with Ram-11. Position of internal elastic lamina (IEL) is indicated. Bar=20 μm. B, Macrophage density is expressed as neointimal Ram-11–positive cells (mean±SE) per mm² total neointimal area. Ad.PlGF2 significantly increased neointimal macrophage content in hypercholesterolemic rabbits. *$P<0.05$ for PlGF2 vs lacZ. C, Flt-1 immunostaining was increased in Ad.PlGF2-infected arteries compared with Ad.LacZ. Immunostaining of macrophages with Ram-11– and Flt-1–specific antibodies in serial sections from Ad.PlGF2-infected collared arteries shows abundant Flt-1 immunostaining in neointima, parts of which are adjacent to regions of Ram-11–positive staining in serial cross section. Position of IEL is indicated. Bar=20 μm.

Upregulation of the EC adhesion molecule, VCAM-1, is thought to play a central role in mediating increased monocyte adhesion to the endothelium and transendothelial monocyte migration leading to neointimal accumulation of macrophages.[19–21] Lumenal endothelial expression of VCAM-1 was increased in collared arteries from cholesterol-fed rabbits compared with either the sham-operated contralateral control arteries in the same animals or collared arteries from normocholesterolemic rabbits (results not shown). In hypercholesterolemic rabbits, Ad.PlGF2-transduced collared arteries displayed increased endothelial VCAM-1 staining compared with Ad.LacZ (Figure 5). Quantification of VCAM-1 immu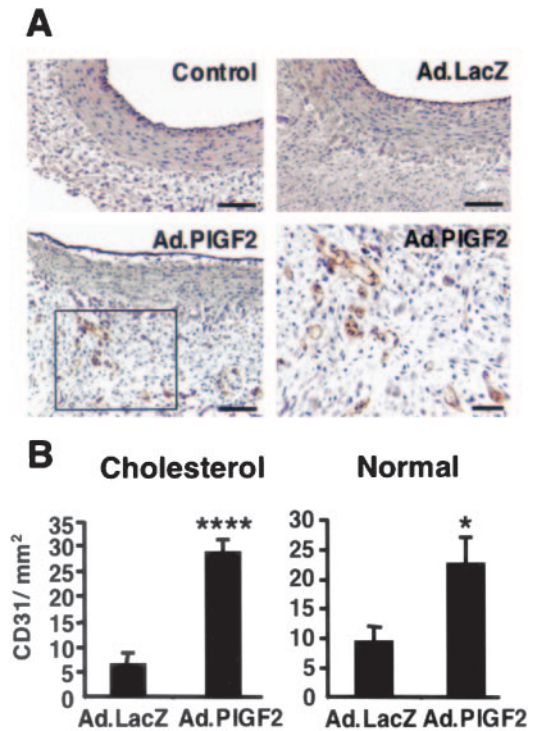nostaining showed that the total percentage of the endothelium positive for VCAM-1 immunostaining was significantly elevated ($P<0.001$) in PlGF2-transduced arteries (Figure 5).

Figure 4. Angiogenic response to Ad.PlGF2 in collared arteries. A, Sections of collared carotid arteries in hypercholesterolemic rabbits infected with Ad.LacZ or Ad.PlGF2 were immunostained with CD31 antibody. CD31 staining is shown at ×10 (upper and lower left photomicrographs; bar=40 μm) and ×40 magnification (lower right; bar=20 μm). Increase in CD31-positive microvessels occurred in adventitia of arteries transduced with Ad.PlGF2. B, Numbers of adventitial CD31-positive microvessels and cells in rabbits fed high cholesterol or normal diets were quantified, and results are presented as mean±SE. CD31-positive vessels/mm². ****$P<0.001$ and *$P<0.05$ for Ad.PlGF2 vs lacZ.

Deficiency of PlGF Reduces Early Atherosclerotic Lesion Development in apoE$^{-/-}$ Mice

The results presented so far indicated that local adenoviral PlGF2 expression induced atherogenic processes that included intimal thickening, macrophage recruitment, and endothelial VCAM-1 expression in the hypercholesterolemic rabbit. To investigate whether endogenous PlGF played a role in neointimal macrophage accumulation in atherosclerosis, plaque formation at the aortic root was analyzed in mice lacking both PlGF and apoE (apoE$^{-/-}$:PlGF$^{-/-}$) and compared with single apoE-deficient littermates (apoE$^{-/-}$:PlGF$^{+/+}$). PlGF deficiency caused a significant reduction in size ($P=0.034$) and macrophage content ($P=0.007$) of early atherosclerotic plaques in apoE$^{-/-}$ mice after 10 weeks on a high cholesterol diet compared with mice deficient only in apoE (Figure 6; Table 2). After 25 weeks of diet, the mean plaque area was reduced in apoE$^{-/-}$:PlGF$^{-/-}$ mice compared with apoE$^{-/-}$ mice, but the effect was not statistically significant (Table 2). In addition, a reduction in plaque macrophages was not observed after 25 weeks on a high-fat diet. At both time points, macrophage distribution within the athero-
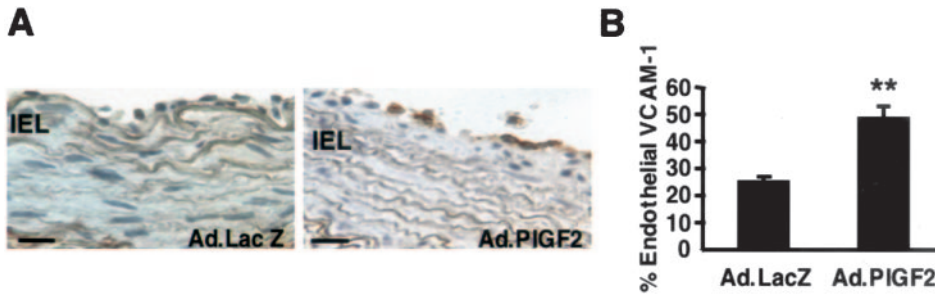
Figure 5. Ad.PlGF2 increases VCAM-1 expression in collared arteries. A, Immunohistochemical staining of VCAM-1 was performed in sections from collared arteries infected with Ad.LacZ or Ad.PlGF2. In hypercholesterolemic rabbits, endothelial VCAM-1 immunostaining is increased in Ad.PlGF2-transduced vessels. Position of internal elastic lamina (IEL) is indicated. Bar=20 μm. B, Quantification of endothelial VCAM-1 immunostaining shows that endothelial VCAM-1 immunostaining is significantly increased in Ad.PlGF2-infected arteries. **$P<0.001$ for Ad.PlGF2 vs Ad.LacZ. Values represent VCAM-1 staining, expressed as percentage of total endothelial area, in sections taken from 3 points in each treated artery (n=8 arteries each for Ad.LacZ and Ad.PlGF2).

sclerotic lesions was similar in both genotypes. Macrophages penetrated throughout the entire early lesions (Figures 6e and 6f), whereas they were more confined to the surface of plaques in advanced lesions (Figures 6i and 6j). Semiquantitative analysis revealed no significant differences in the macrophage distribution between apoE$^{-/-}$:PlGF$^{+/+}$ and apoE$^{-/-}$:PlGF$^{-/-}$ mice in early or late lesions (Table 2). Analysis of plaques in the descending thoracic and abdominal aorta showed that cross-sectional plaque areas were not significantly reduced in apoE$^{-/-}$:PlGF$^{-/-}$ compared with apoE$^{-/-}$:PlGF$^{+/+}$ mice (17.73$\pm$5.63 and 20.19$\pm$3.31 μm^2, respectively; n=10 for each genotype; P=NS). However, PlGF-deficient mice developed significantly fewer lesions in the descending aorta compared with their apoE$^{-/-}$:PlGF$^{+/+}$ littermates: plaque numbers were 1.5$\pm$0.3 and 3.5$\pm$0.6 for apoE$^{-/-}$:PlGF$^{-/-}$ and apoE$^{-/-}$:PlGF$^{+/+}$ mice, respectively (n=10 for each genotype; $P<0.05$). Plaque number and size in the descending aorta were similar in both genotypes after 25 weeks.

In agreement with a possible effect of PlGF on macrophage recruitment and as a potential mechanism for reduced early lesion growth, the total leukocyte count in peripheral blood was slightly reduced in the apoE$^{-/-}$:PlGF$^{-/-}$ mice with early lesions (2.97$\pm$0.26$\times$10^3/μL in apoE$^{-/-}$:PlGF$^{+/+}$ mice versus 2.23$\pm$0.16$\times$10^3/μL in apoE$^{-/-}$:PlGF$^{-/-}$ mice; n=20; $P<0.05$). No difference in leukocyte counts was detected in mice with advanced complex lesions (after 25 weeks of diet), with values of 3.33$\pm$0.70$\times$10^3/μL in apoE$^{-/-}$:PlGF$^{+/+}$ mice versus 3.09$\pm$0.34$\times$10^3/μL in apoE$^{-/-}$:PlGF$^{-/-}$ mice (n=8, P=NS). Unlike macrophages, accumulation of CD3-positive T lymphocytes in early atherosclerotic lesions was very low but similar in both genotypes (percent of CD3-positive area/total plaque area: 0.09$\pm$0.01 in apoE$^{-/-}$:PlGF$^{+/+}$ mice versus 0.08$\pm$0.01 in apoE$^{-/-}$:PlGF$^{-/-}$ mice; n=5; P=NS). In addition, no significant differences in CD3-positive lymphocyte content or distribution were detected in advanced lesions (percent of CD3-positive area/total plaque area: 1.21$\pm$0.64 in apoE$^{-/-}$:PlGF$^{+/+}$ mice versus 0.13$\pm$0.55 in apoE$^{-/-}$:PlGF$^{-/-}$ mice; n=5; P=NS). Consistent with our previous findings,[6] plaque microvessels detected by staining for the endothelial markers von Willebrand factor and CD31 were not found in the early lesions of either apoE$^{-/-}$:PlGF$^{-/-}$ or apoE$^{-/-}$:PlGF$^{+/+}$ mice, which suggests that plaque neovascularization does not have a major role in the early stages of plaque growth. In advanced lesions, the numbers of vessels were similar in the advanced lesions of both types of mice (number of plaque vessels per mouse: 2.0$\pm$0.4 in apoE$^{-/-}$:PlGF$^{+/+}$ mice versus 1.9$\pm$0.6 in apoE$^{-/-}$:PlGF$^{-/-}$ mice; n=8; P=NS).

Discussion

This study shows that targeted periadventitial delivery of an adenoviral vector encoding PlGF2 increased a spectrum of atherogenic processes in the collared carotid arteries of cholesterol-fed rabbits, including arterial intimal thickening, endothelial VCAM-1 expression, and macrophage accumulation. Furthermore, the reduction in the size, number, and macrophage content of plaques in mice doubly deficient in PlGF and apoE supports the conclusion that endogenous PlGF plays an important role in recruitment of macrophages and plaque growth in early atherosclerosis. These findings are consonant with previous work showing that inhibitory Flt-1 antibody reduced plaque growth and macrophage infiltration particularly in early and intermediate lesions of apo E$^{-/-}$ mice.[6] The less significant effect of PlGF deficiency on plaque growth and macrophage content in more advanced lesions may be due to a more important role of other inflammatory factors in the promotion of monocyte/macrophage migration or to a more dominant role for deposition of extracellular matrix (ie, collagen) in plaque growth during the later stages of atherosclerosis. These findings demonstrate that local PlGF delivery can directly promote atherogenic intimal thickening and intimal macrophage recruitment and indicate that PlGF production is required for macrophage migration into early atherosclerotic lesions.

The biological roles of both PlGF and its specific receptor, Flt-1, have remained enigmatic because Flt-1 does not have a clearly defined signaling function, and during embryonic development, it is thought to act primarily as a regulator of VEGF-A functions mediated via KDR. However, recent findings have revealed a role for PlGF in postembryonic angiogenesis in diverse pathophysiological settings. Importantly, Flt-1, unlike Flk-1, is expressed on cells of the myeloid lineage,[6,22] and there is increasing evidence that PlGF-induced mobilization of Flt-1–expressing bone marrow–derived hematopoietic cells and their progenitors is a key mediator of the biological effects of the PlGF-Flt-1 axis.[8] Impaired pathophysiological angiogenesis in PlGF$^{-/-}$ mice can be rescued by transplantation of wild-type bone marrow, and inhibitory anti-Flt-1 antibodies block neovascularization in ischemia, tumor growth, and arthritis in part because of
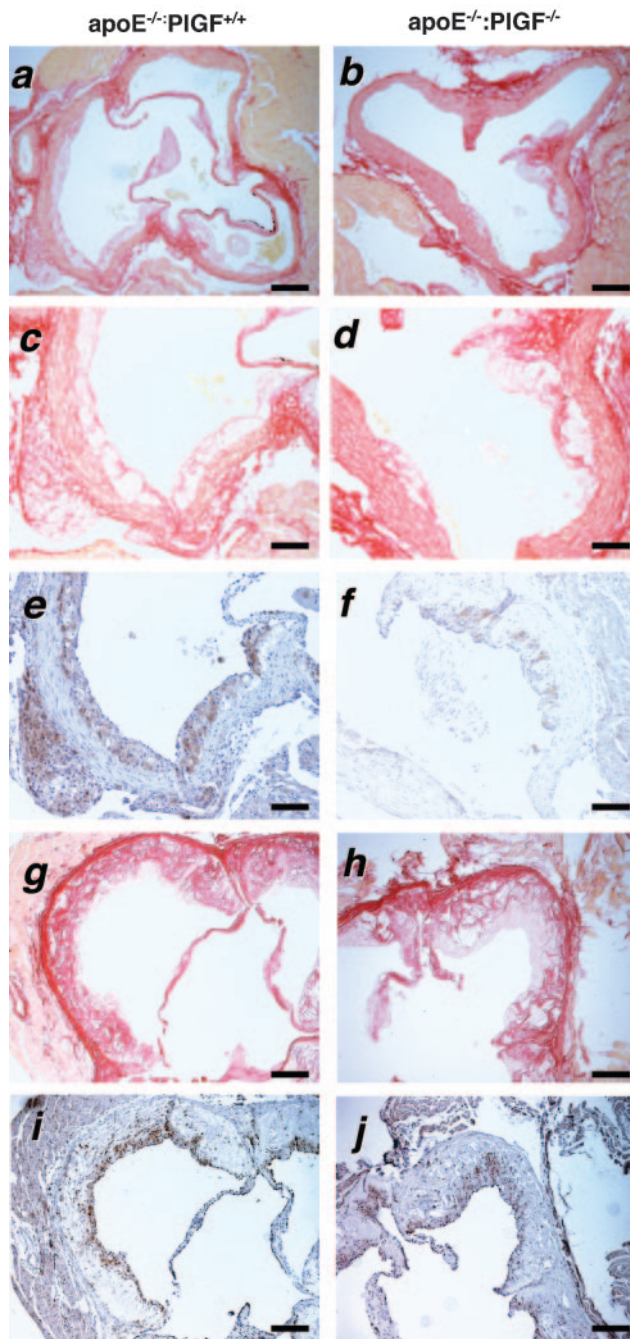
Figure 6. Deficiency of PlGF reduces early atherosclerotic lesion formation in apoE$^{-/-}$ mice. a–d, Sirius red–stained cross sections through aortic root of apoE$^{-/-}$:PlGF$^{+/+}$ (a, c) and apoE$^{-/-}$: PlGF$^{-/-}$ (b, d) mice after 10 weeks of diet reveal reduced early atherosclerotic lesions in apoE$^{-/-}$:PlGF$^{-/-}$ mice. e and f, Mac-3 staining on cross sections through aortic root shows fewer macrophages in apoE$^{-/-}$:PlGF$^{-/-}$ lesions (f) than in apoE$^{-/-}$:PlGF$^{+/+}$ lesions (e) after 10 weeks of diet. Macrophage distribution is similar in both genotypes. g and h, Sirius red–stained cross sections through aortic root of apoE$^{-/-}$:PlGF$^{+/+}$ (g) and apoE$^{-/-}$: PlGF$^{-/-}$ (h) mice after 25 weeks of diet indicate similar-sized lesions in both genotypes. i and j, Mac-3 staining on cross sections through aortic root of apoE$^{-/-}$:PlGF$^{+/+}$ (i) and apoE$^{-/-}$: PlGF$^{-/-}$ mice (j) after 25 weeks of diet indicates similar macrophage infiltration and distribution in advanced lesions of both genotypes. L in all panels indicates vessel lumen. Bar=200 μm in panels a, b, g, h, i, and j and 100 μm in panels c through f.

TABLE 2. Effect of PlGF Deficiency on Plaque Growth and Macrophage Content in apoE$^{-/-}$ Mice

	apoE$^{-/-}$:PlGF$^{+/+}$	apoE$^{-/-}$:PlGF$^{-/-}$
Mean plaque area, μm^2		
10 wk (n=20)	34 262±6330	20 235±3961*
25 wk (n=8)	320 688±28 468	278 619±15 305
Mac3-positive area/total plaque area, %		
10 wk (n=5)	19.61±2.43	9.9±1.59†
25 wk (n=2)	6.01±2.44	6.13±1.71
Mac3-positive distribution		
10 wk (n=5)	2.1±0.04	2.0±0.05
25 wk (n=2)	2.9±0.08	3.0±0.01

Data represent mean±SE of cross-sectional plaque areas (μm^2) and ratios (%) of Mac3-positive areas to plaque areas in apoE$^{-/-}$:PlGF$^{+/+}$ and double apoE$^{-/-}$:PlGF$^{-/-}$ knockout mice after 10 and 25 weeks on high-fat, high-cholesterol diet. Semiquantitative analysis of macrophage distribution (presented as mean±SE) was performed by scoring Mac-positive staining as follows: 0=no macrophages; 1=macrophages confined to subendothelium; 2=macrophages throughout plaque; and 3=macrophages throughout plaque but with more macrophages in the fibrous cap region.

*P=0.034 for plaque area and †P=0.007 for macrophage content by unpaired Student t test in apoE$^{-/-}$:PlGF$^{-/-}$ vs apoE$^{-/-}$:PlGF$^{+/+}$ mice at 10 weeks; P=0.22, and 0.97 for, respectively, mean plaque area and percent of Mac3-positive area/total plaque area after 25 weeks.

reduced mobilization of bone marrow–derived myeloid progenitors.[6,23] PlGF also stimulates hematopoiesis after bone marrow irradiation by recruiting Flt-1–positive, bone marrow–derived stem cells.[24] Although previous findings have implicated Flt-1 in recruitment of monocyte/macrophages to atherosclerotic lesions,[6] a role for its ligand PlGF has not been established. The finding that white blood cells were reduced in mice doubly deficient in PlGF and apoE suggests that PlGF-dependent mobilization of monocytes is likely to contribute to atherosclerosis, and partly mediates the infiltration of macrophages into early lesions in the apoE$^{-/-}$ mouse. The findings presented here also indicate that local PlGF overexpression in the rabbit carotid artery directly stimulates atherogenesis by increasing recruitment of macrophages from the circulation. Because PlGF2 transgene expression was detected only in the collared region of the rabbit carotid artery and was absent from other tissues, it is highly likely that the effects of PlGF in this model are not attributable to increased systemic PlGF leading to increased mobilization of bone marrow–derived stem cells. Furthermore, Ram-11–positive macrophages in collared arteries also expressed Flt-1, which suggests that intra-arterial PlGF acted by directly inducing Flt-1 expression and migration of Flt-1−expressing monocytes into lesions.

Increased endothelial expression of VCAM-1 is likely to be an important mediator of the increased neointimal macrophage influx in Ad.PlGF2-transduced arteries. The cytokine-inducible adhesion molecule VCAM-1 is upregulated in the arterial endothelium at atherosclerosis-prone sites in hypercholesterolemic rabbits and mice,[19,21] and is essential for early atherosclerotic lesion formation in LDL receptor-deficient mice.[20] The mechanism by which PlGF2 increases

endothelial VCAM-1 could involve either direct Flt-1-mediated upregulation of VCAM-1 expression or an indirect pathway mediated by inflammatory cytokines. Consistent with the latter possibility, PlGF treatment of human monocytes increased mRNA expression of tumor necrosis factor-α and interleukin-1β, both strong inducers of endothelial VCAM-1 expression.[25]

Periadventitial transfer of Ad.PlGF2 also markedly increased adventitial neovascularization in collared rabbit arteries. This finding further supports recent reports that PlGF is a key angiogenic mediator in diverse models of adult pathophysiological angiogenesis. However, because neovascularization was not a feature of early lesions in either apoE$^{-/-}$ or apoE$^{-/-}$:PlGF$^{-/-}$ mice, and PlGF deficiency did not alter the numbers of plaque microvessels detected in advanced lesions, atherogenic effects of PlGF appear to be largely independent of angiogenesis, at least in the apoE$^{-/-}$ mouse model. This conclusion is consistent with previous findings showing that an inhibitory anti-Flt-1 antibody reduced atherosclerosis in apoE$^{-/-}$ mice without inhibiting plaque neovascularization.[6] The role of angiogenesis as a mediator of the neointima-promoting effects of PlGF2 in VSMC hyperplasia induced by clinically relevant interventions such as balloon injury or vein grafting is not known, however, and requires further investigation.

In rabbits fed a normal diet, Ad.PlGF2 enhanced intimal thickening without increasing neointimal macrophage accumulation, which indicates that PlGF2 also has a neointima-increasing effect that does not result from intimal macrophage recruitment. Because Flt-1 is expressed in plaque VSMCs in vivo[6] and mediates direct effects of VEGF on VSMCs in vitro,[26,27] this effect could be mediated via PlGF-induced VSMC proliferation or migration, a possibility that warrants further study. The increased adventitial macrophage staining observed in Ad.PlGF2-transduced arteries in normocholesterolemic animals does suggest, however, that PlGF2 promotes monocyte chemotaxis to the adventitia in the absence of high blood cholesterol. The significance of monocyte recruitment to the adventitia for the neointima-increasing effects of PlGF2 is unclear, but this effect could contribute to the adventitial neovascular response to PlGF2.

The present study supports the emergent view that in adult animals, PlGF is a functional cytokine, particularly in pathophysiological settings, able to stimulate angiogenesis and atherogenic migration of monocytes/macrophages into the arterial wall. The finding that PlGF and its receptor, Flt-1, have a potential role in the pathogenesis of atherosclerotic disease has clinical implications. Therapeutic strategies targeting either PlGF or Flt-1 may be an attractive and novel approach to the inhibition of inflammatory macrophage infiltration underlying early atherosclerotic plaque formation. This notion is supported by previous findings that administration of anti-Flt-1 antibody has an atheroprotective effect in atherosclerosis-prone apoE-deficient mice by reducing early atherosclerotic plaque growth and plaque macrophage infiltration.[6] The finding that functional loss of PlGF and Flt-1 selectively inhibits the early stages of atherosclerosis without reducing late lesion development may not militate against a therapeutic role for PlGF-induced arteriogenesis[7,10] in ische-

mic heart disease, because patients suitable for therapeutic arteriogenesis generally have advanced atherosclerosis. Future studies in other animal models of human atherosclerosis and in human lesions will be helpful in elucidating a dual role for PlGF and Flt-1 in cardiovascular disease.

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