Angiogenesis-Dependent and Independent Phases of Intimal Hyperplasia

Rohit Khurana, Zhenwu Zhuang, Shalini Bhardwaj, Masahiro Murakami, Ebo De Muinck, Seppo Yla-Herttuala, Napoleone Ferrara, John F. Martin, Ian Zachary and Michael Simons

Circulation 2004;110;2436-2443; originally published online Oct 11, 2004;
DOI: 10.1161/01.CIR.0000145138.25577.F1
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Angiogenesis-Dependent and Independent Phases of Intimal Hyperplasia

Rohit Khurana, MD; Zhenwu Zhuang, MD; Shalini Bhardwaj; Masahiro Murakami, MD, PhD; Ebo De Muinck, MD, PhD; Seppo Yla-Herttuala, MD; Napoleone Ferrara, MD; John F. Martin, FRCP; Ian Zachary, MD; Michael Simons, MD

Background—Neointimal vascular smooth muscle cell (VSMC) proliferation is a primary cause of occlusive vascular disease, including atherosclerosis, restenosis after percutaneous interventions, and bypass graft stenosis. Angiogenesis is implicated in the progression of early atheromatous lesions in animal models, but its role in neointimal VSMC proliferation is undefined. Because percutaneous coronary interventions result in induction of periadventitial angiogenesis, we analyzed the role of this process in neointima formation.

Methods and Results—Local injury to the arterial wall in 2 different animal models induced periadventitial angiogenesis and neointima formation. Application of angiogenesis stimulators vascular endothelial growth factor (VEGF-A_{165}) or a proline/arginine-rich peptide (PR39) to the adventitia of the injured artery induced a marked increase in neointimal thickening beyond that seen with injury alone in both in vivo models. Inhibition of either VEGF (with soluble VEGF receptor 1 [sFlt1]) or fibroblast growth factor (FGF) (with a dominant=negative form of FGF receptor 1 [FGF-R1DN]), respectively, signaling reduced adventitial thickening induced by VEGF and PR39 to the level seen with mechanical arterial injury alone. However, neither inhibitor was effective in preventing neointimal thickening after mechanical injury when administered in the absence of angiogenic growth factor.

Conclusions—Our findings indicate that adventitial angiogenesis stimulates intimal thickening but does not initiate it. **(*Circulation*. 2004;110:2436-2443.)**

Key Words: growth substances ■ restenosis ■ gene therapy

Normal muscular arteries have no vessels within the inner media or intima, although the adventitial vasa vasorum is responsible for the oxygen and nutrient supply to the walls of large vessels. However, neointima can develop in a variety of pathologic states, such as long-standing hypertension, atherosclerosis, and mechanical injury such angioplasty. A number of factors are thought to contribute to neointima formation, including proliferation of adventitial and medial smooth muscle cells, circulating smooth muscle progenitors, and inflammatory processes in the media adventitia.

See p 2283

Several studies raise the possibility that vessel wall angiogenesis may be involved in the development of neointima. In the apolipoprotein $E^{-/-}$ mouse, administration of vascular endothelial growth factor (VEGF) promoted intraplaque angiogenesis and the development of atherosclerotic plaques,[1] whereas antiangiogenic drugs inhibited lesion formation.[2–4] Increased neovascularization has been observed in the vessel wall at sites of intimal hyperplasia in models of arterial stenting,[5] angioplasty,[6,7] and venous bypass graft failure.[4,8] VEGF and fibroblast growth factor (FGF) protein and gene transfer to the vasculature have been shown to influence vascular remodeling[9] with some but not other studies, suggesting that VEGF enhances postangioplasty restenosis.[10,11] It has been hypothesized that the importance of angiogenesis is dependent on the stage of lesion development, being a critical "trigger" in the early phase but receding within aging lesions.[12] However, despite a considerable amount of experimental and clinical investigation, the specific role of angiogenesis in intimal thickening has not been addressed.

To study this issue, we used 2 distinct animal models in which neointima formation was induced by different mechanical stimuli. Proangiogenic, antiangiogenic, or a combination of the 2 types of stimuli were locally applied to the arterial wall, and the extent of vascular smooth muscle cell (VSMC) accumulation and vascular wall angiogenesis was then determined. The proangiogenic stimuli used included the PR39 peptide and its biologically active fragment PR11[13,14] and

Received April 29, 2004; revision received June 11, 2004; accepted July 12, 2004.

From the Centre for Cardiovascular Biology and Medicine (R.K., J.M., I.Z.), Department of Medicine, University College, London, UK; the Section of Cardiology (R.K., Z.Z., M.M., E.D.M., M.S.), Departments of Medicine and of Pharmacology and Toxicology, Dartmouth Medical School, Lebanon, NH; the Department of Biotechnology and Molecular Medicine (S.B., S.Y.-H.), A.I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland; and the Department of Molecular Oncology (N.F.), Genentech, Inc, South San Francisco, Calif.

Correspondence to Michael Simons, MD, Section of Cardiology, Dartmouth Hitchcock Medical Center, One Medical Center Dr, Lebanon, NH 03756. E-mail Michael.Simons@Dartmouth.edu

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000145138.25577.F1**

$VEGF_{165}$. PR39 is though to initiate angiogenesis by blocking proteasome-mediated degradation of the hypoxia-inducible factor HIF-1α, a regulator of VEGF and VEGF receptor expression, and by activation of FGF receptor expression,[13] thereby stimulating both VEGF and FGF angiogenic cascades. The antiangiogenic stimuli included sFlt-1, an inhibitor of VEGF-dependent angiogenesis,[15] and a dominant-negative form of FGF receptor 1 that is known to inhibit signaling of all FGF receptors.[16]

Methods

Generation of Adenoviruses

Adenoviral (Ad) constructs (E1,E3 deleted) encoding either green fluorescent protein (GFP) (Ad-GFP), PR39 (AdPR39), and a dominant-negative FGFR1 fused with GFP (Ad-GFP-FGFR1DN) were generated by the Vector Core Laboratory of the Harvard Gene Therapy Initiative. VEGF-A_{165} (Ad-VEGF-A_{165}) and LacZ (Ad-LacZ) adenoviruses were produced by Ark Therapeutics. Adenoviruses were desalted on G50 Sephadex columns (Boehringer Mannheim) immediately before their use in animal studies.

Collar Model and Periadventitial Gene Transfer

All protocols involving animals conformed to animal care and use guidelines of Dartmouth College. Male New Zealand White rabbits (3.0 to 3.5 kg; Charles River, Cambridge, Mass) were anesthetized, and a biologically inert, silicone collar (20 mm long; inlet/outlet diameter 1.8 mm; Ark Therapeutics) was placed around the right carotid artery. The contralateral artery was sham-operated by exposing it to similar stretch as the collared artery. Five days after collaring, 100 μL adenoviral gene solution (5×10^9 plaque-forming units [PFU]) and/or soluble flt-1 IgG1-3 (0.4 mg/kg; Genentech, Inc.) was introduced into the periadventitial space between the collar and artery. Treated and control arteries were harvested 4 or 9 days after gene transfer, flushed with ice-cold saline, and divided into 2; the proximal part was immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 hours, rinsed in 15% sucrose (pH 7.4) overnight, and embedded in paraffin; the distal part was either immersion-fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.4) for 30 minutes, rinsed in PBS for 15 minutes, and embedded in OCT compound (Sakura Finetek USA, Inc) or snap-frozen in LN_2 and stored at −70°C for total RNA extraction.

Rat Balloon Carotid Angioplasty Model

Vascular injury of the left common carotid arteries was performed in anesthetized, male, Sprague-Dawley rats (240 to 310 g; Harlan, Indianapolis, Ind) as previously described,[17] with an infiltrated 2F Fogarty embolectomy catheter (Edwards Lifesciences Co). After injury, the external carotid artery was ligated proximal to the cannulation site, and 250 μL 28% pluronic F-127 gel Sigma) containing 0.1 mg/kg PR11 (n=8), 0.1 mg/kg PR11 AAA (n=9), 0.4 mg/kg sFlt-1 (n=7), or 5×10^9 pfu Ad-GFP-FGFR1DN (n=10) was applied around the injured segment. Fourteen days after injury, carotid arteries were perfusion-fixed in situ by incubation for 3 hours in 3.8% formalin in PBS at 120 mm Hg. Arteries were then cut into 3 segments of equal length and embedded in paraffin. Sections used for immunohistochemistry were rinsed with cold PBS and embedded in OCT (Sakura Finetek USA, Inc).

RNA Isolation and RT-PCR

Total RNA was isolated from 100 to 150 mg (pooled from 2 rabbit carotid arteries) frozen tissue by an RNA purification system (Invitrogen) and treated with RNase-free DNase for 1 hour at 37°C. For detection of Ad-PR39, total RNA (500 ng) was reverse-transcribed (RT) to cDNA with 5 U RT and amplified (AccessQuick, Promega) with 100 nmol/L each of forward (5′-CTCTACCGCCTCCTGGAGCT-3′ and reverse (5′-GGCCCTTC-ATAATATCCCCCA-3′) primers. The RT step was carried out at 48°C for 45 minutes. After denaturation at 95°C for 2 minutes, 38 cycles of polymerase chain reaction (PCR) were performed with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Nested PCR was used for the amplification of VEGF-A with the primers 5′-TCGATCCATGAACTTTCTGC-3′, 5′-TTCGTTTAA-CTCAAGCTGCC-3′, 5′-GAGCCTTGCCTTGCTGCTC-3′, and 5′-GGAACATTTACACGTCTGCG-3′. PCR products of predicted size were confirmed by electrophoresis on a 1% agarose gel.

VEGF, FGFR1, and β-actin mRNA expression was quantified by real-time PCR. Two micrograms of total RNA was RT with a superscript first-strand synthesis kit (Invitrogen), and PCR was performed with 12.5 ng RNA, 50 nmol/L of forward and reverse primers (Invitrogen), SYBR Green PCR core reagents (Applied Biosystems), and the GeneAmp 5700 sequence detection system (Applied Biosystems) under the following conditions: denaturation at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Forward and reverse primers for VEGF, FGFR1 (a gift of William A. Paznekas and Ethylin Wang Jabs, NIH P60 DE13078) and β-actin were, respectively, 5′-TCAACGTCACCA-TGCAGATCA-3′ and 5′-AGCTCATCTCCCCTATGTGCTG-3′; 5′-GACAAGGACAAACCCAACCGT-3′ and 5′-GCATCCGACT-TCAACATCTTCAC-3′; and 5′-CTCATGAAGATCCCTCAC-GGAGC-3′ and 5′-GCACAGCTTCTCCTTGATGTCC-3′. The expression of VEGF, FGFR1, and β-actin mRNA within each sample was calculated according to a standard calibration curve constructed for each amplicon (n=3). Values for each gene were normalized to levels of the housekeeping gene β-actin.

Immunohistochemistry

Five-micron arterial sections were deparaffinized and stained with hematoxylin and eosin for morphometric analysis or immunostained with the avidin-biotin-peroxidase system (Vector Laboratories); 3,3′-diaminobenzidine (Biogenex) and a light hematoxylin counterstain were used as substrates for visualization. The following antibodies were used: mouse monoclonal antibody against CD31 (DAKO; 1:50), monoclonal antibody RAM-11 (DAKO; 1:500), VSMC-specific α-actin monoclonal antibody HHF35 (DAKO; 1:200), and mouse anti-rat CD31 clone TLD-3A12 (Pharmingen; 1:25).

β-Galactosidase Staining

Five-micron sections from fixed, OCT-embedded, frozen arteries were incubated in the dark at 37°C overnight in β-galactosidase (β-gal) staining solution containing 1 mg/mL 5-bromo-4-chloro-4-indolyl β-D-galactopyranoside (X-Gal, Sigma), 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L $MgCl_2$, 0.002% Igepal CA160 (Sigma), and 0.01% sodium dodecyl sulfate and titrated with NaH_2PO_4 to pH 7.4. Sections were counterstained with eosin and visualized under light microscopy.

Confocal Microscopy

Four-micron sections of OCT-embedded, GFP-transduced arteries were fixed in acetone for 5 minutes at −20°C, mounted in antifade medium (Biomeda GelMount), and visualized with a Bio-Rad MRC-1024 krypton/argon laser confocal system and an 522DF35 emission filter for GFP.

Morphometry and Image Analysis

Images of stained sections were acquired at ×5 or ×40 with a high-resolution color camera (Olympus BH-2 microscope, Polaroid DMC-1 camera) and analyzed with automated image-analysis software (Image J, National Institutes of Health). Intimal thickening was measured by calculating the intima to media ratio, where the intima was defined as the areas between the luminal endothelium and the internal elastic lamina, and the media was defined as the area between the internal and external elastic laminas. Sections were cut at 6 to 10 500-μm intervals along the length of the arterial segment, stained with hematoxylin and eosin, and then blindly analyzed for each artery to obtain intima-media ratios.

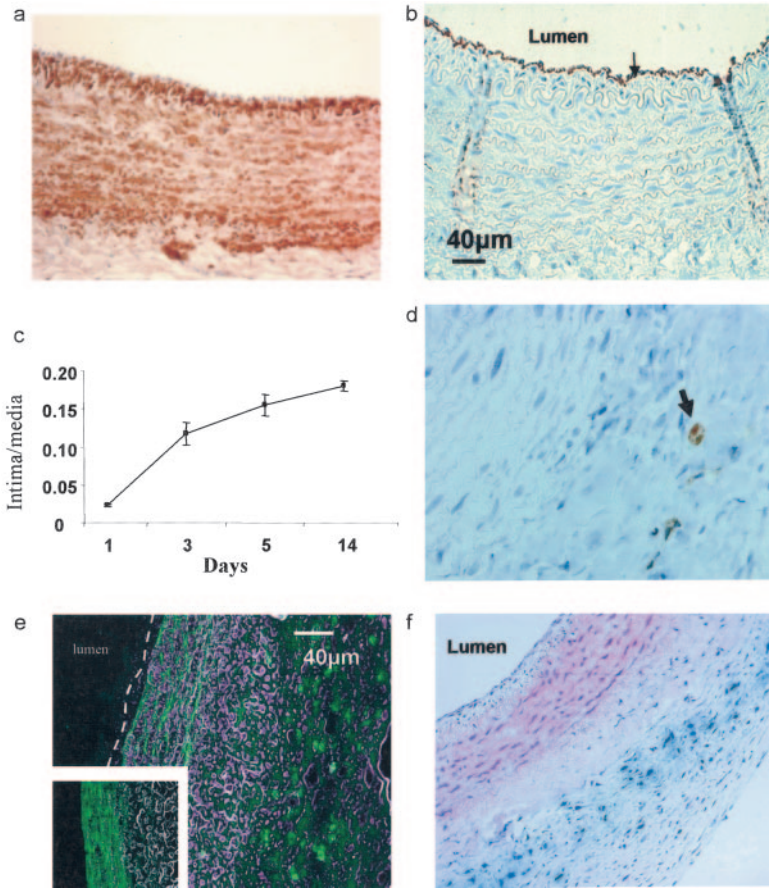

Figure 1. Collared rabbit carotid artery model of neointima formation. a, Anti α-actin immunostain ($\times$400) indicates neointima comprises predominantly VSMCs. b, Anti-CD31 immunostain ($\times$400, hematoxylin counterstain) for endothelial cells indicates that luminal endothelium (arrow) remains intact. c, Time course of intimal thickening, with each time point representing mean intima-media ratio in 6 to 10 random sections from arteries (n=3). d, Anti-CD31 immunostain demonstrates low level of adventitial neovascularization. e, Adenoviral-GFP gene (5×10^9 PFU) transduction demonstrates that adventitial cells of collared arteries are predominantly targeted. Endothelial and neointimal cells are not transfected. Insert: Sham control illustrates autofluorescence within elastin fibers of media. f, X-gal stain after adenoviral-LacZ gene (5×10^9 PFU) transduction demonstrates adventitial targeting of gene in collared artery ($\times$200).

Arterial Delivery of PR11 Peptide

The release kinetics of PR11 from 28% pluronic gel were determined in vitro by dissolving increasing volumes (5 to 40 μL) of ^{125}I-PR11 (260 μCi/mL; Peninsula Labs Inc) in 0.5 mL of 28% pluronic gel prepared in Ca^{2+}/Mg^{2+}-free PBS and incubating the gel in 1 mL of 1% albumin/PBS at 37°C. Radioactivity released from the gel was measured after different times up to 48 hours. Experiments were repeated 3 times. The values are expressed as a mean percentage over the baseline count. To measure arterial delivery of PR11, 10 μL ^{125}I-PR11 (2.6 μCi) in 250 μL of 28% pluronic gel was applied to the adventitia of the left common carotid artery of male Sprague-Dawley rats immediately after balloon injury. After 48 hours, animals were perfusion-fixed at 37°C with 0.9% saline followed by 4% formalin. The tissue was paraffin-embedded, and 4-μm sections were cut and mounted on glass slides. The slides were immersed in developer (Eastman Kodak), air-dried for 45 minutes, and allowed to develop for 7 days at 4°C, after which the sections were fixed (Eastman Kodak). The distribution of silver grains activated by γ-radiation from ^{125}I-PR11 was viewed under dark-field microscopy.

Cell Culture and SMC Migration Assay

Porcine SMCs (passage 3 to 7) were grown to 70% to 90% confluence on noncoated culture dishes in full serum (10%) containing Dulbecco's modified Eagle's medium (DMEM). After overnight starvation in 0.5% fetal calf serum (FCS)/DMEM, the cells were washed twice with Ca^{2+}/Mg^{2+}-free DPBS (Mediatech, Inc), detached with a nonenzymatic cell dissociation buffer (Sigma) to obtain single-cell suspensions, and resuspended in DMEM/0.5% FCS at a concentration of 5×10^5 cells/mL. Aliquots (0.1 mL) of cells (5×10^4 cells) were added to the upper chamber of a noncoated transwell PVF-free membrane with 8-μm pores in 24-well plates (Corning Costar Inc) and allowed to attach for 2 hours. The upper chambers were then transferred to the lower transwell compartment containing 0.6 mL of 0.5% FCS/DMEM and PR39 (Genemed Inc), PDGF-BB (Peprotech), and VEGF (Peprotech) in varying concentrations. Incubation was continued for 1 hour and 4 hours at 37°C, after which the adherent cells were fixed with 4% formalin, washed repeatedly with PBS, and then stained with toluidine blue. The cells on the upper surface of the membrane were scraped off, the membrane was mounted onto a microscope slide, and the number of cells that had migrated to the lower surface of each membrane was counted in 5 separate high-power fields at a magnification of $\times$200 (Olympus BH-2). Experiments were performed in quadruplicate, and SMC migration was averaged and expresses as mean$\pm$SEM.

Statistical Analysis

Intima-media ratios were averaged and expressed as mean$\pm$SEM. CD31 staining was positive when a single cell or a mature vessel with a lumen was present in the adventitia. Numbers of vessels were expressed per mm^2 of the adventitial area. Differences between treatment groups were evaluated by ANOVA and Bonferroni correction (SPSS).

Results

To examine the effect of these agents on intimal thickening, we first used a model in which positioning of a nonocclusive, inert, silicone collar around the rabbit carotid artery induces a VSMC-rich neointima without endoluminal manipulation and in the presence of an intact endothelium (Figure 1, a and b) that reaches a maximum effect after 14 days (Figure 1c). CD31 immunostaining demonstrates that some adventitial neovascularization occurred in the area of the vessel covered by the collar (Figure 1d). The presence of the collar allows localized delivery of biologic agents to the arterial wall. Injection of adenoviruses encoding GFP protein demon-

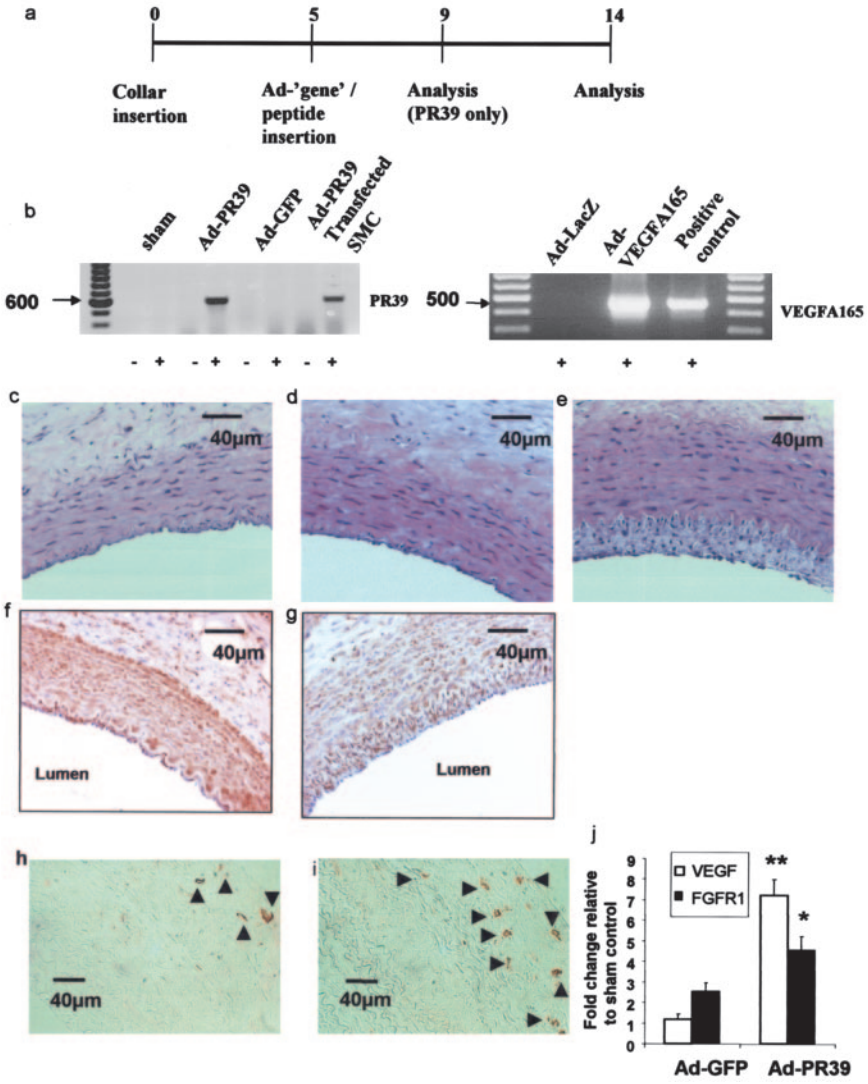

Figure 2. Ad-PR39 and Ad-VEGF$_{165}$ induce adventitial neovascularization and intimal thickening. a, Summary of experimental plan. Periadventitial application of adenoviral PR39, VEGF-A$_{165}$, FGFR1-DN, and sFlt-1 peptide to collared segments of carotid arteries was performed 5 days after collaring; carotid tissue was analyzed 4 or 9 days later. b, RT-PCR analysis reveals presence of PR39 (600-bp band) and VEGF-A$_{165}$ (547-bp band) in total RNA obtained from pooled arteries treated with Ad-PR39 (n=4) and Ad-VEGF-A$_{165}$ (n=2), respectively. Left margin and right margins, molecular size marker; − indicates without RT step; +, with RT. Neointima formation (×400) 9 days after transduction with c, Ad-GFP; d, Ad-PR39 (4 days); e, Ad-PR39; f, Ad-LacZ; and g, Ad-VEGF-A$_{165}$. (c–e, hematoxylin and eosin sections; f,g, HHF stain for VSMCs). Ad-PR39 significantly increases adventitial neoangiogenesis (CD31-positive structures/mm^2 adventitia) at i, 9 days (123 vs 17±10, $P<0.001$) compared with h, Ad-GFP transduction. j, Real-time PCR analysis at 9 days shows that Ad-PR39 significantly increased levels of VEGF-A (7.21±0.78 vs 1.20±0.23, $^{**}P<0.001$) and FGFR1 mRNA (4.54±0.66 vs 2.55±0.4, $^{*}P<0.03$), after normalization to endogenous β-actin levels, relative to quantities detected in sham controls.

strated the strongest expression in the adventitia and expression in isolated medial cells (Figure 1f, left), localized to the region of the arteries surrounded by the collar, and no expression in the adjacent noncollared artery.

The effect of PR39 and VEGF on neointima formation in this model was then tested by injection of adenoviruses encoding these proteins, as well as vector controls (Figure 2a). Periadventitial delivery of Ad-PR39 and Ad-VEGF$_{165}$ to collared arteries resulted in easily detectable expression of both proteins (Figure 2b and 2c), and both genes induced a striking increase in intimal thickening (Figure 2c–2g). Administration of Ad-GFP (Figure 2c) or Ad-LacZ (Figure 2f) constructs had no effect on neointima formation. Ad-PR39 stimulated neovascularization almost exclusively in the adventitia, with no significant medial and intimal CD31 staining (Figure 2i), whereas Ad-GFP gene transfer had no significant effect on adventitial angiogenesis (Figure 2h) or neointima formation.

Because PR39 is thought to mediate its proangiogenic effects by stimulation of VEGF and FGF receptor expression, we assayed expression of these genes in Ad-PR39–treated arteries. Real-time quantitative PCR analysis of Ad-PR39–transduced arteries demonstrated that VEGF-A and FGFR1 mRNAs were increased by 7-fold and 2-fold, respectively, compared with Ad-GFP–transduced control arteries (Figure 2j).

To test whether the stimulation of periadventitial angiogenesis was responsible for increased neointima development after Ad-PR39 or Ad-VEGF administration, we used inhibitors of VEGF and FGF signaling. VEGF activity was inhibited with a recombinant sFlt-1, and FGF signaling was inhibited with a dominant-negative FGFR1 construct, Ad-FGFR1-DN.

Transduction of human umbilical vein endothelial cells with Ad-FGFR1-DN but not with a control adenovirus inhibited FGF2-induced extracellularly regulated kinase-1/2 phosphorylation (Figure 3a). In vivo studies demonstrated that Ad-FGFR1-DN successfully transduced the adventitia of collared arteries (Figure 3b). Treatment of collared arteries with either sFlt-1 or Ad-FGFR1-DN in the absence of proangiogenic stimuli caused no significant decrease in basal neointima formation, which was similar to that of control arteries (Figure 3c, 3d, and 3h). However, coadministration of collared arteries with PR39 and either sFlt-1 or Ad-FGFR1-DN inhibited the proangiogenic effects of PR39 in

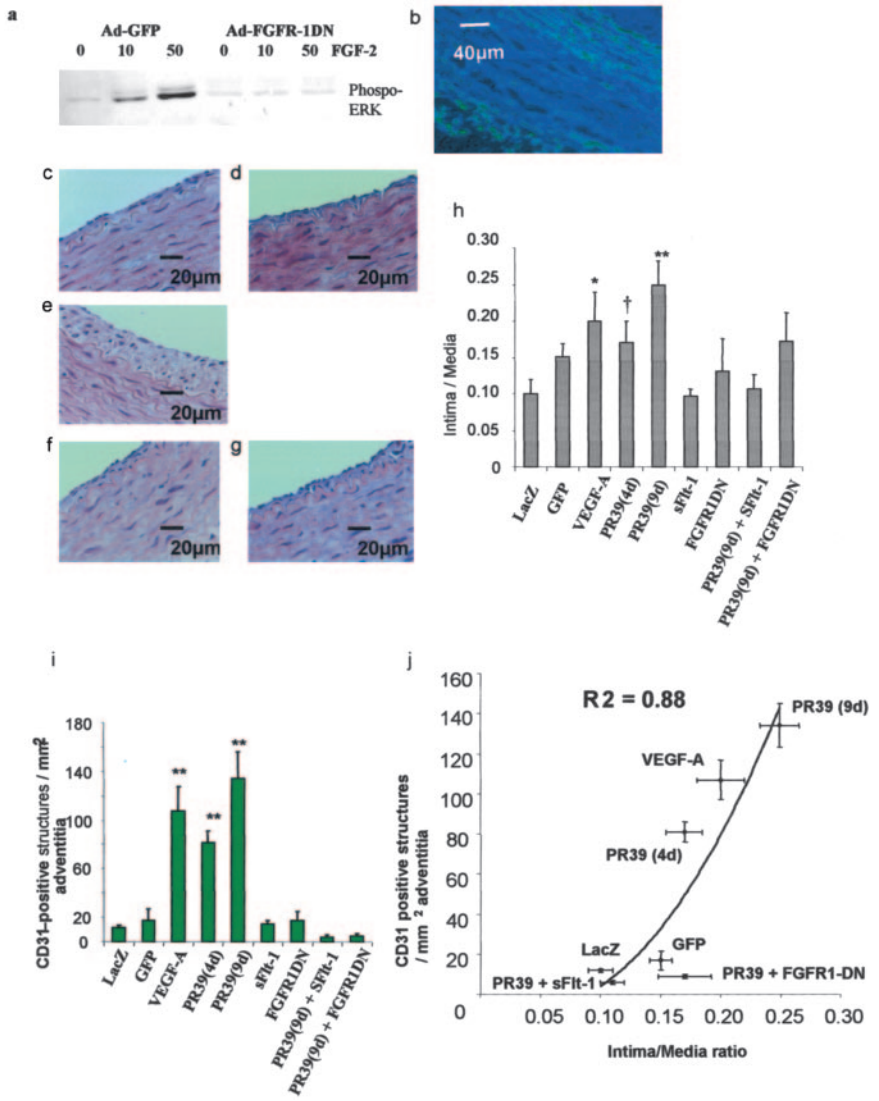

Figure 3. Inhibition of VEGF and FGFR1 block PR39-induced intimal thickening and adventitial angiogenesis. a, Human umbilical vein endothelial cells were transduced with adenoviruses and serum-starved (0.25% fetal bovine serum, 0.2% bovine serum albumin) for 16 hours and then stimulated with FGF_2 (10 or 50 ng/mL) for 10 minutes. Western blot for phospho-ERK1/2 was performed with no response in Ad-FGFR1-DN at increasing concentrations of FGF_2 induction. b, Confocal immunofluorescence image showing adventitial distribution of Ad-FGFR1-DN in collared carotid artery. At 9 days, hematoxylin and eosin stains show that both c, sFlt-1 and d, Ad-FGFR1-DN transfer alone had no effect on neointima formation, whereas e, Ad-PR39 increased intimal thickening after 9 days. Intimal thickening induced by Ad-PR39 was inhibited by cotransfection with f, sFlt-1 or g, Ad-FGFR1-DN to levels comparable to control gene transfection. h, Quantification of intima-media ratios (*$P<0.01$, **$P<0.001$, †P=NS). i, Quantification of adventitial neovascularization shows that PR39 induces marked angiogenic response after 4 and 9 days that is inhibited by cotreatment with either sFlt-1 or FGFR1-DN (**$P<0.001$). j, Adventitial angiogenesis is significantly correlated with degree of intimal thickening in collared arteries after treatment with angiogenesis stimulators and/or inhibitors.

the adventitia and reduced intimal thickening to control levels (Figure 3e–3i). This strongly suggests that PR39-induced adventitial angiogenesis is mediated through upregulation of both VEGF-A_{165} and FGFR1 and promotes neointima formation. Importantly, a comparison of all treatment groups showed a strong positive correlation ($R^2=0.88$) between adventitial blood vessel formation and intima-media ratios in collared arteries (Figure 3j).

To further investigate the role of periadventitial angiogenesis in promoting neointima formation, we used a rat carotid artery injury model. As with the collar model, balloon injury in the rat carotid artery induced a mild degree of periadventitial angiogenesis, as demonstrated by accumulation of CD31-positive cells (Figure 4a). To further stimulate angiogenesis, PR11, a biologically active truncated form of PR39, was delivered to the periadventitial surface of the carotid artery after balloon catheter injury and endothelial denudation. Effective delivery of the peptide in a polaxamer gel applied to the adventitial surface of the rat carotid artery immediately after balloon denudation was demonstrated with the use of ^{125}I-PR11. Autoradiography of arterial sections harvested 48 hours after ^{125}I-PR11 demonstrated the presence of radioactive tracer within the adventitial and outer medial layers (Figure 4b). Furthermore, virtually all of the ^{125}I-PR11 was released from the gel in vitro within 10 hours at all peptide concentrations tested (data not shown). Periadventitial gel delivery of PR11 at the time of balloon injury significantly enhanced intimal hyperplasia compared with application of a biologically inactive control peptide, PR11AAA, or balloon angioplasty alone after 14 days (Figure 4c). CD31 immunostaining showed that the extent of luminal reendothelialization was similar in control and PR11-treated arteries (results not shown). Similar to results in the collar model, periadventitial administration of sFlt-1 or Ad-FGFR1-DN alone had no effect on the extent of basal neointimal formation while reducing the extent of adventitial angiogenesis (Figure 4a and 4c).

The results in both models suggest that induction of angiogenesis further promotes neointimal growth. One potential mechanism here, in addition to stimulation of adventitial angiogenesis, is the ability of VEGF and PR39 to induce SMC migration. In a transwell migration assay, both

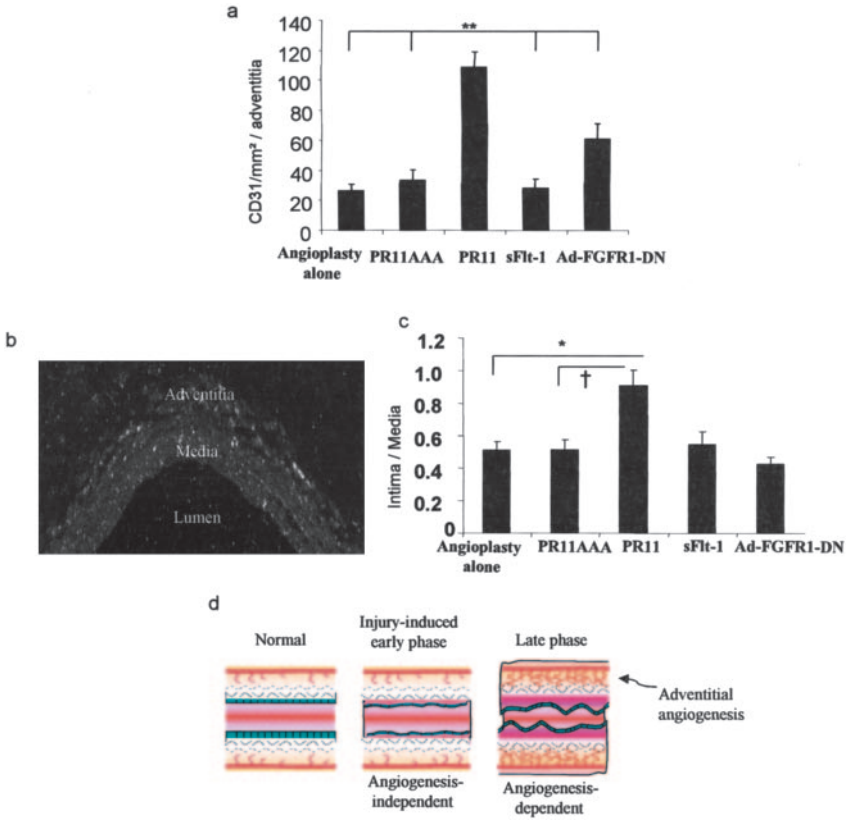

Figure 4. Adventitial angiogenesis and neointimal proliferation in rat balloon injury model. a, Adventitial angiogenesis as determined by CD31 staining is significantly increased by PR11 but unaffected by PR11AAA (**$P<0.001$). b, Dark-field autoradiography of 4-μm section of balloon-injured rat carotid artery treated with periadventitial 0.65 μCi ^{125}I-PR11 in 250 μL pluronic gel (28%) shows adventitial and medial retention of peptide up to 48 hours after treatment c, Periadventitial PR11 peptide (0.1 mg/kg in 250 μL pluronic gel) (28%) increases neointima formation compared with percutaneous intervention alone (0.9 vs 0.55,*$P<0.02$) and PR11AAA treatment (0.9 vs 0.52, †$P<0.01$). Neither sFlt-1 nor Ad-FGFR1-DN significantly altered intimal thickness. d, Schematic illustrating proposed 2-stage model of intimal thickening: after initial injury-induced and angiogenesis-independent early phase, adventitial angiogenesis contributes to later phase of intimal hyperplasia.

VEGF-A and PR39 peptides exerted a significant promigratory effect on aortic SMCs in both a time- and concentration-dependent fashion (Figure 5). The effect of PR39 was substantially greater than the highest concentration of VEGF-A assayed, after both 1 and 4 hours. PR39 at the higher concentration was not significantly greater than platelet-derived growth factor-BB, which is known to be a potent SMC chemoattractant.

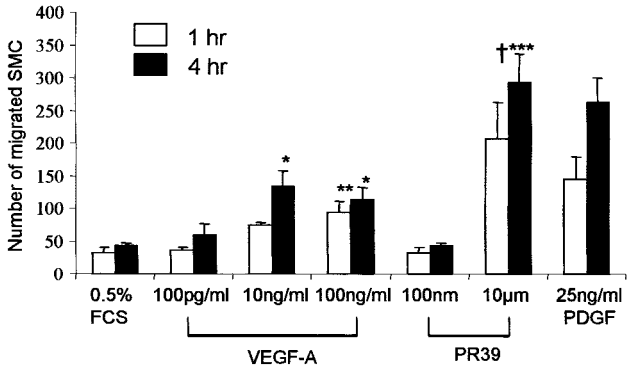

Figure 5. PR39 and VEGF-A stimulate SMC migration in time- and concentration-dependent manner. VEGF-A significantly promotes migration of aortic SMCs through 8-μm pore-size transwell migration chamber at highest concentration after 1 hour (**$P<0.001$). Migration is significantly enhanced after 4 hours at both 10 and 100 ng/mL (*$P<0.05$). PR39 also exerts significant promigratory effect at higher but not lower concentration after both 1 and 4 hours (***$P<0.0001$). Effect of PR39 is much greater compared with VEGF-A (†$P<0.01$) with no significant difference compared with PDGF-BB.

Discussion

This study demonstrates that neointimal formation after mechanical injury of an artery consists of angiogenesis-dependent and -independent components. The mechanical injury to the artery by itself induces a mild adventitial angiogenic response. The augmentation of this response leads to a greatly increased neointima formation, whereas even full inhibition of adventitial neovascularization does not eliminate the injury-induced component of neointima development.

Several lines of evidence support these conclusions. Both Ad-PR39 and Ad-VEGF$_{165}$ induced a strong neovascular response specifically in the adventitia and enhanced intimal thickening induced by periadventitial collar placement. Furthermore, a strong positive correlation was observed between the degree of adventitial angiogenesis and intimal thickening. These neointima-inducing effects were completely abolished by coadministration of angiogenesis inhibitors sFlt-1 and FGF-R1DN. This effect was seen in 2 distinct models: a collar model that preserves the endothelium and the balloon injury model that does not. At the same time, administration of these inhibitors by themselves, in the absence of angiogenesis stimulators, had no effect on injury-induced neointima formation in either model.

Although adventitial neovascularization was responsible for promotion of intimal thickening, the early phase of neointimal VSMC accumulation was largely independent of angiogenic stimulation. Two lines of evidence support this conclusion. First, transduction of collared arteries for shorter times induced a striking adventitial neovascular response but had little effect on neointimal thickness, thus suggesting that

periadventitial angiogenesis has little effect on the extent or time course of the injury phase of neointima development. Second, as already mentioned, inhibition of angiogenesis by sFlt1 and Ad-FGFR1-DN did not suppress basal intimal thickening induced in the absence of an angiogenic stimulus in either the collar or balloon injury model. This leads us to conclude that angiogenesis is not a sufficient trigger for the initiation of neointima formation but that proangiogenic agents can enhance or promote growth of an intimal lesion that was initiated by another stimulus.

Endogenous adventitial angiogenesis in the absence of an exogenous angiogenic stimulus was low and was not significantly reduced by either sFlt-1 or Ad-FGFR1-DN. However, the fact that the much greater level of angiogenesis induced in PR39-transduced arteries was completely suppressed by these angiogenic inhibitors argues that basal angiogenesis does not play an important role in neointima formation in the absence of an angiogenic stimulus.

The occurrence of a strong adventitial neovascular response in collared and balloon-injured vessels transduced with angiogenic factors indicates that the adventitia may play a major role in vascular remodeling and intimal thickening.[18] At least 2 mechanisms can explain the close association between adventitial neovascularization and intimal thickening in response to angiogenic stimuli. On the one hand, the local supply of nutrients and oxygen to the inner layers of the artery may become rate limiting after the initial phase, and the growth of adventitial new vessels may create a microenvironment permissive of vascular remodeling.[19] On the other, local production of growth and chemotactic factors, such as VEGF, FGFs, and platelet-derived growth factor-BB, may directly stimulate VSMC accumulation.[20,21] In accordance with this notion, both PR39 and VEGF-A exerted a direct chemotactic effect on SMCs. Of note, although the ability of VEGF to induce SMC migration has been previously reported,[22,23] the same has not been reported for PR39. This process may well be amplified by new vessels in the adventitia that provide an additional source of VSMC mitogens or circulating smooth muscle progenitor cells.[24]

Most accounts of the formation of VSMC-rich neointimal lesions stress the role of either resident subendothelial or medial VSMCs, whereas the involvement of the adventitia has received little attention. Recent studies suggest that migration of myofibroblasts that are either resident within the adventitia or recruited as progenitors from the circulation contribute significantly to neointimal VSMC accumulation.[25,26] The possibility that the adventitial vasa vasorum of human coronary arteries plays a role in the formation of atherosclerotic plaques was first advanced by Barger et al,[27] and it has subsequently been postulated that the density of newly formed vasa vasorum in response to injury is proportional to vessel stenosis.[7]

On the basis of the result of the present study, we propose a 2-step model of neointima formation: an early angiogenesis-independent phase initiated by injury or a mechanical stimulus and a later angiogenesis-dependent phase, during which the stimulation of adventitial angiogenesis further augments neointima formation (Figure 4d).

The demonstration of an angiogenesis-dependent phase of neointima formation may explain progression of arteriopathy in a number of disease processes, including atherosclerosis, and may also explain a marked increase in postangioplasty restenosis in patients treated with the bone marrow stem cell–releasing agent G-CSF observed in a recent clinical trial.[28] Our findings may have particular relevance for vasculitis, a disease of the vessel wall associated with intimal hyperplasia and thought to be triggered by invasion of the outer media and adventitia by T cells, dendritic cells, monocytes, and granulocytes.[29] The pathogenic mechanisms underlying vasculitis, specifically the roles of adventitial cells and neovascularization, have not been completely defined. Increased serum and local production of VEGF has been reported in Kawasaki disease, a vasculitis preferentially affecting large vessels,[30] but a direct pathogenic role of VEGF has not been demonstrated. Further investigation of the roles of angiogenesis and angiogenic cytokines in vasculitis could generate insight into the underlying pathogenic mechanism(s). The appreciation of the contributory role of adventitial neovascularization in the pathogenesis of vascular disease is of particular importance in the context of angiogenic cytokine gene therapy trials, where safety issues are a foremost concern.[31]

Acknowledgment

This study was supported in part by NIH grants HL70247, HL53793, and HL62289 (to M.S.).

References

1. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*. 2001;7:425–429.
2. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation*. 1999;99:1726–1732.
3. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies S, Javaherian K, Folkman J. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc Natl Acad Sci U S A*. 2003;100:4736–4741.
4. Shigematsu K, Yasuhara H, Shigematsu H. Topical application of antiangiogenic agent AGM-1470 suppresses anastomotic intimal hyperplasia after ePTFE grafting in a rabbit model. *Surgery*. 2001;129:220–230.
5. Shibata M, Suzuki H, Nakatani M, Koba S, Geshi E, Katagiri T, Takeyama Y. The involvement of vascular endothelial growth factor and flt-1 in the process of neointimal proliferation in pig coronary arteries following stent implantation. *Histochem Cell Biol*. 2001;116:471–481.
6. Edelman ER, Nugent MA, Smith LT, an Karnovsky MJ. Basic fibroblast growth factor enhances the coupling of intimal hyperplasia and proliferation of vasa vasorum in injured rat arteries. *J Clin Invest*. 1992;89:465–473.
7. Kwon HM, Sangiorgi G, Ritman EL, Lerman A, McKenna C, Virmani R, Edwards WD, Holmes DR, Schwartz RS. Adventitial vasa vasorum in balloon-injured coronary arteries: visualization and quantitation by a microscopic three-dimensional computed tomography technique. *J Am Coll Cardiol*. 1998;32:2072–2079.
8. Westerband A, Gentile AT, Hunter GC, Gooden MA, Aguirre ML, Berman SS, Mills JL. Intimal growth and neovascularization in human stenotic vein grafts. *J Am Coll Surg*. 2000;191:264–271.
9. Yla-Herttuala S, Martin JF. Cardiovascular gene therapy. *Lancet*. 2000;355:213–222.
10. Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF, Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal

hyperplasia in balloon- injured rat carotid artery [see comments]. *Circulation*. 1995;91:2793–2801.
11. Lazarous DF, Shou M, Scheinowitz M, Hodge E, Thirumurti V, Kitsiou AN, Stiber JA, Lobo AD, Hunsberger S, Guetta E, et al. Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation*. 1996;94:1074–1082.
12. Ross JS, Stagliano NE, Donovan MJ, Breitbart RE, Ginsburg GS. Atherosclerosis: a cancer of the blood vessels? *Am J Clin Pathol.* 2001; 116(suppl):S97–S107.
13. Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, et al. PR39, a peptide regulator of angiogenesis. *Nat Med*. 2000;6:49–55.
14. Bao J, Sato K, Li M, Gao Y, Abid R, Aird W, Simons M, Post MJ. PR-39 and PR-11 peptides inhibit ischemia-reperfusion injury by blocking proteasome-mediated IκBα degradation. *Am J Physiol Heart Circ Physiol*. 2001;281:H2612–H2618.
15. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669–676.
16. Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J*. 1994;13:3296–3301.
17. Simons M, Edelman ER, DeKeyser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature*. 1992;359:67–70.
18. Wilcox JN, Waksman R, King SB, Scott NA. The role of the adventitia in the arterial response to angioplasty: the effect of intravascular radiation. *Int J Radiat Oncol Biol Phys*. 1996;36:789–796.
19. Stupack DG, Cheresh DA. ECM remodeling regulates angiogenesis: endothelial integrins look for new ligands. *Sci STKE*. 2002:PE7.
20. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9: 653–660.
21. Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med*. 2003;9:604–613.
22. Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES. Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol*. 2001;188:359–368.
23. Grosskreutz CL, Anand-Apte B, Duplaa C, Quinn TP, Terman BI, Zetter B, D'Amore PA. Vascular endothelial growth factor-induced migration of vascular smooth muscle cells in vitro. *Microvasc Res* 1999;58:128–136.
24. Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM. Smooth muscle progenitor cells in human blood. *Circulation*. 2002;106: 1199–1204.
25. Sartore S, Chiavegato A, Faggin E, Franch R, Puato M, Ausoni S, Pauletto P. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circ Res*. 2001;89:1111–1121.
26. Wilcox JN, Okamoto EI, Nakahara KI, Vinten-Johansen J. Perivascular responses after angioplasty: which may contribute to postangioplasty restenosis: a role for circulating myofibroblast precursors? *Ann N Y Acad Sci* 2001;947:68–90; discussion 90–62.
27. Barger AC, Beeuwkes RD, Lainey LL, Silverman KJ. Hypothesis: vasa vasorum and neovascularization of human coronary arteries: a possible role in the pathophysiology of atherosclerosis. *N Engl J Med*. 1984;310: 175–177.
28. Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK, Kim YJ, Soo Lee D, Sohn DW, Han KS, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet*. 2004;363:751–756.
29. Weyand CM, Goronzy JJ. Medium- and large-vessel vasculitis. *N Engl J Med*. 2003;349:160–169.
30. Terai M, Yasukawa K, Narumoto S, Tateno S, Oana S, Kohno Y. Vascular endothelial growth factor in acute Kawasaki disease. *Am J Cardiol*. 1999;83:337–339.
31. Simons M, Wave SA. Therapeutic angiogenesis in cardiovascular disease. *Nat Rev Drug Discov*. 2003;2:863–871.