Oral Imatinib Mesylate (STI571/Gleevec) Improves the Efficacy of Local Intravascular Vascular Endothelial Growth Factor-C Gene Transfer in Reducing Neointimal Growth in Hypercholesterolemic Rabbits

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Background—Platelet-derived growth factor (PDGF) antagonists have demonstrated beneficial effects on neointima formation, but in studies using PDGF inhibitors and extended follow-up, the lesions reoccur. These findings implicate a need to combine targeting of PDGF with other strategies. Stimulation of reendothelialization by treatment with endothelial cell mitogens of the vascular endothelial growth factor (VEGF) family counteracts restenosis, but there are also concerns regarding the durability of the effect with this approach.

Methods and Results—To explore whether a combined use of PDGF antagonist and stimulation of reendothelialization confers better results than each therapy alone, we combined systemic administration of imatinib mesylate (STI571/Gleevec, 10 mg/kg per d), a tyrosine kinase inhibitor with activity against PDGF receptors, with local intravascular adenovirus-mediated VEGF-C gene transfer (1.15 × 10^10 pfu) in cholesterol-fed, balloon-injured rabbits. Throughout the course of the STI571 therapy, the circulating concentrations were able to suppress PDGF receptor phosphorylation. At 3 weeks, the treatment with STI571 led to a transient decrease in intralesion macrophages and to an increase in intimal smooth muscle cell apoptosis. VEGF-C application reduced neointima formation and accelerated reendothelialization. However, none of the therapies alone reduced intimal thickening at a 6-week time point, whereas the combined treatment led to a persistent reduction (55% versus control) in lesion size at this time point.

Conclusions—Our study provides one of the first successful examples of gene therapy combined with a pharmacological treatment to modulate 2 distinct ligand-receptor signaling systems and suggests combination of local VEGF-C gene therapy with systemic inhibition of PDGF signaling as a novel principle to prevent intimal hyperplasia after vascular manipulations. (Circulation. 2004;109:1140-1146.)

Key Words: restenosis ■ gene therapy ■ platelet-derived factors

Locally targeted therapies such as drug-eluting stents have been successfully developed to deliver high concentrations of antimitotic agents at the site of vascular injury, but other therapies are still highly warranted, because these approaches may interfere with endothelial cell integrity and delay healing. It is hypothesized that rapid reendothelialization of the vessel wall after angioplasty should reduce restenosis. Regrowth of the endothelial monolayer inhibits the replication of underlying smooth muscle cells (SMCs) after injury, and local application of the endothelial cell mitogens of the vascular endothelial growth factor (VEGF) family have been shown to reduce neointima formation in animals. However, with extended follow-up, the therapeutic effect is attenuated, and in recent randomized placebo-controlled clinical trials, no reduction in restenosis was observed at target coronary or femorotibial lesions.

We hypothesized that better long-term results could be obtained with VEGF gene therapy by simultaneously inter-
ferring with additional distinct ligand-receptor systems and thereby targeting other components in neointima formation as well. For VEGF gene transfer, local transfection with VEGF-C encoding replication-deficient adenoviruses was performed. VEGF-C does not bind to VEGF receptor (VEGFR)-1 and thereby does not induce monocyte/macrophage infiltration into the transsected vessel segment. To inhibit platelet-derived growth factor (PDGF)-induced growth of macrophage and SMC populations in neointima, we used imatinib mesylate (STI571/Gleevec). This compound is a potent inhibitor of PDGF receptor kinase and also Bcr-Abl and c-kit oncoproteins and was recently approved for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors. STI571 has been used to block PDGF-driven processes in experimental tumor models and human malignancies. Various antagonists have been successfully used to interrupt PDGF-dependent neointima growth in animals (see references in the studies by Östman and Heldin and Leppänen et al). However, recent studies indicate that a single treatment with PDGF inhibitors does not result in beneficial long-term results, and results in humans have also been discouraging with agents that target PDGF either exclusively or together with other cytokines.

To evaluate the combined effects of STI571 therapy and VEGF-C gene transfer on intimal thickening, a hypercholesterolemic rabbit balloon injury model was used where the animals were subjected to a moderate (0.25%) cholesterol supplementation. The drug therapy was administered orally over the first 3 weeks after the injury. It was found that a brief STI571 therapy with VEGF-C adenovirus led to a long-lasting inhibition of neointima formation, whereas none of the therapies alone produced such an effect.

Methods

Cell Culture Experiments
Porcine aortic endothelial cells, transfected with PDGF β-receptor (PAE/PDGFβR) or PDGF α-receptor (PAE/PDGFαR), were maintained in F12 medium, and rabbit abdominal aortic smooth muscle cells (RAASMScs) were cultured in DMEM. Receptor expression and tyrosine phosphorylation were analyzed as described. Semi-quantitative determination of receptor phosphorylation was determined by scanning and analyses with the NIH ImageJ free-ware.

Adenoviruses
Replication-deficient E1-partially-E3-deleted clinical GMP-grade adenovirus vectors encoding hVEGF-C or nuclear-targeted lacZ under control of the cytomegalovirus promoter were produced as described and confirmed to be free from helper viruses, lipopolysaccharide, mycoplasma, and other bacterial contaminants.

Denudation Injury and Gene Transfer
The protocol was approved by the Kuopio University Ethics Committee. New Zealand White rabbits (National Laboratory Animal Center, Kuopio, Finland) weighing 2.3 to 2.8 kg were fed a 0.25% cholesterol diet 2 weeks before denudation of the entire aorta by a 3F embolectomy catheter (Sorin) introduced by a femoral artery cut-down. Three days after the injury, the animals were randomized to adenoviral gene transfer (1.15×10^10 pfu in 10 mL sterile saline) performed with local drug delivery catheter (Dispatch, Boston Scientific) introduced from a carotid artery cutdown to a 2-cm-long infrarenal abdominal aortic segment free of side branches, as documented by angiography. Intravascular procedures were performed using fentanyl/propofol (Hypnorm vet, Janssen Pharmaceutical) and midazolam (Dormicum, Roche) anesthesia. Imatinib mesylate (STI571/Gleevec; 10 mg/kg i.p. d−1; Novartis) was dissolved in sterile water (2 mg/mL) and given with an orogastric tube 1 hour before the gene transfer and every 12 hour thereafter for the following 3 weeks.

Blood Sampling and Diagnostic Autopsy
Plasma drug concentrations were determined by HPLC. Blood lipids were determined with commercial kits. Complete blood count was analyzed in the 3-week end point cohort. Three animals from each of the 8 groups were subjected to a diagnostic veterinarian autopsy (Dr P. Syrjälä, National Veterinary and Food Research Institute, Kuopio, Finland) with histopathological analysis from aorta, liver, kidney, lung, spleen, heart, adrenal gland, thyroid, testis, epididymis, bone marrow, jejunum, gastric ventricle, pancreas, and cerebrum.

Histopathology, Immunostaining, and Morphometry
Three hours before euthanasia, animals received an intravenous injection of 50 mg bromodeoxyuridine (BrdU) in 40% ethanol. The 2-cm-long vessel segment subjected to gene transfer was identified by comparing the angiography films with the anatomical landmarks visualized under an operating microscope. The vessel was dissected free and flushed gently with saline and processed additionally under the dissecting microscope. Four evenly spaced segments (4 to 5 mm apart) of the target vessel were fixed in 4% paraformaldehyde and 15% sucrose (pH 7.4) for 4 hours, rinsed in 15% sucrose (pH 7.4), embedded in paraffin, serially sectioned (5-μm sections), and stained for the detection of endothelium (CD31, DAKO, 1:50), SMCs (HHF35, DAKO, 1:50), macrophages (RAM-11, DAKO, 1:50), and BrdU-positive cells (Bu20a, DAKO, 1:50). Controls for the immunostainings included incubations with class- and species-matched immunoglobulins and omission of the primary antibodies. The remaining proximal, intermediate, and distal portions of the transfected vessel segment were processed for cryosections, X-Gal staining, and analysis of the transgene expression by reverse transcription–polymerase chain reaction (RT-PCR), respectively. Specimens from 3-week end point animals were stained for β-galactosidase activity with X-Gal. TUNEL reaction was performed on serially sectioned cryosections from the transfected vessel segment.

Intima-to-media ratio (I/M), cell counts, endothelial cell coverage, and proliferation index measurements were each performed from all animals from 4 randomly selected sections, taken 4 to 5 mm apart from each other, from the transfected vessel segment by an individual (I/M by M.C.; other measurements by O.L.) blinded to the treatment allocation. The mean values calculated from the 4 measurements were used in statistical analysis. Two observers controlled the obtained values from randomly selected multiple samples.

Reverse Transcription–Polymerase Chain Reaction
RNA was extracted from transfected aortic segments with Trizol reagent (Gibco-BRL), and 2 μg of RNA was used for cDNA synthesis. VEGF-C primers were 5′-CTGCTTACTGCGTTATCG-3′ and 5′-CCTGTTCTCTGTTATGTTGC-3′. Five microliters of the first PCR product was used for the second PCR with primers 5′-TCTCCAAAAAGCTACACC3′ and 5′-CAAATGTCATGTTGGAAG-3′.

Statistical Analyses
Stat View 5.0 (Abacus Concepts) and SPSS (SPSS Inc) software were used for statistical calculations. Values are expressed as mean±SE and compared with the double-placebo-treated control group by ANOVA with Dunnett’s post-hoc analysis. P<0.05 was considered statistically significant.

Results
To evaluate a short course of therapy with STI571 and adenovirus-mediated VEGF-C gene transfer on neointima formation, cholesterol-fed rabbits were randomized into local intravascular VEGF-C or lacZ adenovirus gene transfer and...
oral therapy with STI571 or carrier. The 4 treatment groups were analyzed at 2 different time points after balloon injury (Figure 1A).

To document the ability of STI571 to block rabbit PDGF receptor, we performed experiments with rabbit aortic SMCs and cells stably transfected with human PDGF α- and β-receptors. Analysis revealed that cultured rabbit aortic SMCs expressed PDGF β-receptors but not PDGF α-receptors (Figure 1B). Pretreatment of rabbit cells with STI571 induced a dose-dependent inhibition of PDGF-BB–induced PDGF β-receptor phosphorylation (Figure 1C). Scanning of immunoblots was performed to provide semi-quantitative data on PDGF receptor inhibition (Figure 1D). At 3 μmol/L STI571, 70.7% and 13.6% inhibition of the human receptor and 53.5% inhibition of the rabbit receptor were achieved.

Combination Treatment, in Contrast to Single Treatments, Results in Persistent Reduction in I/M

The extent of neointima was quantified by planimetry (Figure 2A). Control-treated rabbits displayed an I/M of 0.38±0.05 at the 3-week end point (Figure 2A, left). At this time point, the 2 groups subjected to VEGF-C gene transfer or VEGF-C together with the kinase inhibitor demonstrated a reduction in lesion formation, with I/M of 0.23±0.02 and 0.24±0.05, respectively (P<0.05). No significant reduction was seen after treatment with STI571 alone.

At the 6-week end point, the I/M in the control group was 0.51±0.08 (Figure 2A, right, and Figure 2B). Single therapy with VEGF-C or STI571 failed to induce a persistent reduc-
tion in lesion formation. In contrast, the group that had received both treatments displayed a decreased I/M (0.23 ± 0.05, P < 0.05). Compared with the control group, this corresponds to a 55% reduction in lesion size. The effect was completely attributable to a decrease in neointima size at both time points and did not involve vascular remodeling (data not shown).

The experimental groups were characterized with regard to the extent of balloon injury (internal elastic lamina [IEL] damage), animal weights, blood lipid profiles, and basic hematological parameters. No differences between the groups were observed (Table 1). Transgene expression was verified in the 2 lacZ-treated groups at 3 weeks by β-galactosidase staining and by RT-PCR of VEGF-C mRNA in the VEGF-C–transfected animals (data not shown). No differences were observed in circulating drug levels between the animals expressing the lacZ or VEGF-C transgene (Table 2). Finally, compared with the control animals, none of the experimental therapy groups displayed at the site of gene transfer any increases in inflammatory cell or foam cell accumulation or atherosclerotic lesion formation. No signs of toxicity to internal organs were observed in examination performed by a veterinarian pathologist (data not shown).

VEGF-C Gene Transfer Is Associated With Accelerated Endothelial Cell Coverage and Reduced Macrophage Infiltration

At the 3-week end point, no significant differences in reendothelialization were detected (Figure 3A, left). At 6 weeks, an increase in endothelial cell coverage was observed in both groups that had received VEGF-C gene transfer (Figure 3A, right, and Figure 3B). A decrease in the number of vessel wall macrophages, as determined by counting of RAM-11–positive cells, was seen only in STI571–treated animals at the end of drug therapy (Figure 4A, left). Three weeks later, significantly fewer vessel wall macrophages were seen in both groups that had received VEGF-C (Figure 4A, right, and Figure 4B). An inverse relationship between macrophage infiltration and endothelial cell coverage was thus observed at this end point.

Combination Treatment, But Not the Mono Treatments, Leads to a Reduced Intimal SMC Number at the Late End-Point, With No Associated Signs of Catch-Up Growth

A reduction in intimal SMC population occurred in the 2 VEGF-C–treated groups at 3 weeks and in the combination

### Table 1. Vessel Injury, Animal Weights, Lipid Profile, and Hematological Parameters

<table>
<thead>
<tr>
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<th>Three-week end point</th>
<th>Six-week end point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IEL damage, %</td>
<td>Weight, g</td>
</tr>
<tr>
<td>VEGF-C/STI571</td>
<td>+/−</td>
<td>−/−</td>
</tr>
<tr>
<td></td>
<td>23.6 ± 2.4</td>
<td>2551 ± 90</td>
</tr>
<tr>
<td></td>
<td>29.3 ± 1.1</td>
<td>2624 ± 150</td>
</tr>
<tr>
<td></td>
<td>27.6 ± 2.6</td>
<td>2618 ± 124</td>
</tr>
<tr>
<td></td>
<td>24.0 ± 2.0</td>
<td>2625 ± 66</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>10.2 ± 3.4</td>
<td>13.5 ± 2.3</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.51 ± 0.08</td>
<td>0.50 ± 0.08</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>9.3 ± 3.3</td>
<td>12.2 ± 2.3</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.0 ± 0.1</td>
<td>1.9 ± 0.5</td>
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<tr>
<td>Leukocytes, 10⁹/L</td>
<td>5.4 ± 0.2</td>
<td>5.3 ± 2.0</td>
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<tr>
<td>Neutrophils, %</td>
<td>19.0 ± 4.0</td>
<td>26.7 ± 3.2</td>
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<tr>
<td>Lymphocytes, %</td>
<td>77.7 ± 5.8</td>
<td>71.0 ± 3.5</td>
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<tr>
<td>Monocytes, %</td>
<td>1.3 ± 0.7</td>
<td>1.3 ± 0.8</td>
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<tr>
<td>Eosinophils, %</td>
<td>2.0 ± 1.5</td>
<td>1.0 ± 0.6</td>
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<tr>
<td>Erythrocytes, 10¹²/L</td>
<td>4.8 ± 0.3</td>
<td>4.9 ± 0.2</td>
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<tr>
<td>Hematocrit, %</td>
<td>32.2 ± 2.7</td>
<td>33.1 ± 1.4</td>
</tr>
</tbody>
</table>

### Table 2. Determination of Plasma STI571 Concentration (μmol/L)

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 10</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ animals</td>
<td>Peak</td>
<td>Nadir</td>
<td>Peak</td>
</tr>
<tr>
<td></td>
<td>10.27 ± 1.28</td>
<td>3.57 ± 0.74</td>
<td>16.63 ± 1.99</td>
</tr>
<tr>
<td>VEGF-C animals</td>
<td>11.08 ± 1.10</td>
<td>3.11 ± 1.13</td>
<td>14.26 ± 1.26</td>
</tr>
</tbody>
</table>
group at 6 weeks (Figures 5A and 5B), in line with the I/M data. None of the treatment groups displayed, at either end point, any changes in medial SMC number.

To additionally characterize the effects on SMCs, we determined the fraction of SMCs undergoing apoptosis and proliferation. At 3 weeks, an increase in SMC apoptosis was observed in the intima after the treatment with STI571 alone or in combination with VEGF-C gene transfer and a reduction in the fraction of proliferating SMCs was seen in both groups treated with VEGF-C (Figure 5C). No effects on the fraction of SMCs undergoing proliferation or apoptosis were observed in intima at the later time point or in media.

**Discussion**

Angioplasty disrupts the vessel wall and triggers subsequent stimulation of migration and proliferation of medial SMCs, infiltration of inflammatory cells, and deposition of matrix. Simultaneous stimulation of reendothelialization with local VEGF-based therapy and inhibition of PDGF stimulation of SMCs thus seem a rational strategy for interference. Our study is the first to combine these 2 therapeutic approaches.

The lack of absolute specificity of the presently available tyrosine kinase inhibitors creates intrinsic differences in the identification of targets involved. Besides abundant evidence for the importance of PDGF signaling in restenosis, 2 particular observations suggest that STI571 in this study predominantly, if not exclusively, acts through PDGF receptor inhibition. First, the SMC phenotype with increased apoptosis is identical to what was seen in a study with PDGF-AB/BB neutralizing aptamers. Second, the reduction in macrophage infiltration, which is the other phenotypic effect detected after STI571 treatment alone, reproduces the effects seen in apolipoprotein E-deficient mice after bone marrow grafting from PDGF B-chain-deficient mice. The findings in the present investigation are thus well in line with the existing literature regarding PDGF-dependent processes in the injured vessel wall. Inhibition of c-abl could possibly contribute to some of the effects, because c-abl has been described as a downstream mediator of PDGF signaling. It should also be mentioned that c-kit is expressed on hematopoietic cells, which have been shown to contribute to hyperplastic tissue over prosthetic surfaces. However, evidence...
that these cells contribute significantly to the neointima of native vessels are still lacking.

Side-by-side comparisons of the effects of STI571 on VEGFR-1 and -2 and on PDGF receptor phosphorylation to background levels without affecting VEGFR-1 or -2 phosphorylation. The in vivo concentrations of STI571 in the present study ranged between 2.4 and 16.6 \( \mu \text{mol/L} \) (Table 2). It is thus unlikely that in this study the effects of STI571 treatment involved effects of the drug on VEGF receptor activity. Furthermore, Bergers et al. have recently demonstrated in a mouse tumor model that PDGF kinase inhibitors, such as STI571, used in similar plasma concentrations as in the present study can be used successfully to target PDGFR-positive pericytes that are not targeted by VEGF kinase inhibitors.

The reendothelialization process after VEGF gene transfer in these hypercholesterolemic rabbits seems to be slower than that reported for rabbits fed a normal chow. Thus, the present model may also involve some degree of endothelial dysfunction caused by the increased cholesterol levels that may not be present in normocholesterolemic animals. At 6 weeks, the lesions displayed an increased endothelial coverage, and a reduction in vessel wall macrophages was seen that correlated with the degree of reendothelialization. Accordingly, no signs of increased inflammatory cell infiltration or foam cell formation were seen in the analyzed tissue sections. This may be a benefit for VEGF-C in cardiovascular gene therapy compared with VEGF-A, which can stimulate monocyte influx into tissues.

The lesions from the 6-week end point were also characterized by fewer intimal SMCs. Although static histological data cannot fully explain dynamic processes such as reendothelialization and lesion growth, our findings support the notion that the beneficial effects of the combination treatment occurred as a consequence of the VEGF-C–mediated reendothelialization and STI571 effects on SMCs. Because no effects on neointima formation occurred with VEGF-C treatment alone at 6 weeks, we conclude that the reendothelialization induced by VEGF-C can only lead to a lasting inhibition if the development of the initial lesion is suppressed. A reduction in macrophage content was also observed. Whether this finding contributes to the reduced I/M ratio is presently unclear. However, reduced macrophage content and increased reendothelialization were also observed in the group treated only with VEGF-C, and we thus conclude that these changes alone are not sufficient to reduce neointima formation.

Catheter-mediated transduction can lead to \( \leq 5\% \) gene transfer efficiency in blood vessels in large experimental animals and humans. Thus, gene transfer can only be used for secreted compounds to obtain a local effect. On the other hand, inhibition of intracellular signaling requires delivery of the therapeutic agent into most target cells, which cannot presently be achieved by gene therapy. Therefore, the administration modes of VEGF-C and STI571 are well suited for their respective modes of action. Both STI571 and VEGF-C have also been well tolerated in clinical trials.

In conclusion, our study demonstrates that the combination of oral STI571 therapy with local VEGF-C gene transfer results in a prolonged reduction of neointima formation and suggests a new approach for the prevention of restenosis after vascular manipulations.

**Acknowledgments**

Financial support was received from the Finnish Academy, Sigrid Juselius Foundation, and Ludwig Institute for Cancer Research. Dr Bergqvist was partly supported by grants from the Swedish Medical Research Council (MFR 00759). The authors thank E. Batt, M. Nieminen, and staff at National Laboratory Animal Center, Kuopio, Finland for skillful technical assistance; I. Schiller and M. Poiko-
lainen for preparation of the manuscript; and Dr P. Syrjälä for veterinary pathology evaluation.

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Circulation. 2004;109:1140-1146; originally published online February 9, 2004; doi: 10.1161/01.CIR.0000117234.08626.7C

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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