

# Blockade of Vascular Endothelial Growth Factor Suppresses Experimental Restenosis After Intraluminal Injury by Inhibiting Recruitment of Monocyte Lineage Cells

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**Background**—Therapeutic angiogenesis by delivery of vascular endothelial growth factor (VEGF) has attracted attention. However, the role and function of VEGF in experimental restenosis (neointimal formation) after vascular intraluminal injury have not been addressed.

**Methods and Results**—We report herein that blockade of VEGF by soluble VEGF receptor 1 (*sFlt-1*) gene transfer attenuated neointimal formation after intraluminal injury in rabbits, rats, and mice. *sFlt-1* gene transfer markedly attenuated the early vascular inflammation and proliferation and later neointimal formation. *sFlt-1* gene transfer also inhibited increased expression of inflammatory factors such as monocyte chemoattractant protein-1 and VEGF. Intravascular VEGF gene transfer enhanced angiogenesis in the adventitia but did not reduce neointimal formation.

**Conclusions**—Increased expression and activity of VEGF are essential in the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. (*Circulation*. 2004;110:2444-2452.)

**Key Words:** restenosis ■ remodeling ■ inflammation ■ endothelium-derived factors ■ gene therapy

Vascular endothelial growth factor (VEGF) has attracted attention for endothelial regeneration and angiogenesis.<sup>1-3</sup> VEGF is one of the most potent vascular permeability factors known, is thought to function as an endogenous regulator of endothelial integrity after injury, and thus, protects the artery from disease progression.<sup>4</sup> Previous animal studies have reported that local delivery of VEGF after endothelial injury promotes endothelial regeneration, accelerates the recovery of endothelium-dependent relaxation, and reduces neointimal formation (see review<sup>4</sup>), suggesting the close correlation between accelerated endothelial integrity and reduced neointima after balloon injury. Increased expression of VEGF and its 2 receptors (VEGFR-1, Flt-1; VEGFR-2, Flk-1) in atherosclerotic and restenotic lesions has been reported.<sup>5-7</sup> However, there is still considerable debate over the vasculoprotective versus atherogenic effects of VEGF.<sup>8</sup> Emerging evidence suggests that (1) VEGF induces migration and activation of monocytes<sup>9</sup>; (2) VEGF induces adhesion molecules<sup>10</sup> and monocyte chemoattractant protein-1 (MCP-1)<sup>11</sup>; and (3) VEGF enhances neointimal formation and atherogenesis by stimulating intraplaque angiogenesis in hypercholesterolemic animals without balloon injury<sup>12,13</sup> or by increasing monocyte infiltration into atheroscle-

rotic lesions.<sup>14</sup> Therefore, it remains unclear whether VEGF protects the artery from vascular disease or accelerates vascular disease.

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Clinical and experimental studies involving arterial gene transfer of VEGF showed that it failed to reduce restenosis after balloon angioplasty.<sup>15-17</sup> The role of VEGF in restenotic changes (neointimal formation and negative remodeling) after injury therefore remains a mystery. This is mainly because the inhibitor of VEGF has not been tested for experimental restenosis, although inhibitors of VEGF are currently being evaluated for tumor angiogenesis and other treatment-intractable inflammatory disorders.<sup>3</sup> It is practically impossible to investigate the role of VEGF in postnatal life in mice lacking VEGF or its receptors, because the absence of VEGF function leads to embryonic lethality owing to vascular defects.<sup>4</sup> A soluble form of the VEGF receptor-1 (*sFlt-1*) is expressed endogenously by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor against VEGF.<sup>18</sup> We and others have demonstrated that intramuscular transfection of the *sFlt-1* gene effectively and specifically

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blocks VEGF and thus, "quenches" the activity of VEGF in remote organs *in vivo*.<sup>19,20</sup>

The aim of this study was to decisively determine a role for VEGF in restenotic changes after intraluminal injury. We report herein that blockade of VEGF by systemic *sFlt-1* gene transfer attenuates the development of neointimal formation after intraluminal injury by inhibiting inflammation, which suggests an essential role for VEGF in the pathogenesis of restenosis after injury. Our present data are clinically important because VEGF gene therapy for therapeutic angiogenesis and restenosis has been attempted in clinical studies.<sup>16,17,21</sup>

## Methods

### Expression Vector

The 3.3-kb mouse *sFLT-1* gene, originally obtained from the mouse lung DNA library,<sup>22</sup> was cloned into the *Bam*HI (5') and *Nor*I (3') sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen). Plasmid cDNA3 encoding the luciferase gene was used to detect gene transfection.

### Rat and Rabbit Models of Balloon Injury

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the guidelines of American Physiological Society. A portion of this study was performed at the Kyushu University Station for Collaborative Research.

Twenty-week-old male, normal chow-fed Wistar-Kyoto rats were anesthetized, and their right common carotid arteries were injured by passage (3 times) of an infiltrated 2F Fogarty balloon catheter.<sup>23</sup> Male Japanese white rabbits weighing 3.0 to 3.5 kg were fed a high-cholesterol diet for 2 weeks. Their right common carotid arteries also were injured by passage (3 times) of an inflated 2F Fogarty catheter.<sup>24</sup> After injury, all rabbits were fed the same high-cholesterol diet. Three days before balloon injury, the animals were randomly divided into 2 groups: the empty-plasmid group was injected with the empty plasmid, and the *sFlt-1* group was injected with the *sFlt-1* gene into femoral muscle (150  $\mu$ g/50  $\mu$ L TE buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0] in rats, 1500  $\mu$ g/0.5 mL TE buffer in rabbits). To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator (CUY21, BTX) as previously described.<sup>19,23-25</sup>

### Morphometric and Immunohistochemical Analyses

All animals were euthanized by intravenous injection of a lethal dose of sodium pentobarbital. Tissue sections from rabbits and rats were prepared as described<sup>23,24</sup> and either (1) stained with Masson's trichrome or elastica van Gieson's stains or (2) subjected to immunostaining with antibodies against macrophages/monocytes (ED1, Serotec, for rats; RAM11, Dako, for rabbits), proliferating cells (proliferating cell nuclear antigen for rats from Dako, Ki-67 for rabbits from Dako), endothelial cells (CD31, Dako), VEGF (Santa Cruz), VEGFR-1 (Santa Cruz), VEGFR-2 (Santa Cruz),  $\alpha$ -smooth muscle actin (Dako), MCP-1 (R&D Systems), interleukin-1 $\beta$  (IL-1 $\beta$ ; R&D Systems), or nonimmune mouse IgG (Zymed). After avidin-biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. Immunofluorescence double staining was performed to localize VEGF and its receptors by the use of fluorescence-conjugated antibodies in rats. Morphometric analysis was performed by microscopy with a computerized digital image-analysis system by a single observer who was blinded to the treatment protocol.<sup>23,24</sup>

### Real-Time Quantitative Reverse Transcription-PCR

Real-time polymerase chain reaction (PCR) amplification was performed with rabbit cDNA by using the ABI PRISM 7000 sequence

detection system (Applied Biosystems) as described previously.<sup>23</sup> The respective PCR primers and TaqMan probes were designed from GenBank databases aided by a software program (Applied Biosystems; online Table 1). Results were analyzed by sequence detection software (Applied Biosystems), expressed in arbitrary units, and adjusted for glyceraldehyde 3-phosphate dehydrogenase mRNA levels.

### Mouse Femoral Wire-Injury Model With Bone Marrow Reconstitution

Intraluminal injury of the femoral artery of wild-type mice whose bone marrow had been replaced with that of ROSA26 mice, which expresses  $\beta$ -galactosidase (LacZ) ubiquitously, was performed.<sup>26</sup> Four weeks after bone marrow transplantation, transluminal arterial injury was induced by inserting a straight spring wire (0.38 mm in diameter) into the femoral artery as described.<sup>26</sup> The femoral artery was excised and stained with X-gal solution for 7 hours and then fixed in 4% paraformaldehyde. LacZ-positive cells were counted and expressed as a proportion of the total number of cells. The paraffin-embedded sections were stained with elastica van Gieson's stain.

Peripheral blood was obtained from the retro-orbital venous plexus of the mice. Fluorescence-conjugated antibodies against CD31 (PharMingen) and c-Kit (PharMingen) were used as bone marrow-derived monocyte-lineage markers. A fluorescein isothiocyanate-conjugated antibody against Mac-1 (PharMingen) was used as a circulating monocyte-lineage marker after gating for monocyte cell size with exclusion of granulocytes. Data were analyzed by flow cytometry and appropriate software (Becton Dickinson).

### Blood Measurements

Plasma total cholesterol levels in rabbits were determined with commercially available kits (Wako Pure Chemicals). To measure sFlt-1 released by the transfected skeletal muscle, plasma concentrations of sFlt-1 were measured by use of an sFlt-1 ELISA kit (R&D Systems) in rabbits. Concentrations of VEGF in plasma and femoral arterial tissues were also measured in mice by use of an ELISA kit (R&D Systems).

### Statistical Analysis

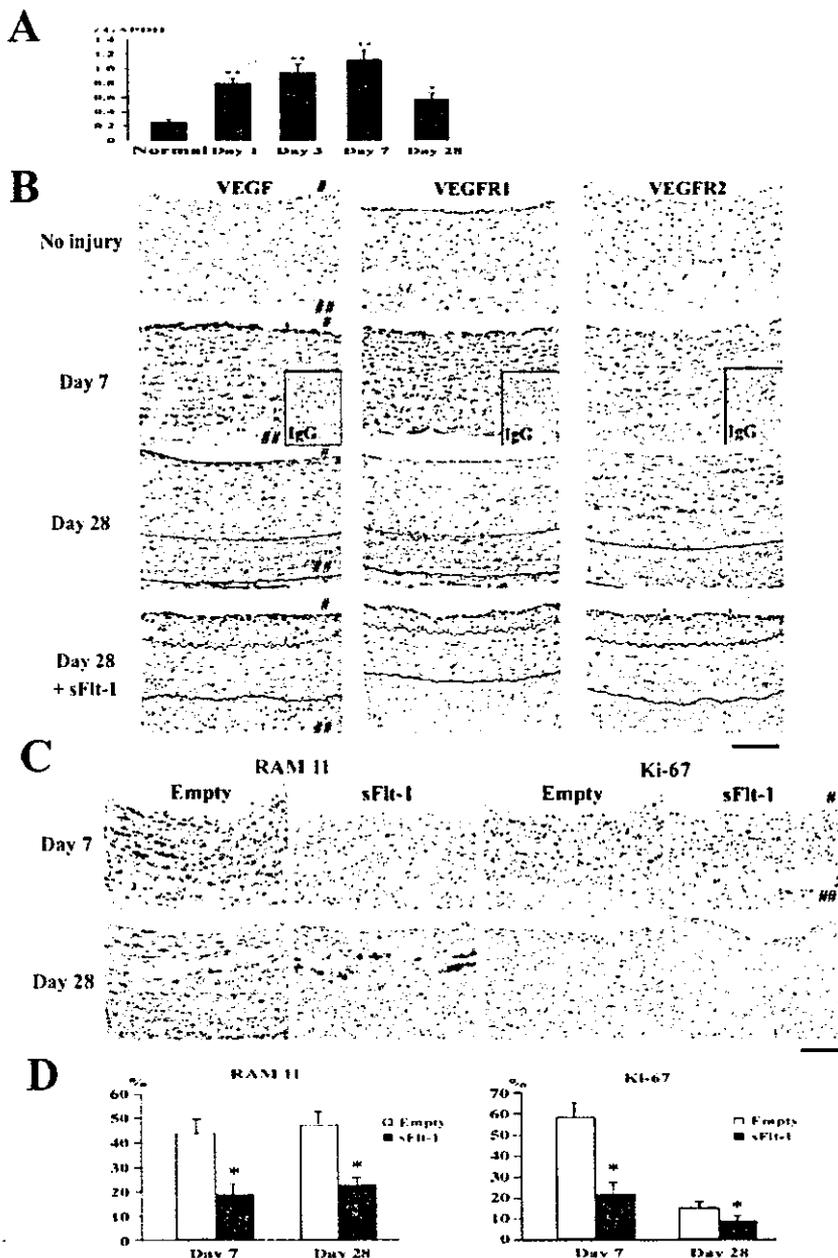
Data are expressed as mean  $\pm$  SE. Statistical analysis of differences was compared by ANOVA and Bonferroni's multiple-comparison tests. A level of  $P < 0.05$  was considered statistically significant.

## Results

### Increased Expression of VEGF and Its Receptors in Rabbits and Rats

Significant increases in VEGF mRNA levels were detected after balloon injury in rabbits, which peaked on day 7 and persisted until day 28 (Figure 1A). Immunohistochemical staining revealed that VEGF and VEGFR-1 increased in vascular smooth muscle cells in the media and regenerated endothelial layer during the early phase (day 7) and in cells in the neointima, media, and adventitia during the later phase (day 28) after balloon injury in rabbits (Figure 1B). VEGFR-2 did not increase on day 7 but did increase in the injured artery on day 28. *sFlt-1* gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (Figure 1B).

The localization of VEGF, VEGFR-1, and VEGFR-2 was studied in rats by immunohistochemistry. As observed in rabbits, immunoreactive VEGF and VEGFR-1 increased in the media on days 3 and 7 and in the neointima, media, and adventitia on day 28 (Figure 2A). The increase in immunoreactive VEGFR-2 was less prominent during the early phase but became apparent on day 28 (Figure 2A).



**Figure 1.** Expression of VEGF after balloon injury and inhibitory effects of *sFlt-1* gene transfer on inflammatory-proliferative changes in rabbits. **A**, Time course of VEGF mRNA levels in rabbits. mRNA levels were assessed by real-time PCR at indicated times. Expression of VEGF mRNA in each sample was normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA expression in that sample. Each bar has n=6 to 8. \* $P < 0.05$ , \*\* $P < 0.01$  vs control (uninjured) artery. **B**, Immunohistochemistry of rabbit carotid artery. Arterial cross sections were stained immunohistochemically with VEGF, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), or nonimmune IgG. # and ## indicate lumen and adventitia, respectively. Internal and external elastic layers are highlighted with blue and black lines, respectively, in photographs taken 28 days after injury. Bar indicates 100  $\mu$ m. **C**, Carotid artery sections from empty-plasmid and *sFlt-1* groups 7 and 28 days after balloon injury were stained immunohistochemically with antibody against monocytes/macrophages (RAM11) or proliferating cells (Ki-67). # and ## indicate lumen and adventitia, respectively. Bar=100  $\mu$ m. **D**, Effect of *sFlt-1* gene transfer on inflammation (RAM11-positive monocytes/macrophages) and proliferation (Ki-67-positive cells) 7 and 28 days after balloon injury (n=8 each). \* $P < 0.01$  vs control group.

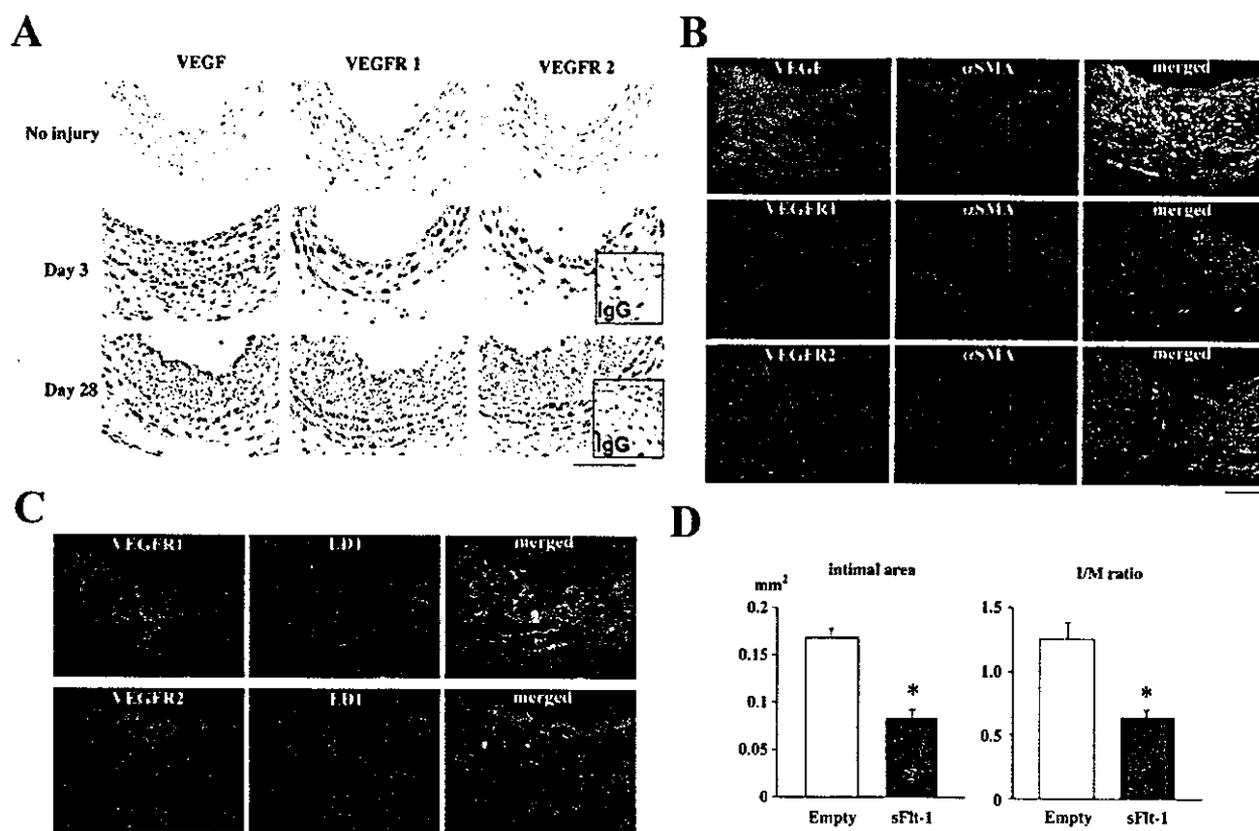
Fluorescence double immunohistochemistry revealed that VEGF and VEGFR-1 were expressed predominantly in  $\alpha$ -smooth muscle actin-positive cells in the media and neointima on day 28 (Figure 2B). During the early phase, ED1-positive monocytes recruited into the intima and adventitia expressed VEGFR-1 but not VEGFR-2 (Figure 2C). *sFlt-1* gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (data not shown).

**Inhibitory Effects of *sFlt-1* Transfer on Inflammatory and Proliferative Changes in Rabbits**

As we reported,<sup>23,24</sup> inflammatory and proliferative changes became evident by 3, 7, and 28 days after balloon injury in rabbits (Figure 1C and 1D). *sFlt-1* gene transfer reduced these inflammatory and proliferating changes.

**Inhibitory Effects of *sFlt-1* Transfer on Neointimal Formation and/or Negative Remodeling in Rabbits and Rats**

The carotid arteries in the control and empty-plasmid groups developed significant neointimal formation and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) in rabbits by day 28 (Figure 3A and 3B). The arteries from the *sFlt-1* group showed less neointimal formation, negative remodeling, perivascular fibrosis, and adventitial vasa vasorum; an adventitial VEGFR-2-positive vasa vasorum; and a larger lumen area. There was no significant difference in plasma levels of total cholesterol between the 2 groups (online Table II), indicating that the observed effects of *sFlt-1* gene transfer were not caused by changes in serum cholesterol levels. In rats, neointimal



**Figure 2.** Expression of VEGF and its receptors after balloon injury and inhibitory effects of *sFlt-1* gene transfer on neointimal formation in rats. **A**, Immunohistochemistry of arterial cross-sections stained immunohistochemically with VEGF, VEGFR-1 (Flt-1), or VEGFR-2 (Flk-1). Bar indicates 100  $\mu$ m. **B**, Fluorescence double immunohistochemistry 28 days after balloon injury. Photomicrographs show injured arteries stained with VEGF, VEGFR-1 (Flt-1), or VEGFR-2 (Flk-1) in green. Photomicrographs show injured arteries also stained with  $\alpha$ -smooth muscle actin in red. Single-fluorescence-positive cells were stained in green or red, whereas double-positive cells were stained in yellow. Scale bar indicates 100  $\mu$ m. **C**, Fluorescence double immunohistochemistry 7 days after balloon injury. Photomicrographs show injured arteries stained with VEGFR-1 or VEGFR-2 in green. Photomicrographs show injured arteries also stained with ED1 in red. Single-fluorescence-positive cells were stained in green or red, whereas double-positive cells were stained in yellow. Scale bar indicates 100  $\mu$ m. **D**, Neointimal formation (neointimal area and intima-media ratio) on 28 day after balloon injury. \* $P < 0.01$  vs empty-plasmid group,  $n = 8$  or 9.

formation was also less in the *sFlt-1* group than in the empty-plasmid group on day 28 (Figure 2D).

To assess transfection efficacy of *sFlt-1*, plasma sFlt-1 concentration was measured in rabbits. The plasma sFlt-1 levels were  $96 \pm 14$ ,  $377 \pm 25$  ( $P < 0.01$  versus before),  $413 \pm 20$  ( $P < 0.01$ ),  $284 \pm 15$  ( $P < 0.05$ ), and  $113 \pm 16$  ( $P > 0.1$ ) pg/mL before and at 3, 7, 14, and 28 days after *sFlt-1* transfection, respectively, indicating that sFlt-1 was released from the transfected muscle to the circulation.

#### No Significant Effects of *sFlt-1* Gene Transfer on Endothelial Regeneration in Rabbits and Mice

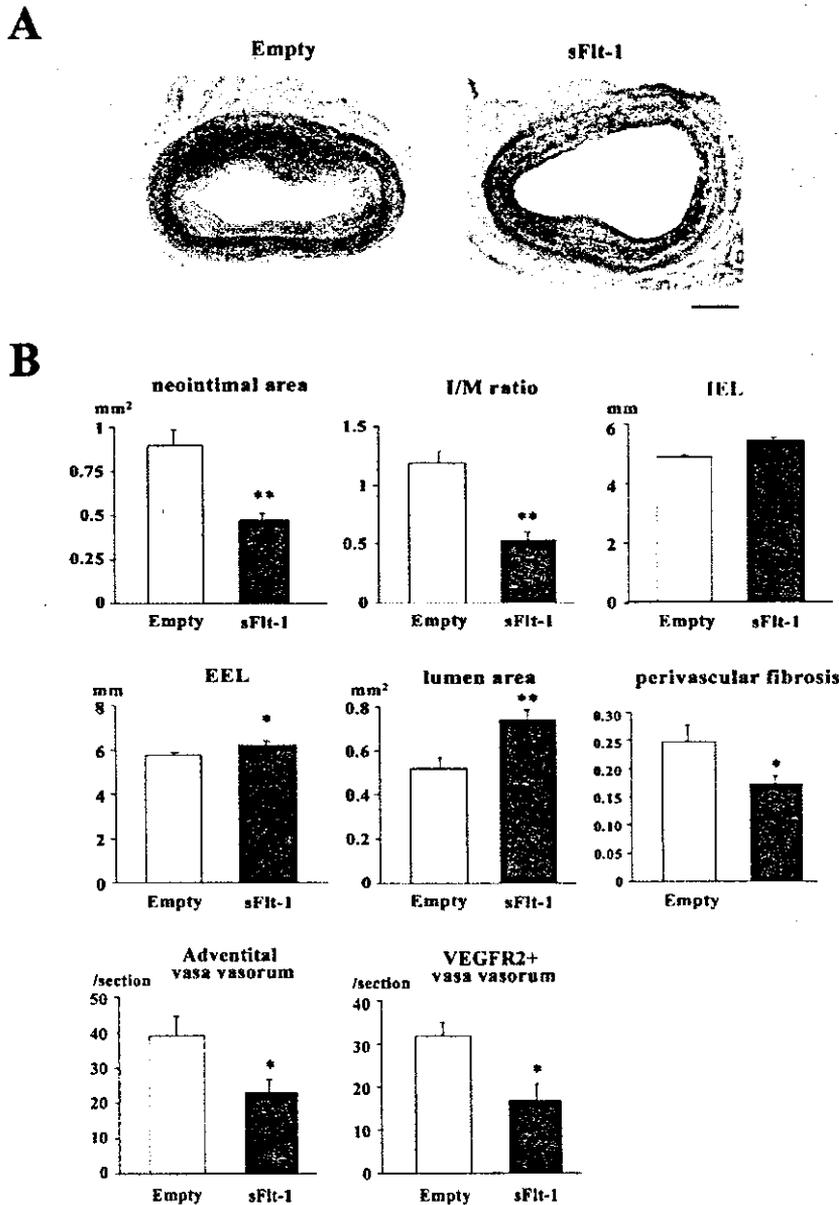
In rabbits, there were no significant differences between the empty-plasmid and *sFlt-1*-transfected groups in the ratio of luminal surface area covered with endothelium (Figure 4A) and that of the CD31-positive endothelial layer 7 days after injury (Figure 4B). In mice, endothelial recovery was scarcely observed on day 7 (data not shown) but was noted equally in the 2 groups on day 14 (Figure 4C).

#### Inhibitory Effects of *sFlt-1* Transfer on Expression of Proinflammatory Factors

*sFlt-1* transfection reduced the increased gene expression of MCP-1, platelet-derived growth factor, transforming growth factor- $\beta$ , IL-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$ , matrix metalloproteinase-9, and VEGF (Figure 5A). *sFlt-1* transfer did not affect the increased gene expression of matrix metalloproteinase-1 and tissue factor. Immunohistochemical staining performed 7 days after balloon injury revealed increased immunoreactive MCP-1 and IL-1 $\beta$  in cells in the neointima and smooth muscle cells in the media, which were attenuated by *sFlt-1* gene transfer (Figure 5B).

#### Contribution of Bone Marrow Cells to the Effect of *sFlt-1* Gene Transfer in Mice

As reported,<sup>26</sup> a considerable proportion of neointimal and medial cells were LacZ-positive 28 days after injury in mice whose bone marrow expressed LacZ ubiquitously. Intimal area, intima-media ratio, and LacZ-positive cells were decreased in *sFlt-1*-transfected mice than in empty plasmid-



**Figure 3.** Inhibitory effect of *sFlt-1* gene transfer on restenotic changes (neointimal formation and negative remodeling) in rabbits. **A**, Carotid artery sections from empty-plasmid and *sFlt-1* groups 28 days after balloon injury stained with elastica van Gieson's stain. Bar=200  $\mu$ m. **B**, Neointimal formation (neointimal area and intima-media ratio), negative remodeling (length of internal elastic lamina, length of external elastic lamina, and lumen area), perivascular fibrosis, adventitial vasa vasorum (number of CD31-positive vessels in adventitia), and VEGFR-2-positive vasa vasorum (number of VEGFR-2-positive vessels in adventitia) on day 28 after balloon injury are shown. \* $P < 0.05$ , \*\* $P < 0.01$  vs empty-plasmid group, n=8 or 9.

transfected mice (Figure 6A and 6B). Wire injury also increased recruitment of bone marrow-derived monocytes (CD31-positive and c-Kit-positive) and circulating monocytes (Mac-1-positive) into peripheral blood (Figure 6C). *sFlt-1* gene transfer attenuated such changes in cell distribution, suggesting that wire injury increased such cell lineages in peripheral blood through VEGF. Plasma and femoral arterial concentrations of VEGF increased after wire injury, which was partly attenuated by *sFlt-1* transfection (online Tables III and IV).

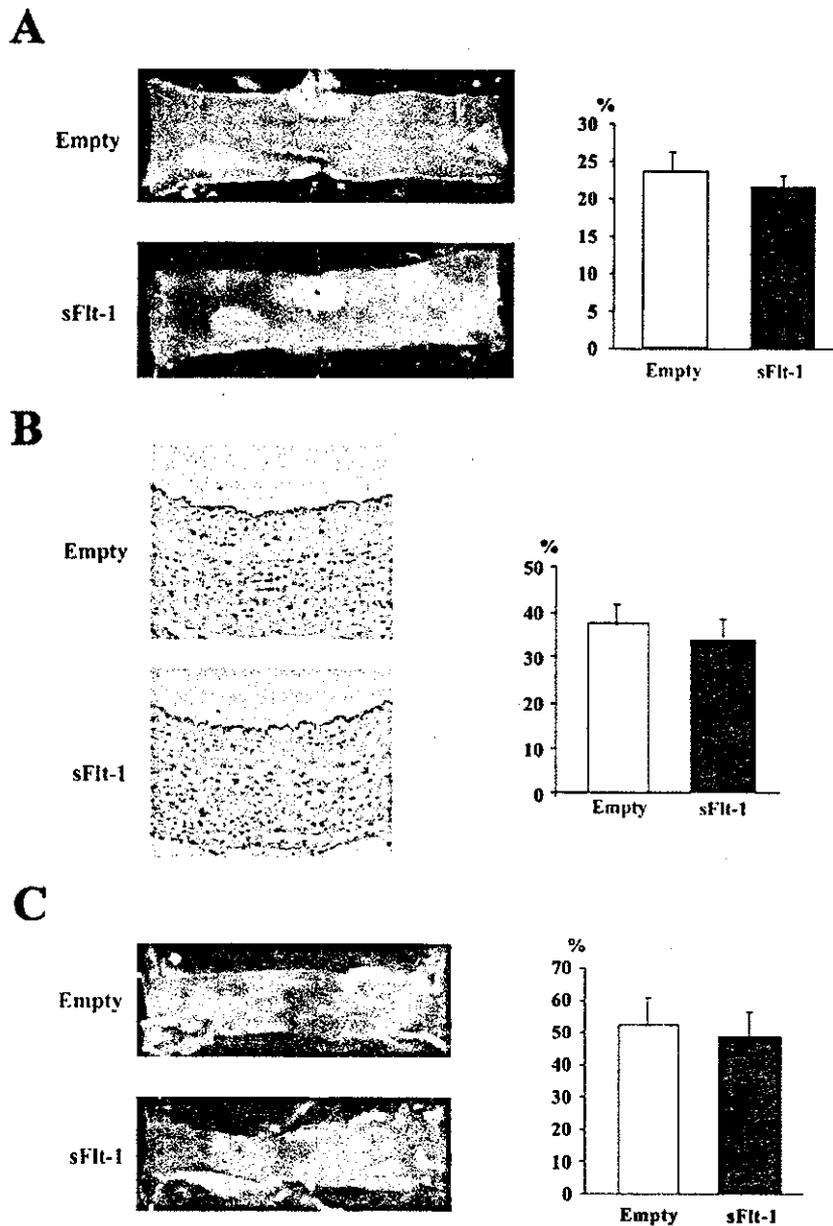
**Effects of *VEGF*<sub>165</sub> Gene Transfer on Neointimal Formation and Adventitial Angiogenesis in Rabbits**

A recombinant adenoviral vector containing human *VEGF*<sub>165</sub> or the *LacZ* gene was produced. Gene transfer was performed by administering adenovirus solution (1 mL,  $2 \times 10^9$  plaque-forming units) by a channel balloon catheter (Remedy,

Boston Scientific Inc) immediately after balloon injury of rabbit carotid arteries (online Data Supplement and Figure). There were no significant differences between the empty-plasmid and *VEGF*-transfected groups in terms of neointimal formation, perivascular fibrosis, and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) on day 28. In contrast, the number of adventitial vasa vasorum (the degrees of adventitial angiogenesis) was markedly increased in the *VEGF*-transfected group.

**Discussion**

VEGF has conventionally been thought to be an endothelial cell-specific growth factor and that it inhibits vascular pathological processes by accelerating endothelial proliferation and regeneration through endothelial VEGFR-2. If so, blockade of VEGF would suppress endothelial regeneration



**Figure 4.** No significant effect of *sFlt-1* gene transfer on endothelial regeneration. **A**, Reendothelialization of rabbit carotid artery determined by Evans blue staining (deendothelialized areas are stained with blue) 7 days after balloon injury in rabbits. Endothelial regeneration of injured arteries was identified by intravenous injection of Evans blue dye 30 minutes before harvesting of vessels from rabbits and mice. Ratios of surface area covered by endothelium to total area in empty-plasmid and *sFlt-1* groups ( $n=7$  each) are shown. **B**, Cross sections of rabbit femoral arteries stained with CD31 antibody 7 days after injury. Degrees of endothelial recovery (length of CD31-positive layer, length of internal elastic lamina in cross sections) in both groups are also shown ( $n=7$  each). **C**, Reendothelialization of mouse femoral arteries 14 days after injury as determined by Evans blue staining ( $n=9$  each).

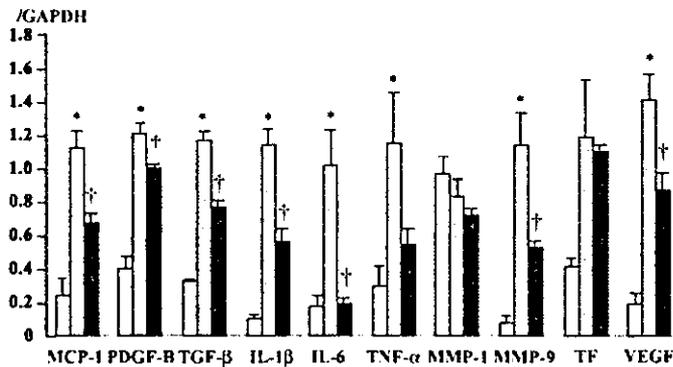
and enhance neointimal formation after injury. In contrast to the conventional assumption, we here demonstrated that blockade of VEGF by *sFlt-1* gene transfer attenuated neointimal formation in rabbits, rats, and mice, indicating the essential role of VEGF in experimental restenosis.

As previously reported by others,<sup>5-7,15</sup> we demonstrated persistent increases in VEGF in arterial wall cells after injury. Emerging evidence suggests expression of functional VEGFR-1 and VEGFR-2 in cell types other than endothelial cells. We showed herein an increased expression of VEGFR-1 in lesional monocytes and medial smooth muscle cells during the early stage and in smooth muscle cells in the neointima and media during later stages. Increased VEGFR-2 expression was noted only at later stages. *sFlt-1* gene transfer attenuated the increased expression of inflammatory and growth factors such as VEGF, MCP-1, IL-1 $\beta$ , and so forth at

early stages. Expression of VEGFR-1 in monocytes mediates chemotaxis,<sup>9</sup> and VEGFR-1 in smooth muscle cells mediates migration.<sup>27</sup> VEGFR-1 has been shown to act as an important mediator of VEGF-induced inflammation.<sup>9,10,13</sup> More recently, Yamada et al<sup>28</sup> showed that VEGF-mediated angiogenesis and inflammation are mediated by MCP-1. We also demonstrated the central role of MCP-1 in the mechanism of neointimal formation after vascular injury.<sup>23-25,29</sup> It is likely, therefore, that VEGF might cause vascular inflammation and migration of vascular smooth muscle cells and thus, cause neointimal formation after injury. Further studies are needed to elucidate the relative roles of VEGFR-1 and VEGFR-2 in the mechanisms of neointimal formation.

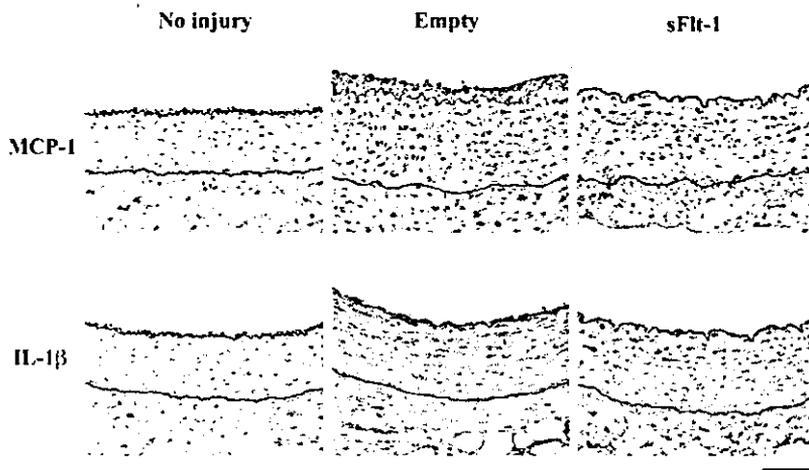
This study also demonstrated in rabbits the role of VEGF in the development of negative remodeling, another major cause of human restenosis after balloon angioplasty.<sup>30</sup> Fibro-

**A**



**Figure 5.** Inhibitory effect of *sFlt-1* gene transfer on expression of various inflammatory factors in rabbits. **A**, Effect of *sFlt-1* gene transfer on mRNA levels of various proinflammatory factors 1 day after injury. Quantitative real-time PCR was performed. \* $P < 0.01$  vs uninjured control (uninjured) artery; † $P < 0.05$ , †† $P < 0.01$  vs empty-plasmid group. **B**, Carotid artery sections from control uninjured animals and those from empty-plasmid and *sFlt-1* groups 7 days after balloon injury stained immunohistochemically with MCP-1 and IL-1β. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=100 μm. Immunohistochemical experiments were repeated 5 times, all with representative results.

**B**



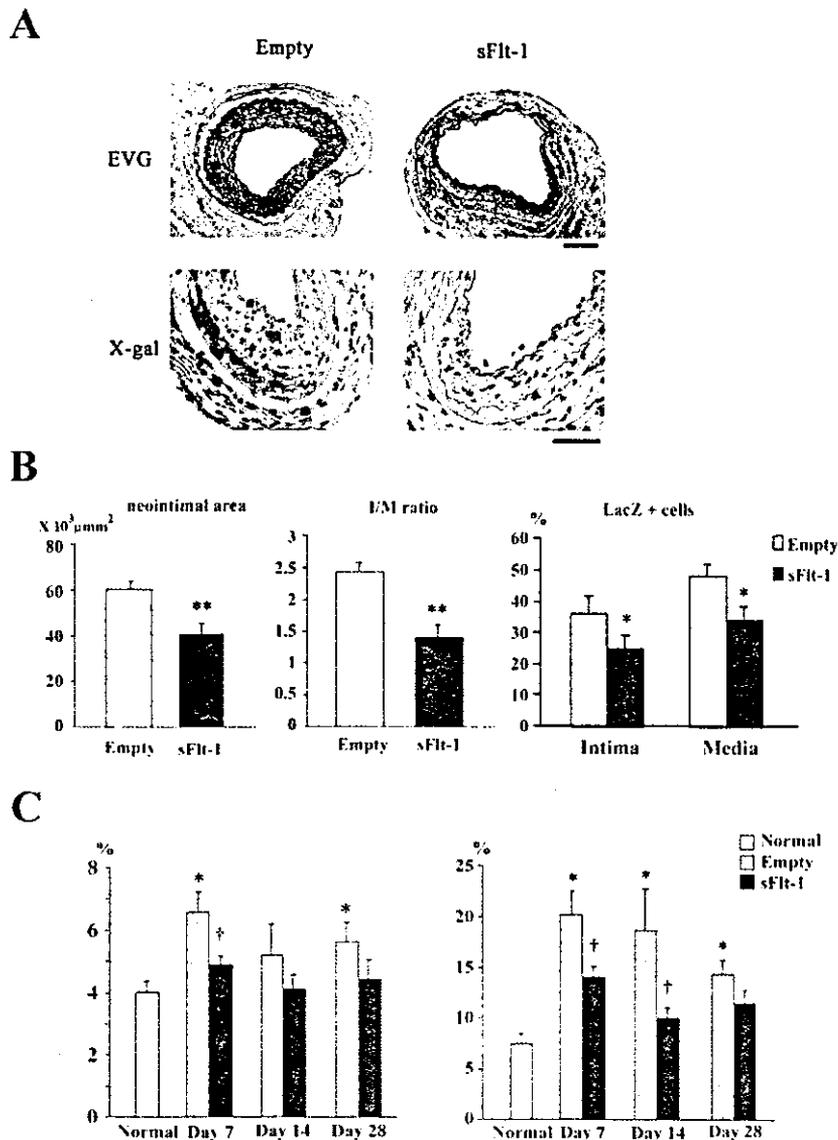
sis and vasa vasorum in the adventitia have been implicated to be the central pathological processes leading to constrictive negative remodeling after balloon injury. Therefore, our present data suggest that *sFlt-1* gene transfer inhibited the development of negative constrictive remodeling by limiting adventitial fibrosis and angiogenesis.

VEGFR-1 has been shown to be an important mediator of stem cell recruitment and differentiation.<sup>13</sup> Sata and colleagues<sup>26</sup> have shown that a considerable proportion of neointimal and medial cells were bone marrow-derived progenitor cells. However, the role of VEGF in the recruitment and differentiation of progenitor cells into neointimal cells after vascular injury has not been addressed. We showed here that *sFlt-1* gene transfer inhibited recruitment of bone marrow-lineage cells into the peripheral circulation and injured arterial wall and thus, reduced neointimal formation after injury. These data suggest that VEGF might contribute to neointimal formation by recruiting bone marrow-derived and circulating monocytes.

Surprisingly, *sFlt-1* gene transfer did not affect endothelial regeneration after endothelial injury, suggesting a minor role of endogenous VEGF in endothelial regeneration after endothelial injury. It remains to be determined whether inhibition of VEGFR-2-mediated activity of endothelial regeneration

by *sFlt-1* gene transfer might have been overshadowed by other stimuli (eg, basic fibroblast growth factor, angiopoietins, etc). On the contrary, adenovirus-mediated gene transfer of *VEGF* enhanced adventitial angiogenesis but did not reduce neointimal formation after balloon injury in rabbits. The latter observation is consistent with previous reports demonstrating that exogenous VEGF does not reduce neointimal formation in animals and humans.<sup>15-17</sup> Taken together, the role or mechanisms of action of VEGF may differ between exogenous and endogenous VEGF and between angiogenesis and neointimal formation.

This study may have significant clinical implications. First, *sFlt-1* gene transfer might be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. However, local delivery of *sFlt-1* must be preferable for clinical use to avoid potential side effects by systemic delivery. Second, our finding indicates that more research is required, especially on the safety of VEGF, before translational clinical research proceeds. Deleterious effects associated with overexpression of VEGF have recently been reported: (1) injection of VEGF-expressing skeletal muscle myoblasts into the murine heart caused the formation of hemangiomas and lethagenic heart failure<sup>31</sup> and (2) *VEGF* gene transfer into rabbit carotid arteries induced neointimal



**Figure 6.** Contribution of bone marrow-derived cells to effect of *sFlt-1* gene transfer in mice. **A**, Arterial sections from empty-plasmid and *sFlt-1* groups 28 days after wire injury stained with X-gal or elastica van Gieson's (EVG) stains. Bar=100  $\mu\text{m}$ . **B**, Inhibitory effects of *sFlt-1* gene transfer on neointimal formation (neointimal area and intima-media ratio) and percentage of LacZ-positive cells (100 $\times$ LacZ-positive nuclei/total nuclei) in neointima and media. \* $P < 0.05$ , \*\* $P < 0.01$  vs phosphate-buffered saline,  $n = 8$  each. **C**, Summary of fluorescence-activated cell sorting analysis of recruitment of bone marrow-derived monocytes (left) and circulating monocytes (right) into peripheral circulation in normal mice and mice transfected with empty plasmid or *sFlt-1* after wire injury,  $n = 7$  or 8. \* $P < 0.05$  vs normal mice (no injury); † $P < 0.05$  vs empty-plasmid group.

formation with angiomatoid proliferation of endothelial cells.<sup>12</sup> These studies highlight the potential side effects or toxicity that would be against the clinical use of VEGF for therapeutic angiogenesis and restenosis.

In conclusion, this study has provided direct in vivo evidence suggesting that increased expression and activity of VEGF are essential for the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. Although there is no clinical evidence suggesting enhancement of atherosclerosis or neointimal formation by VEGF therapy,<sup>16,17</sup> our present data raise the question of whether VEGF therapy is useful without serious risks in patients with advanced atherosclerosis.

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# Essential Role of Vascular Endothelial Growth Factor and Flt-1 Signals in Neointimal Formation After Periadventitial Injury

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**Objective**—Vascular endothelial growth factor (VEGF) is upregulated after arterial injury. Its role in the pathogenesis of neointimal formation after periadventitial injury, however, has not been addressed.

**Methods and Results**—Expression of VEGF and its receptors but not that of placental growth factor markedly increased with the development of neointimal formation in hypercholesterolemic mice after cuff-induced periarterial injury. Transfection with the murine soluble Flt-1 (sFlt-1) gene to block VEGF in vivo in mice inhibited early inflammation and later neointimal formation. The sFlt-1 gene transfer did not affect plasma lipid levels but attenuated increased expression of VEGF, Flt-1, Flk-1, monocyte chemoattractant protein-1, and other inflammation-promoting factors. Mice with Flt-1 kinase deficiency also displayed reduced neointimal formation.

**Conclusions**—Inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. VEGF might promote neointimal formation by acting as a proinflammatory cytokine. (*Arterioscler Thromb Vasc Biol.* 2004;24:2284-2289.)

**Key Words:** remodeling ■ growth substances ■ inflammation ■ arteriosclerosis ■ gene therapy

Neointimal formation is a major cause of restenosis after coronary intervention.<sup>1,2</sup> Vascular endothelial growth factor (VEGF) and its receptors (VRGFR-1: Flt-1, VEGFR-2: Flk-1) are upregulated in vascular inflammatory and proliferative disorders such as atherosclerosis and restenosis.<sup>3-6</sup> VEGF is thought to protect the artery from such disorders by inducing endothelial regeneration and improving endothelial function.<sup>7</sup> VEGF gene transfer or administration of its protein induces endothelial regeneration and attenuates neointimal formation after endothelial injury.<sup>7-9</sup> VEGF is reported to inhibit leukocyte infiltration through hemoxygenase-1.<sup>10</sup> There is still considerable debate, however, over the role of VEGF in the development of neointimal formation after injury.<sup>11,12</sup> Emerging evidence suggests that VEGF causes or promotes the development of atherosclerosis or neointimal formation after injury. VEGF induces migration and activation of monocytes,<sup>13</sup> adhesion molecules,<sup>14</sup> or monocyte chemoattractant protein-1 (MCP-1)<sup>15</sup> through its receptor Flt-1. Moreover, administration of VEGF protein to hypercholesterolemic animals enhances atherogenesis by inducing monocyte infiltration and activation.<sup>16</sup> In addition, VEGF might promote migration of vascular smooth muscle cells through Flt-1.<sup>17,18</sup> Angiogenesis inhibitors are shown to reduce

intimal neovascularization and plaque growth in hyperlipidemic mice.<sup>19</sup>

One major reason for the inconsistent reports regarding the role of VEGF might be because there are no selective VEGF inhibitors tested. The only known endogenous VEGF inhibitor is a soluble form of the VEGF receptor-1, Flt-1 (sFlt-1).<sup>20</sup> This isoform is mainly expressed by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor.<sup>20</sup> We and others previously demonstrated that intramuscular transfection of the sFlt-1 gene blocks VEGF signaling and thus quenches VEGF activity in vivo.<sup>21,22</sup> Therefore, sFlt-1 gene transfer can be used as an inhibitor against VEGF and its receptors (Flt-1, Flk-1). In addition, Flt-1 tyrosine kinase-deficient mice can be used to determine the role of Flt-1 signals.<sup>23</sup>

In this study, we investigated the role of VEGF and Flt-1 signals in the pathogenesis of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. Several animal models for evaluation of neointimal formation after injury have been reported, including balloon injury, wire injury, chemical injury, and cuff injury, among others. The ideal animal model for human neointimal forma-

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tion is uncertain. The cuff model was chosen because cuff placement in the presence of hypercholesterolemia offers the advantage of inducing reproducible site-controlled neointimal formation and stenosis.<sup>24,25</sup> In addition, the cuff-induced injury triggers vascular inflammation and induces neointimal lesions that are partly similar to the restenotic and atherosclerotic lesions observed in humans.<sup>24,25</sup> Our present data provide direct evidence suggesting that inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periaortic injury.

## Methods

### Expression Vector

The 3.3-kb mouse sFlt-1 gene was obtained from a mouse lung cDNA library<sup>26</sup> and cloned into the BamHI(5') and NotI(3') sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen).

### Experimental Animals

The study protocol was reviewed and approved by the Committee on Ethics for Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Apolipoprotein E-deficient (apoE-KO) and wild-type mice (8 to 10 weeks old,  $n=5$  to 9 each group) with a genetic background of C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, Me) and fed with commercial standard chow. Placement of cuff and gene transfer were performed as previously described.<sup>21,27</sup> A nonconstrictive polyethylene cuff (1.5 mm long; PE20, 0.38-mm inner diameter, 1.09-mm outer diameter) was placed loosely around the left femoral artery. Either empty plasmid or sFlt-1 plasmid (300  $\mu\text{g}/100 \mu\text{L}$  PBS) was injected into the right femoral muscle using a 27-gauge needle immediately and 10 days after cuff placement. To enhance transgene expression, these animals received electroporation at the injected site immediately after injection.<sup>21,27-29</sup> It has been shown that electroporation-mediated gene transfer is useful to introduce genes into muscle tissues in vivo with no serious tissue injury.<sup>30</sup> To determine the role of flt-1 signals, Flt-1 tyrosine kinase-deficient mice with a genetic background of C57BL/6J were used.<sup>23</sup>

### In Vivo Matrigel Plug Assay

An in vivo matrigel plug assay was used to determine the effect of sFlt-1 gene transfer on VEGF activity.<sup>21,27</sup> Matrigel matrix alone (300  $\mu\text{L}$ ) or mixed with recombinant VEGF protein (100 ng/mL) was injected subcutaneously into the flanks of C57BL/6J mice. The matrigels were then removed 7 or 14 days after injection, and angiogenesis and inflammation were examined by histopathologic analysis.

### Histopathology, Immunohistochemistry, and Morphometry

Mice were anesthetized with pentobarbital, and the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded.<sup>27</sup> Serial cross sections (5  $\mu\text{m}$  thick) throughout the entire length of the cuffed femoral artery were used for histological analysis. Cryosections were made from 2 mice in each condition. All sections were routinely stained with hematoxylin-eosin or van Gieson. Mac-3 (PharMingen) staining was used to detect monocytes/macrophages, and CD3 (Santa Cruz Biotechnology) was used for T cells. Proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology) was used to detect vascular proliferation. An antibody against von Willebrand factor (vWF; Sigma Chemical Co) was used to mark endothelial cells. Antibodies against VEGF (Santa Cruz Biotechnology) and placental growth factor (PlGF;

R&D Systems Inc) were also used. Indirect immunofluorescence double-staining with matched primary and fluorescein-conjugated secondary antibodies was used to stain for colocalization with VEGF receptors in smooth muscle cells or monocytes as follows: Rabbit anti-mouse Flt-1 (Santa Cruz Biotechnology), rabbit anti-mouse Flk-1 (Santa Cruz Biotechnology), rat anti-mouse Mac-3, anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; Boehringer Mannheim Corp), anti-rabbit IgG conjugated with fluorescein isothiocyanate or rhodamine, and anti-rat IgG conjugated with fluorescein isothiocyanate or rhodamine (Santa Cruz Biotechnology).

Ten equally-spaced cross sections were examined in all mice to quantify intimal lesions. Using image analysis software, the total cross-sectional medial area was measured between the external and internal elastic lamina; the total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

### Plasma Measurements

Plasma total cholesterol and triacylglycerol levels were determined with commercially available kits (Wako Pure Chemicals). Plasma concentrations of sFlt-1, VEGF, and PlGF were measured by the use of ELISA kit (R&D Systems Inc).

### RT-PCR and RNase Protection Assay

RNA was prepared from the pooled samples (5 to 7 arteries for 1 sample).<sup>21</sup> First-strand DNA was synthesized using reverse transcriptase with random hexamers from 1  $\mu\text{g}$  total RNA in a 20- $\mu\text{L}$  reaction volume according to the manufacturer's protocol (GeneAmp RNA polymerase chain reaction Kit; Perkin-Elmer). Primers used for amplification of VEGF were 5'-GGA TCC ATG AAC TTT CTG CT-3' and 5'-GAA TTC ACC GCC TCG GCT TGT C-3' with expected sizes of 654, 582, and 450 bp for the 3 VEGF isoforms (VEGF 188, 164, and 120, respectively). Primers for PlGF were 5'-CCC ACA CCC AGC TCA CGT ATT TA-3' and 5'-TCC CCT CTA CAT GCC TTC AAT GC-3'. Primers for Flk-1 were 5'-ACT GCA GTG ATT GCC ATG TTC T-3' and 5'-GCT CAT CCA AGG GCA ATT CAT-3'. Primers for the internal control,  $\beta$ -actin, were 5'-ATG GAT GAC GAT ATC GCT-3' and 5'-ATG AGG TAG TCT GCT AGG T-3' with an expected product of 550 bp.

RNase protection assays were performed using 5  $\mu\text{g}$  total RNA with 2 custom template sets according to the manufacturer's protocol (PharMingen).<sup>27</sup>

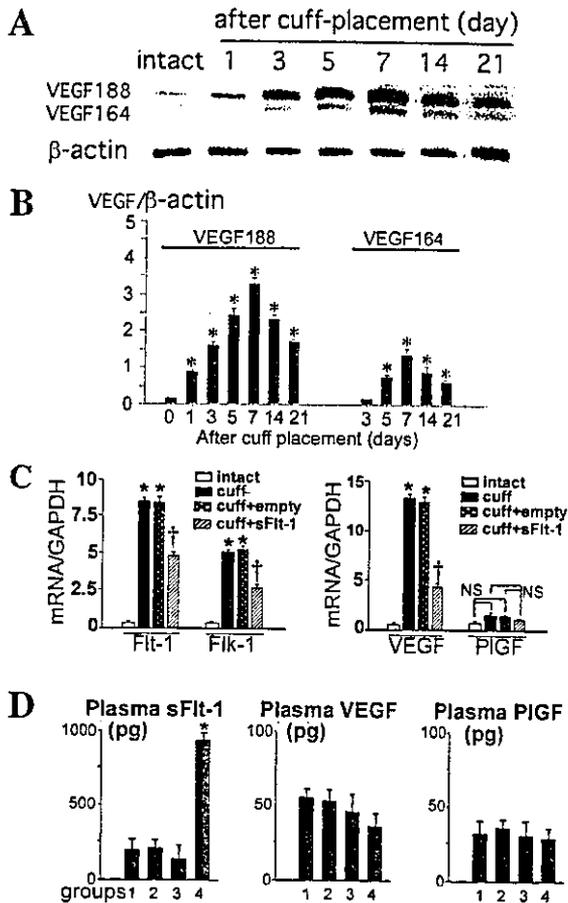
### Statistical Analysis

Data are expressed as the mean  $\pm$  SE. Statistical analysis of differences was compared by ANOVA. Post hoc analyses were performed using Bonferroni correction for multiple comparison tests.  $P < 0.05$  was considered to be statistically significant.

## Results

### In Vivo Matrigel Plug Assay in ApoE-KO Mice

Seven days after injection of matrigel, there were significant angiogenic (number of CD31-positive cells per  $\text{mm}^2$ ) and inflammatory (number of Mac3-positive cells per  $\text{mm}^2$ ) reactions in the matrigel plugs containing recombinant VEGF protein compared with matrigel alone. Soluble Flt-1 gene transfer but not injection of an empty plasmid suppressed both the angiogenic and inflammatory reactions to VEGF to a level similar to that of matrigel plugs without VEGF (Figure 1, available online at <http://atvb.ahajournals.org>). This suppression of angiogenic and inflammatory reactions to VEGF was noted on day 14 but not on day 21 of sFlt-1 gene transfer (data not shown).

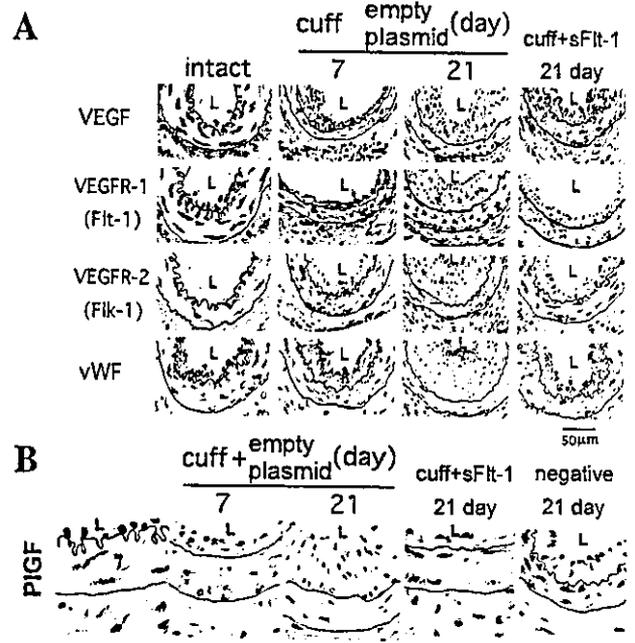


**Figure 1.** Gene expression VEGF, Flt-1, Flk-1, and PIGF in cuffed femoral artery. **A**, Time course of VEGF mRNA levels (RT-PCR) and expression of arterial VEGF and  $\beta$ -actin mRNA after cuff placement. mRNA levels were assessed at the indicated times. This is a representative assay from 5 separate experiments. **B**, Densitometric analysis of data in **A**. Expression of VEGF mRNA in each sample was normalized by  $\beta$ -actin mRNA expression in the same sample.  $N=5$  for each bar.  $*P<0.01$  vs control intact artery. **C**, Gene expressions of Flt-1, Flk-1, PIGF, and VEGF (RT-PCR) in femoral arteries before or 7 days after cuff placement/sFlt-1 gene transfer.  $*P<0.01$  vs intact control. **D**, Plasma levels of sFlt-1, PIGF, and VEGF 7 days after cuff placement in 4 animal groups: (1) untreated control, (2) mice with cuff alone, (3) cuff+empty plasmid, and (4) cuff+sFlt-1 plasmid.  $N=6$  for each.  $*P<0.01$  vs control group.

**Gene Expression and Immunoreactivity in ApoE-KO Mice**

The mRNA levels of 2 VEGF isoforms (188 and 164) markedly increased after cuff placement, whereas they were undetectably low in control intact artery (Figure 1A and 1B). Peak expression was observed on day 7. VEGF 121 mRNA was undetectable before and after cuff placement. Gene expression of Flt-1, Flk-1, VEGF, and PIGF was also increased on day 7 (Figure 1C). Plasma concentrations of sFlt-1, VEGF, and PIGF were measured on day 7 in several groups of animals (Figure 1D). Plasma sFlt-1 was increased in sFlt-1 transfection group. There was no significant change in plasma VEGF nor in PIGF among groups (Figure 1D).

Immunohistochemical staining indicated that compared with faint staining in the control artery, VEGF increased in the vicinity of inflammatory lesions (mononuclear cell infil-



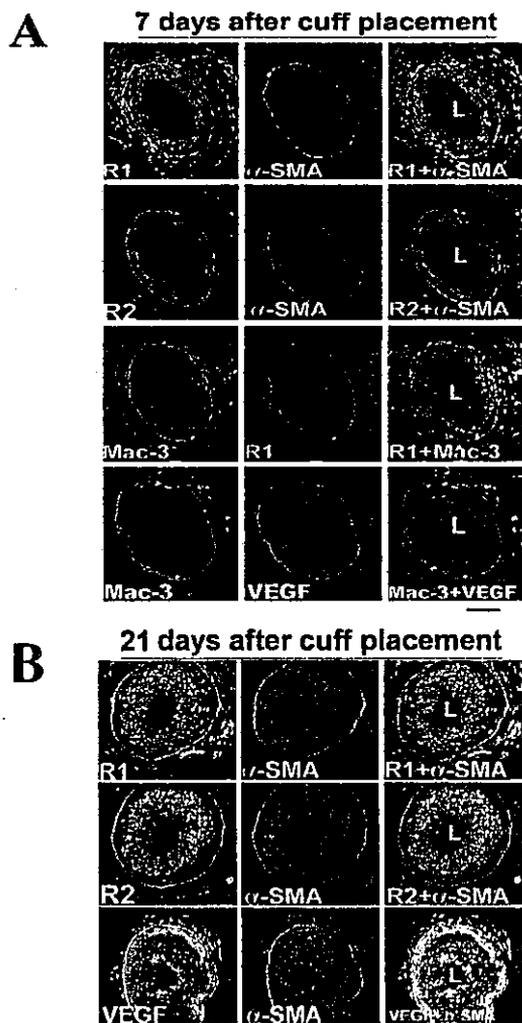
**Figure 2.** Immunostaining of VEGF, Flt-1, Flk-1, and PIGF in cuffed femoral artery. **A**, Cross sections of intact or cuffed femoral arteries were stained immunohistochemically against VEGF, VEGF receptor 1 (Flt-1), VEGF receptor 2 (Flk-1), or vWF 7 and 21 days after cuff placement in empty plasmid group. Immunohistochemical sections of cuffed arteries on day 21 in sFlt-1 group are also shown. Black lines indicate internal and external elastin laminae. L indicates lumen. Scale bar=50  $\mu$ m. **B**, Immunohistochemical staining of PIGF in femoral arteries before or after cuff placement/sFlt-1 gene transfer. Black lines indicate internal or external elastin laminae. L indicates lumen. Scale bar=50  $\mu$ m.

tration) in the intima and adventitia on day 7 and in cells of 3 layers of cuffed artery on day 21 (Figure 2A). The endothelial layer, as detected by vWF staining, was preserved before and after cuff placement (Figure 2A). No detectable increase in PIGF staining was observed before and after cuff placement (Figure 2B).

Both Flk-1 and Flt-1 were undetectable, except in endothelial layers in control intact arteries, but both were increased in the intima, media, and adventitia 7 and 21 days after cuff placement (Figure 2A). Compared with Flt-1 staining, Flk-1 staining was less impressive on day 7 but was apparently noted on day 21. To localize VEGF receptors, immunofluorescent double-staining was performed (Figure 3). On day 7,  $\alpha$ -SMA-positive cells in the media and neointima expressed little Flk-1, whereas they did express Flt-1 (Figure 3A). Also, some  $\alpha$ -SMA-positive cells in the adventitia (possibly adventitial myofibroblasts) expressed Flt-1. Mac-3 positive cells recruited to the neointima, media, and adventitia expressed VEGF and Flt-1. On day 21, most  $\alpha$ -SMA-positive cells in the neointima and media expressed VEGF and its receptors (Figure 3B).

**Time Course of Development of Neointimal Hyperplasia in ApoE-KO Mice**

As published,<sup>24,27,31,32</sup> within 7 days of cuff placement, mononuclear leukocytes, most of which were Mac3-positive monocytes, were recruited into the adventitia, media, and

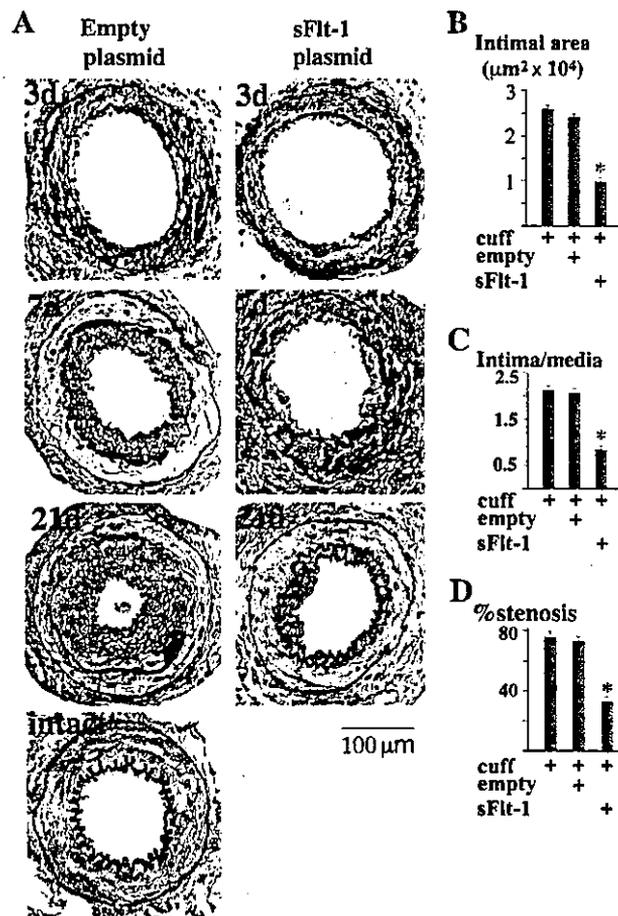


**Figure 3.** Immunofluorescence double-staining of VEGF receptors, monocytes, and  $\alpha$ -SMA in cuffed femoral artery. **A**, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1, green) and  $\alpha$ -SMA (red), with Flk-1 (VEGF-R2, green) and  $\alpha$ -SMA (red), and with Mac-3 (green) and Flt-1 (VEGF-R1, red) 7 days after cuff placement. Scale bar=50  $\mu$ m. **B**, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1) and  $\alpha$ -SMA, Flk-1 (VEGF-R2) and  $\alpha$ -SMA, and VEGF and  $\alpha$ -SMA in the cuffed femoral arteries 21 days after cuff placement. Single fluorescence-positive cells were stained green or red, whereas double-positive cells were stained yellow. White lines indicate external elastin laminas. L indicates lumen. Scale bar=50  $\mu$ m.

intima (Figure 3). After 7 days, neointimal lesions developed and became thick over time (Figure 4A). Monocyte infiltration declined spontaneously and  $\alpha$ -SMA-positive cells appeared predominantly in the neointima. On day 21, significant neointimal formation with luminal stenosis developed (Figure 4A). Endothelial staining with vWF antibody showed that no significant neointimal neovascularization was observed during the course of experiments (Figure 2A).

**Effects of Soluble Flt-1 Gene Transfer on Cuff-Induced Neointimal Hyperplasia in ApoE-KO Mice**

As we previously reported,<sup>27</sup> Mac3-positive monocytes/macrophages and PCNA-positive cells were detected mainly in



**Figure 4.** Histopathology of cuffed femoral artery. **A**, Time course of cuff injury-induced neointimal formation and effect of sFlt-1 gene transfer. Micrographs of cross sections of control (intact) and cuffed arteries stained with van Gieson Elastica on days 3, 7, and 21 are shown. Scale bar=100  $\mu$ m. **B** through **D**, Effects of sFlt-1 gene transfer on neointimal thickening (**B**), intima/media ratio (**C**), and % stenosis (**D**) 21 days after cuff placement. \* $P < 0.01$  vs cuff only and cuff+empty plasmid group.

the adventitia and intima. There was markedly less inflammation (Mac3-positive cells) and proliferation (the PCNA index) in sFlt-1-transfected mice than in empty plasmid-transfected mice on day 7 (Figure II, available online at <http://atvb.ahajournals.org>). There was no detectable change in the number of CD3-positive T cells in empty plasmid or sFlt-1-transferred mice (Figure II). The sFlt-1 gene transfer significantly reduced neointimal formation (increases in neointimal area, intima/media ratio, and luminal stenosis) 21 days after cuff placement (Figure 4A through 4D).

Because sFlt-1 gene transfer markedly reduced monocyte-mediated inflammation, gene expression of a battery of inflammatory cytokines, chemokines, and chemokine receptors was examined by RNase protection assays (Figure III, available online at <http://atvb.ahajournals.org>) or by RT-PCR (Figure 1C) 7 days after cuff placement. The sFlt-1 gene transfer did not affect gene expression of RANTES, macrophage inflammatory protein-1 $\alpha$ , transforming growth factor- $\beta$ , macrophage inflammatory protein-2, and PlGF, but prevented or attenuated the increased gene expression of CCR1, interleukin-6, CCR2, MCP-1, Flt-1, CXCR2, eotaxin,

vascular cell adhesion molecule-1, intercellular adhesion molecule-1, Flk-1, and VEGF. The sFlt-1 gene transfer reduced increased immunostainings against VEGF, Flt-1, and Flk-1, but did not affect staining against vWF (Figure 2A).

Time course of plasma concentrations of sFlt-1 after sFlt-1 gene transfer was determined. Plasma sFlt-1 levels before and 3, 7, and 14 days after sFlt-1 transfection were  $467 \pm 37$ ,  $1037 \pm 132$  ( $P < 0.01$  versus baseline),  $927 \pm 215$  ( $P < 0.01$ ), and  $649 \pm 83$  pg/mL ( $P < 0.05$ ,  $n = 6$  each), indicating that sFlt-1 was released from the transfected muscle.

#### Plasma Lipid Levels in ApoE-KO Mice

Total cholesterol and triacylglycerol levels were  $503 \pm 11$  and  $38 \pm 6$  mg/dL in the control group,  $512 \pm 16$  and  $40 \pm 5$  mg/dL in the empty plasmid group, and  $497 \pm 10$  and  $39 \pm 3$  mg/dL in the sFlt-1 group, indicating that the observed effects of sFlt-1 gene transfer were not caused by changes in serum lipid levels.

#### Effects of Flt-1 Tyrosine Kinase Deficiency on Neointimal Hyperplasia

Wild-type and Flt-1 tyrosine kinase-deficient mice were fed a high-fat diet for 2 weeks, and cuff was placed as mentioned above. Mice received a high-fat diet for an additional 3 weeks. Neointimal formation was noted 21 days after cuff placement in wild-type mice. Compared with wild-type mice, Flt-1 tyrosine kinase-deficient mice displayed reduced neointimal formation (Figure IV, available online at <http://atvb.ahajournals.org>). Total cholesterol levels at 5 weeks of high-fat diet were  $520 \pm 21$  and  $511 \pm 18$  mg/dL in wild-type and Flt-1 tyrosine kinase-deficient mice, respectively.

#### Discussion

This study is the first to demonstrate the essential role of VEGF and Flt-1 signals in the development of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. VEGF is conventionally thought to be an endothelial cell-specific growth factor and to attenuate vascular disease by inducing endothelial proliferation and regeneration mainly through the endothelial type 2 receptor Flk-1.<sup>7</sup> Recent evidence, however, suggests that functional Flt-1 and Flk-1 are expressed in injured arterial wall cells other than endothelial cells. In this study, Flt-1, Flk-1, and VEGF were increased in lesional monocytes and medial smooth muscle cells at early stages and in neointimal and medial smooth muscle cells at later stages. Flt-1 is demonstrated to act as an important mediator of chemotaxis through vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and MCP-1.<sup>13–15</sup> We demonstrated that sFlt-1 gene transfer reduced the early inflammatory and proliferative changes and thus attenuated the development of neointimal formation. It is speculated, therefore, that VEGF might cause inflammation (monocyte recruitment and activation) and proliferation through Flt-1-mediated signals and thus cause neointimal formation after cuff-induced periadventitial injury. In addition, Flt-1 in smooth muscle cells is reported to mediate migration and proliferation *in vitro*.<sup>18,33</sup> An alternative interpretation is that VEGF directly caused migration and proliferation of smooth muscle cells resulting in neointimal

formation. In any way, our present finding of Flt-1 tyrosine kinase-deficient mice suggests the involvement of Flt-1-related signals in the pathogenesis of neointimal formation after periadventitial injury.

Periadventitial inflammation has a major role in the pathogenesis of cuff-induced neointimal formation.<sup>27,32</sup> To gain insight into the mechanism of VEGF-mediated inflammation and neointimal formation, we assessed gene expression of various inflammatory genes. The sFlt-1 gene transfer attenuated increased gene expression of inflammatory cytokines, adhesion molecules, chemokines, and chemokine receptors. Yamada et al<sup>34</sup> showed that MCP-1 is essential in VEGF-induced angiogenesis, vascular leakage, and inflammation. An essential role of MCP-1 in the development of neointimal formation after arterial injury has also been reported.<sup>27–29,35,36</sup> The sFlt-1 gene transfer attenuated increased VEGF, Flk-1, and Flt-1 gene expression, indicating that VEGF regulates its activity by an autocrine loop mechanism within diseased arterial wall cells, such as smooth muscle cells, endothelial cells, and lesional monocytes. A positive feedback effect of VEGF is supported by previous studies that demonstrated enhanced VEGF production by monocytes through Flt-1 stimulation.<sup>37</sup> Therefore, sFlt-1 gene transfer might attenuate cuff-induced neointimal formation mainly by suppressing inflammation (monocyte recruitment and activation).

This study has potentially significant clinical implications. Blockade of VEGF by sFlt-1 gene transfer can be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. The efficacy of this strategy for experimental tumor angiogenesis has already been tested.<sup>22</sup> Lutun et al<sup>38</sup> recently reported that treatment with anti-Flt-1 antibody attenuated the development of experimental tumor angiogenesis, arthritis, and atherosclerosis. One limitation of the present finding is that the pathogenesis of neointimal formation after periadventitial injury differs from that after endothelial injury/denudation and from that in humans. The endothelial integrity is preserved in cuff-induced periadventitial injury. For clinical application of our findings to human vascular disease, future studies are needed to examine the efficacy and safety of anti-VEGF strategy with sFlt-1 in experimental atherosclerosis and restenosis.

In conclusion, inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. These data support the notion that VEGF promote neointimal formation by acting as a proinflammatory cytokine after cuff-induced periadventitial injury.

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**Diagnostic Value of the Recovery Time-Course of ST Slope  
on Exercise ECG in Discriminating False- From  
True-Positive ST-Segment Depressions**

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## Diagnostic Value of the Recovery Time-Course of ST Slope on Exercise ECG in Discriminating False- From True-Positive ST-Segment Depressions

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**Background** Using the exercise ECG for diagnosing coronary artery disease (CAD) is hampered by the occurrence of false-positive (FP) ST-segment depression. Because it is known that the recovery ST-T time-course in CAD differs from that in FP subjects, the ST slope may help discriminate FP from true-positive (TP) results.

**Methods and Results** Treadmill digitized ECG from patients with significant ST-segment depressions and normal resting ECG were analyzed in 134 patients with CAD on angiography (>50% narrowing) and reversible perfusion defects (TP group), and 64 subjects with normal perfusion (FP group) on exercise single photon emission computed tomography. The ST slope between the J-point and J<sub>80</sub> was measured every minute up to 6-min postexercise. The ST slope was significantly higher in FP than in TP at peak exercise, and at postexercise 1-, 2- and 3-min ( $p < 0.01$ , all). Thereafter, it gradually increased in TP, while monotonically decreasing in FP. Its decrease from 3- to 6-min could correctly diagnose 88% of FP subjects, whereas it was found in only 19% of TP patients (total accuracy 83%).

**Conclusions** The ST slope change from early to late recovery is a simple yet reliable marker for discriminating FP from TP ST-segment responses in subjects with a normal resting ECG. (Circ J 2004; 68: 915–922)

**Key Words:** Coronary disease; Electrocardiography; Exercise; Ischemia

**G**ender and several anatomical and functional characteristics are associated with an increased incidence of abnormal ST-segment responses to exercise in the absence of coronary artery disease (CAD). Subsets with an increased incidence of false-positive (FP) exercise ECG responses with respect to ischemia include middle-aged women,<sup>1–3</sup> and patients with mitral valve prolapse.<sup>4,5</sup> Patients with left ventricular hypertrophy (LVH) form a separate subset with the potential for inadequate myocardial perfusion in the absence of significant CAD.<sup>4,6</sup> Advanced ECG analysis approaches applicable to the clinical evaluation of the standard exercise ECG in these subjects are currently unavailable and need to be developed.

The ST slope, usually computed as the slope between the J-point and the J<sub>80</sub>, has been mainly used for differentiating the configuration of ST-segment depression (ie, upsloping, horizontal, and downsloping). The latter two are considered more specific for myocardial ischemia than the upsloping shape.<sup>1,7</sup> However, in CAD, the shape often changes with time during the test and a relatively character-

istic time-course can be seen. ST-segment depression during exercise changes from upsloping (or horizontal) to downsloping in the early recovery at 2–4 min; that is, the ST slope decreases in early recovery (Fig 1A).<sup>7,8</sup> Subsequently, both ST-segment depression and the ST slope return gradually toward the baseline; that is, the ST slope increases in late recovery. On the other hand, in many subjects with FP ST-segment depression (FPD) (Fig 1B), the downsloping shape appears, not in the early, but in the late recovery. Unlike in CAD, the ST slope monotonically decreases from early to late recovery, possibly serving as the differentiating characteristic.<sup>5,8</sup>

We thus hypothesized that the temporal changes in the ST slope from early to late recovery are capable of differentiating these 2 groups and we performed high-resolution computer analysis of the ST slope in the recovery in a relatively large population with normal resting ECGs. Because the majority of subjects with normal exercise single-photon emission computed tomography (SPECT) results are no longer referred for coronary angiography in the clinical practice, we used this technique for the selection of FPD subjects. True-positive (TP) results were defined when both angiographic and exercise SPECT images were abnormal.

### Methods

#### Study Population

From the digitized ECG recordings consecutively obtained during routine treadmill testing for the evaluation of CAD, 198 patients (62±9 years) with an exercise-induced significant ST-segment depression were recruited. Patients were excluded if they had resting ECG abnor-

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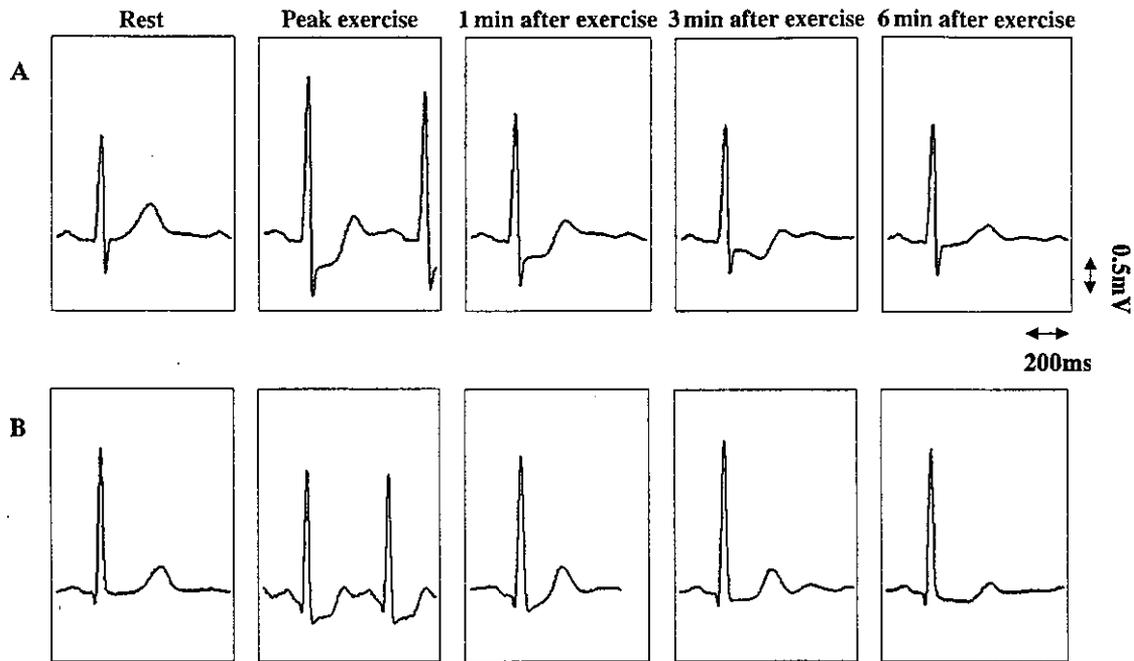


Fig 1. Representative exercise ECG from a patient with (A) true-positive (TP:  $V_5$ ) and (B) false-positive (FP:  $V_6$ ) ST-segment response. In the patient with a TP response, slow-rising ST-segment depression at peak exercise and at 1 min after exercise became downsloping at 3 min followed by its recovery at 6 min. That is, the ST slope transiently decreased soon after exercise but increased with time in late recovery. In the subject with a FP response, a horizontal ST-segment depression at postexercise 3 min became downsloping at 6 min; that is, the ST slope decreased in this late recovery.

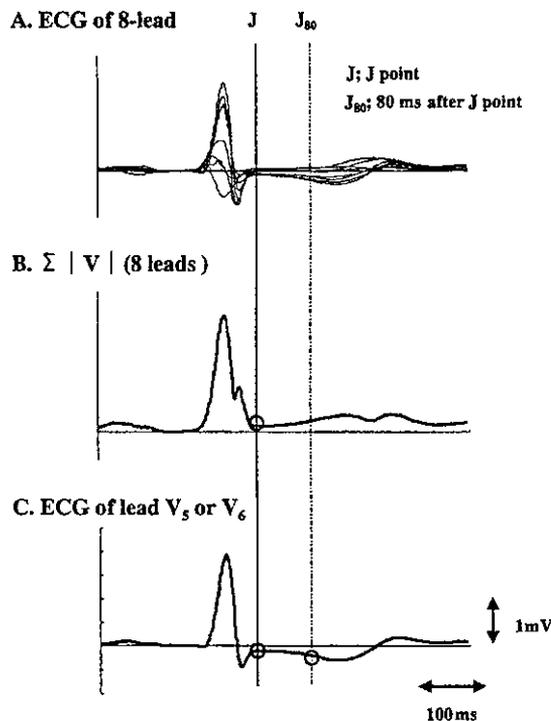


Fig 2. Computer measurements of the ST slope. From ECG data for all 8 leads (A), we computed the algebraic sum ( $\sum |V|$ ) of the absolute value of voltage relative to the isoelectric Q-Q baseline (B), which was used to determine the J-point. Once the J-point was determined, ST-segment displacements from the isoelectric Q-Q baseline at the J-point and at  $J_{80}$  were measured automatically (C). The ST slope is derived as the slope connecting these 2 points. Vertical solid and broken lines indicate the J-point and  $J_{80}$ , respectively.

malities such as prior myocardial infarction, ST-segment depression, T-wave inversion, LVH, or bundle branch block. We also excluded patients with atrial fibrillation or frequent extrasystole ( $>10$  beats/min) and those with exercise-induced ST-segment elevation ( $\geq 0.1$  mV). All subjects gave informed consent.

Patients were divided into the 2 groups according to the results of coronary angiography and exercise SPECT performed within 3 months after treadmill testing. The TP group comprised 134 patients ( $62 \pm 9$  years) who had angiographical coronary artery stenosis ( $>50\%$  luminal narrowing). Inducible ischemia was confirmed by the presence of reversible defects corresponding to coronary lesions. The FP group included 64 subjects ( $63 \pm 10$  years) with normal exercise SPECT images. Of them, 19 (30%) had undergone coronary angiography and none had significant CAD.

We investigated the presence of a history of hypertension, which may be associated with a high incidence of FPD in patients with mild LVH only discernible by echocardiographic study.<sup>9,10</sup>

#### Treadmill Exercise ECG

Exercise testing was performed according to our protocol, which was similar to the modified Bruce protocol.<sup>11</sup> We performed the test using a treadmill system (Formula, Esaote, Italy), while simultaneously digitizing the ECG data at 500 Hz (12-bit resolution) from rest, during exercise, and into recovery for at least 6 min. A hard copy of the standard 12-lead ECG at a paper speed of 25 mm/s at rest, at the end of each stage, at peak exercise, and every minute into recovery was produced. Termination of exercise was decided by the occurrence of exhaustion, ST-segment depression  $>0.3$  mV, significant arrhythmias, moderately severe angina, inadequate blood pressure response, or the attainment of 90% predicted maximal heart rate.

**Table 1** Clinical Characteristics of the Study Population

	FP (n=64)	TP (n=134)
M/F (n, %)	34/30 (53/47)	118/16 (88/12)**
Age (years)	63±10	62±9
Coronary risk factors		
Hypertension (%)	44	60*
Diabetes mellitus (%)	14	40*
Medications		
Nitrates (%)	11	60**
β-blockers (%)	16	57**
Calcium-antagonists (%)	27	68**
Coronary artery disease		
LMT (%)	-	1
SVD (%)	-	46
DVD (%)	-	33
TVD (%)	-	20

Values are mean ± SD or numbers (percentage). \*\* $p < 0.01$  vs FP group. \* $p < 0.05$  vs FP group. FP, false-positive; TP, true-positive; LMT, left main trunk disease; SVD, single vessel disease; DVD, double vessel disease; TVD, triple vessel disease.

On the ECG hard copies, we identified a significant ST-segment depression according to the following criteria: (1) horizontal or downsloping ST-segment displacement at the J-point  $\geq 0.1$  mV; and (2) upsloping ST-segment displacement at  $J_{80} \geq 0.15$  mV in at least 3 consecutive beats at peak exercise. Arterial blood pressure was measured by a sphygmomanometer at the end of each stage, peak exercise and recovery.

#### ECG Analysis

For the standard 12-lead ECG, only 2 of 6 limb leads are actually recorded, and the other 4 leads are derived mathematically from these 2 leads.<sup>12</sup> Our stress system capable of reproducing the 12-lead ECG actually records 2 unique limb leads (leads I and II) and 6 precordial leads in a similar manner. We performed the following analysis using the digitized ECG data set of 8 leads (I, II, and V<sub>1-6</sub>; Fig 2A).

To determine the ST slope, we found the peak of each R wave in lead V<sub>5</sub>, with which the QRS-T complex was averaged over 5 beats to improve the signal-to-noise ratio. On the QRS-T complex, we measured ST-segment displacement at peak exercise and at every minute during the first 6 min postexercise, in a lead with a greater ST-segment depression at peak exercise of either lead V<sub>5</sub> or V<sub>6</sub>. Because the J-point determination is occasionally difficult, we derived the algebraic sum ( $\sum |V_1|$ ) of the absolute value of voltage relative to the isoelectric Q-Q baseline from all 8 leads (Fig 2B). With reference to this curve, we determined the J-point by detecting the point at which the curve most closely approached the baseline (the lowest point of the trough). If the curve did not have a distinct trough, the J-point was assumed to be the inflection point at which a steep descent of the curve was terminated. Once the J-point was determined, ST-segment displacement from the Q-Q baseline at the J-point and  $J_{80}$  was measured automatically (Fig 2C). From these 2 values, we calculated the ST slope (mV/s). The ST slope, treated as a continuous variable ranging from negative to positive values, was used for the later intra- and intergroup comparisons. The time-courses of ST-segment depression and ST slope from peak exercise to the 6-min recovery (every minute, 7 time points) were assessed.

**Table 2** Exercise Test Results

	FP (n=64)	TP (n=134)	p value
Rest HR (beats/min)	72±11	67±11	<0.01
Rest SBP (mmHg)	139±22	132±19	<0.05
Peak HR (beats/min)	149±19	127±21	<0.01
Peak SBP (mmHg)	178±27	160±24	<0.01
Exercise time (s)	526±150	480±136	<0.05
Exercise-induced chest pain (%)	9	46	<0.01
ST-segment depression (mV)	-0.20±0.11	-0.22±0.12	<0.01

FP, false-positive; TP, true-positive; HR, heart rate; SBP, systolic blood pressure.

#### Exercise Thallium-201 Scintigraphy

All subjects underwent bicycle exercise testing according to the same end points as defined earlier. At near-maximal exercise, thallium-201 was intravenously injected and the patient was encouraged to exercise for another 1 min. SPECT images were obtained at 15 min (initial images) and 4 h (delayed images). The images were reconstructed into transaxial tomograms, which were assessed by 2 experienced physicians unaware of the coronary anatomy. Thallium uptake in myocardial segments was classified as normal, mildly, moderately or severely reduced, or absent. A reversible defect was defined when the classification of uptake improved by at least one category from the initial to the delayed image.

#### Statistical Analysis

All data are presented as mean ± SD. Unpaired t-test was used for comparisons between 2 groups. Differences in categorical variables were analyzed by chi-square analysis. The time-course of ST-segment depression and the ST slope were compared by analysis of variance for repeated measurement. When this test was significant, the Newman-Keuls post-hoc multiple comparison was performed.

For convenience in presentation, the term 'sensitivity' is used to measure the percentage of subjects who met the criteria for identifying FP results in the FP group. The term 'specificity' is used to measure the percentage of subjects who did not fulfill the criteria in the TP group.

Performance of diagnostic criteria was evaluated using receiver operating characteristic (ROC) curve analysis.<sup>13,14</sup> The area under the curve (AUC), which is a measure of the discriminatory power of the index, was calculated for each variable and these areas were compared statistically.<sup>15</sup> A p-value <0.05 was considered significant.

## Results

#### Patient Characteristics

Although age was similar between the groups, the FP group included more females than the TP group ( $p < 0.01$ , Table 1). Hypertension, common to both groups, was found more frequently in the TP (60%) group than in the FP group (44%,  $p < 0.05$ ). Approximately half of the TP patients (46%) had single-vessel disease.

#### Treadmill Exercise Test

Resting heart rate and systolic blood pressure were higher in the FP group than in the TP group ( $p < 0.01$  and  $p < 0.05$ , respectively; Table 2). Exercise time, peak heart rate and peak systolic blood pressure were also higher in the FP group ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.01$ , respectively). Chest

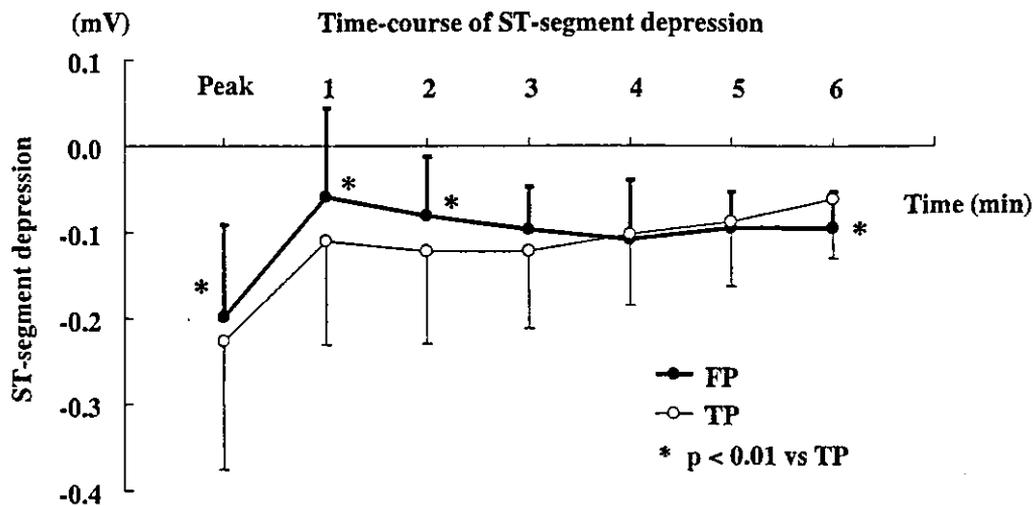


Fig 3. Comparison of the recovery time-course of ST-segment depression changes at the J<sub>80</sub> between the false-positive (FP: closed circle) and true-positive (TP: open circle) groups. \* $p < 0.01$  vs TP.

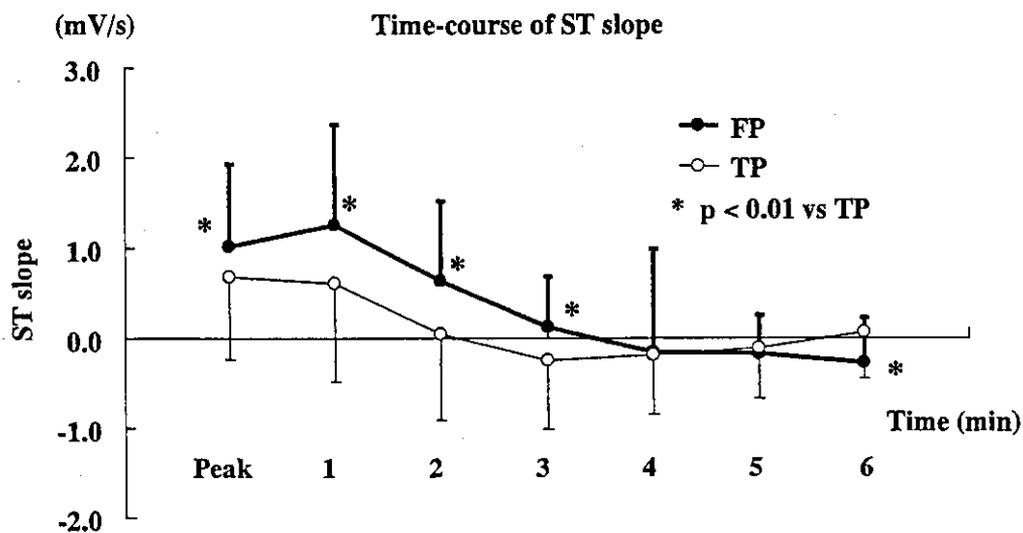


Fig 4. Comparison of the recovery time-course of changes in the ST slope between the false-positive (FP: closed circle) and true-positive (TP: open circle) groups. \* $p < 0.01$  vs TP.

pain, including atypical, equivocal, or typical features for angina, occurred in 46% of the TP group and 9% of the FP group ( $p < 0.01$ ) during the test. Maximal ST-segment depression (J<sub>80</sub>) was marginally but significantly greater in the TP ( $-0.23 \pm 0.15$  mV) than in the FP group ( $-0.20 \pm 0.11$  mV,  $p = 0.002$ , unpaired t-test).

#### Time-Course of ST-Segment Depression

Fig 3 shows the time-course of the recovery of ST-segment depression (J<sub>80</sub>). At peak exercise and in early recovery (at 1 and 2 min), ST-segment depression was significantly greater in the TP group ( $p < 0.01$ , all) than in the FP group. No difference was observed at 3, 4, and 5 min. In the late recovery, ST-segment depression gradually recovered toward the baseline in the TP group, but remained unchanged in the FP group. Consequently, it was greater in the FP group than in the TP group at 6 min postexercise ( $p < 0.01$ ). Despite these differences, ST-segment depression at any time point hardly differed between the 2 groups because of a considerable overlap, evidenced by the large

standard deviation.

#### Time-Course of the ST Slope

Fig 4 shows the time-course of the recovery of the ST slope. It decreased with time up to 3 min postexercise in each group. During this period, the ST slope was significantly lower in the TP group than in the FP group (all  $p < 0.01$ ). Thereafter it continued to decrease in the FP group, whereas it gradually recovered toward the baseline in the TP group. Thus, in the late recovery, the ST slope in the FP group changed in the opposite direction to that in the TP group.

Fig 5 shows the changes in the ST slope for each subject. In the FP group, the ST slope decreased between 3 and 6 min ( $0.12 \pm 0.55$  to  $-0.28 \pm 0.50$  mV/s,  $p < 0.001$ ), whereas it increased in the TP group ( $-0.25 \pm 0.77$  to  $0.06 \pm 0.52$  mV/s,  $p < 0.001$ ). Most FP subjects (56/64, 88%) showed a decrease during this period, which was observed in only 19% of TP patients (25/134).

The time-course difference in the ST slope ( $\Delta$ ST Slope,