Local Delivery of Imatinib Mesylate (STI571)—Incorporated Nanoparticle Ex Vivo Suppresses Vein Graft Neointima Formation

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Background—Clinical outcome of surgical revascularization using autologous vein graft is limited by vein graft failure attributable to neointima formation. Platelet-derived growth factor (PDGF) plays a central role in the pathogenesis of vein graft failure. Therefore, we hypothesized that nanoparticle (NP)-mediated drug delivery system of PDGF-receptor (PDGF-R) tyrosine kinase inhibitor (imatinib mesylate: STI571) could be an innovative therapeutic strategy.

Methods and Results—Uptake of STI571-NP normalized PDGF-induced cell proliferation and migration. Excised rabbit jugular vein was treated ex vivo with PBS, STI571 only, FITC-NP, or STI571-NP, then interposed back into the carotid artery position. NP was detected in many cells in the neointima and media at 7 and 28 days after grafting. Significant neointima was formed 28 days after grafting in the PBS group; this neointima formation was suppressed in the STI571-NP group. STI571-NP treatment inhibited cell proliferation and phosphorylation of the PDGF-R-β but did not affect inflammation and endothelial regeneration.

Conclusions—STI571-NP–induced suppression of vein graft neointima formation holds promise as a strategy for preventing vein graft failure. (Circulation. 2008;118[suppl 1]:S65–S70.)

Key Words: nanotechnology ■ drug delivery system ■ grafting ■ platelet-derived factors ■ signal transduction

Clinical outcome of surgical revascularization using autologous vein graft is limited by vein graft failure resulting from accelerated neointima formation: 30% to 50% of vein grafts fail within 10 years.1 Because platelet-derived growth factor (PDGF), expressed by proliferating vascular smooth muscle cells (VSMCs) and infiltrating monocytes, plays a central role in the pathogenesis of vein graft failure,2 targeted molecular blockade of PDGF signaling is a potential strategy for preventing vein graft failure. Imatinib mesylate (STI571),3 a potent inhibitor of the c-Abl tyrosine kinase (TK), the c-Kit receptor kinase, and the PDGF-R TK, is approved for the treatment of patients with chronic myeloid leukemia. It has been shown that c-Kit–positive progenitor cells can differentiate into α-actin–positive VSMCs and may contribute to neointima formation after vascular injury.4 It has also been reported that c-Abl TK is involved in angiotensin II–induced VSMC hypertrophy.5 In contrast, STI571 has been shown to have little antiproliferative effects on endothelial cells.6 These data suggest that STI571 appropriately inhibits neointima formation without negative effects on endothelial regeneration/vascular healing, and thus provide a rationale for the use of STI571 as a VSMC-selective molecular targeting drug in the prevention of neointima formation associated with vein graft failure.

STI571 has been reported to inhibit balloon injury–induced neointima formation in rats7 when dosages beyond the clinical norm were used (50 mg/kg per day). In contrast, STI571 had no effect on neointima formation in rabbits when administered in a clinically relevant dosage (10 mg/kg per day).8 Recent clinical studies in humans have detected no beneficial effects of oral administration of STI571 (600 mg/d for 10 days)9 on in-stent restenosis. These data suggest that systemic administration of STI571 at clinical dosages may not be sufficient to antagonize PDGF-induced vascular responses at the site of vascular injury. It has been suggested that STI571 administered at standard dosages (400–800 mg/d) may not reach sufficient serum concentrations (maximum concentration: <10 μmol/L) to function as an inhibitor of PDGF-R signaling.10 Furthermore, long-term administration of STI571 causes cardiac mitochondrial dysfunction that results in cardiotoxicity and ventricular dysfunction.11
Therefore, preventing vein graft failure via STI571-mediated PDGF-R signaling blockade requires an efficient local drug delivery system. Ex vivo local delivery of drugs or genes to the vein has been used as a clinically relevant approach. We have recently developed bio-absorbable polymeric nanoparticles (NP) formulated from the polymer polyethylene-glycol (PEG)-modified poly(DL-lactide-co-glycolide) (PLGA).12,13 PEG-PLGA NP offers the advantages of safety, efficient intracellular delivery of different classes of therapeutic agents, and the capacity for sustained intracytoplasmic release.14,15 Therefore, we hypothesized that STI571-incorporated NP could be an innovative therapeutic strategy for preventing vein graft failure. We investigated whether our NP-based drug delivery system worked as an intracellular ex vivo delivery system to the excised vein, and whether blockade of PDGF-R TK by STI571-incorporated NP suppressed vein graft neointima formation in vivo.

Materials and Methods

Preparation of PEG-PLGA NP
PEG-PLGA NP encapsulated with fluorescence marker or STI571 was prepared using an emulsion solvent diffusion method, as previously reported.12,13 Additional details can be found in the online Data Supplement.

Cellular Uptake and Intracellular Distribution of NP In Vitro
We cultured rat aortic smooth muscle cells (SMCs) and evaluated the cellular uptake of PEG-PLGA NP by fluorescence microscopy. Additional details can be found in the online Data Supplement.

Measurement of In Vitro FITC Release Kinetics From NP
To measure FITC release kinetics, FITC-NP (n=8) was immersed in Tris-EDTA buffer, and the released FITC from NP was measured.

Cell Proliferation, Migration, Cytotoxicity, and TUNEL Assay
We cultured human coronary artery SMCs and evaluated proliferation, migration,6 cytotoxicity, and apoptosis. Additional details can be found in the online Data Supplement.

Experimental Animal Models
Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 2.5 to 3.0 kg were fed a high-cholesterol diet for 2 weeks before the operation. Animals were anesthetized, a midline neck incision was made, and an approximately 3-cm segment of the jugular vein was dissected free; all side branches were ligated. The vein segments were gently flushed, and placed in a buffer alone (n=11) or in a solution containing either FITC-encapsulated PEG-PLGA NP at 0.5 mg/mL (n=11), STI571-encapsulated PEG-PLGA NP at 0.5 mg/mL containing STI571 at 100 µmol/L (n=11), or STI571 alone at 100 µmol/L (n=11) for 30 minutes at room temperature. The treated vein segments were interposed into ipsilateral carotid arteries in an end-to-side fashion. The animals were maintained on the same high-cholesterol diet throughout experimental period. All animals received aspirin at 20 mg/day from 3 days before the graft procedure until euthanasia. After venous blood samples were taken, animals were killed with a lethal dose of anesthesia on days 7 (n=5 each) and 28 (n=6 each). The vein grafts were harvested, flushed with saline, and used for histopathologic, immunohistochemical, and biochemical studies.

Ex Vivo NP Delivery in Human Vein
Segments of internal thoracic vein were obtained from patients undergoing coronary arterial bypass surgery. Additional details can be found in the online Data Supplement.

Histopathologic and Immunohistochemical Analysis
Tissue sections from the grafts were harvested and prepared for analysis. Additional details can be found in the online Data Supplement.

Western Blot Analysis
Protein was extracted from cultured SMCs or frozen vein graft tissues. Western blot analysis was performed with antibodies against human PDGF-R-β, phospho-PDGFR-β, phosphotyrosine, phospho-p44/42 MAPK (ERK 1/2), ERK 1/2, c-Abl TK, or actin (additional details can be found in the online Data Supplement).

Statistical Analysis
Data are expressed as the mean±standard error of the mean (SEM). Statistical analysis of differences was performed by ANOVA and Bonferroni’s multiple comparison tests. Statistical significance was set at P<0.05.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

In Vitro Cell Uptake and Intracellular Distribution of NP and NP Release Kinetics

In Vitro
When incubated with rat aortic and human coronary artery SMCs, the fluorescence-encapsulated NP showed excellent capacity of intracellular distribution (Figure 1A). In contrast, no fluorescence was detected when the SMCs were incubated with blank NP or fluorescent molecules only. A large fraction (>90%) of the NP rapidly entered into the cells, and this incorporation rate sustained until 24 hours (Figure 1B). An endocytosis inhibitor (chlorpromazine hydrochloride) attenuated the NP-mediated intracellular incorporation of fluorescence (supplemental Figure I). Fluorescence confocal microscopic study revealed the intracellular retention of NP (Figure 1C). Transmission electron microscopy of the cellular cross-sections revealed the intracellular localization of NP at 1 hour of incubation (Figure 1D).

An analysis of the in vitro FITC release kinetics from FITC-NP showed an early burst of FITC release such that approximately 40% of the total amount ultimately released was present on day 1, followed by sustained release of the remaining FITC over the next 28 days (Figure 1E).

In Vitro Effects of STI571-NP on PDGF-Induced Proliferation and Migration of VSMCs and on Receptor Phosphorylation
As reported by other investigators,6,17 non–NP-STI571 attenuated PDGF-induced proliferation and migration in a dose-dependent manner (Figure 2A and B). STI571 at a concentration of 10 µmol/L prevented the PDGF-induced cell proliferation and migration. The PEG-PLGA NP-containing STI571 at 10 µmol/L also prevented the PDGF-induced responses of VSMCs (Figure 2A and B). Results of a TUNEL assay showed no detectable increase in STI571-induced increase in apoptotic cells (data not shown). Results of a
cytotoxicity assay showed that human coronary artery SMCs incubated with blank PEG-PLGA NP at a concentration of 1 mg/mL remained 100% viable relative to control (data not shown).

Western blot analysis showed that in human coronary artery SMCs, PDGF-induced phosphorylation of PDGF-R-β was suppressed by STI571 at 10 μM as well as by STI571-NP (Figure 2C).

Efficacy of NP-Mediated Drug Delivery System to Vein Grafts
Ex vivo incubation of NP with excised rabbit jugular vein or human internal thoracic vein for 30 minutes resulted in high SMC uptake in the media and in some cells in the adventitia (supplemental Figure IIA and IIB). After 7 days of grafting in rabbits when a thin neointima was formed, FITC-positive cells were detected in the neointima and media (% positive area: 51±9%). After 28 days, many FITC-positive cells were still noted in the neointima and media (% positive area: 12±5%). In contrast, no FITC immunoreactivity was noted in veins incubated with PBS.

Effects of STI571-NP on Vein Graft Failure in Rabbits
As we previously reported,18 significant neointima developed 28 days after grafting in animals interposed with control PBS-treated vein grafts. Ex vivo treatment with STI571-NP, but not with STI571 only or FITC-NP, markedly attenuated neointima formation at day 28 (Figure 3).

Increased monocyte infiltration and PCNA-positive proliferating cells were observed in the intima-media and adventitia of STI571-NP treated grafts as compared to control PBS. The effects of STI571-NP were also observed in other mediators of neointima formation such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO).

Figure 1. In vitro cellular uptake and intracellular distribution of NP. A, Fluorescence microscopic pictures of rat aortic SMCs incubated with blank PEG-PLGA NP, nonencapsulated coumarin-6, and coumarin-6–encapsulated PEG-PLGA NP (0.5 mg/mL) for 60 minutes. Nuclei were counterstained with propidium iodide (PI). Scale bar=200 μm. B, In vitro time course of the percentage of cellular uptake of NP (100× fluorescence-positive cells/total cells per field) are shown. Data are means±SEM (n=4). C, Confocal fluorescence microscopy photographs (left: an X–Y axis image, right: a Z axis image of cross-sections from yellowish dashed line displayed on an X–Y axis image) of rat aortic SMCs incubated with medium containing coumarin-6 encapsulated NP at 0.5 mg/mL. Coumarin-6 fluorescence is green. Nuclei are stained red. Scale bar=10 μm. D, Transmission electron microscopic picture of cross-section of human coronary artery SMCs incubated with NP for 60 minutes. Arrows indicate NP in the cytoplasm. Scale bar=500 nm. E, In vitro time course of cumulative FITC release from the FITC-incorporated NP (n=8 each). The percentage of incremental quantities of released FITC was plotted against time.
We demonstrated for the first time that PEG-PLGA NP is an effective means of delivering drugs or genes that target intracellular proteins involved in the pathogenesis of vein graft neointima formation in rabbits. A, Low-powered micrographs of whole vessel cross-sections of vein grafts from PBS-, FITC-NP-, STI571 only-, and STI571-NP–treated vein grafts after 28 days, stained with Elastica Van Gieson. White line shows internal elastic lamina. Scale bar=1 mm. B, Expanded high-powered micrographs from boxed area in A. Scale bar=100 μm. C, Effects of STI571-NP on neointima area 28 days after grafting (n=6 each). *P<0.05 vs PBS-treated group.

Figure 3. Effects of ex vivo treatment with STI571-NP on vein graft neointima formation in rabbits. A, Low-powered micrographs of whole vessel cross-sections of vein grafts from PBS-, FITC-NP-, STI571 only-, and STI571-NP–treated vein grafts after 28 days, stained with Elastica Van Gieson. White line shows internal elastic lamina. Scale bar=1 mm. B, Expanded high-powered micrographs from boxed area in A. Scale bar=100 μm. C, Effects of STI571-NP on neointima area 28 days after grafting (n=6 each). *P<0.05 vs PBS-treated group.

Effects of STI571-NP on PDGF, PDGF-R, PDGF-R Phosphorylation, and MAPK Pathway

Immunohistochemical studies showed that no PDGF was detected in normal veins. In contrast, intense immunohistochemical staining for PDGF was noted in vein graft tissues 7 days after grafting. There were no significant differences in the degrees of the positive staining area among the 4 groups (supplemental Figure IV). Western blot analysis showed that ex vivo treatment with STI571-NP, but not with STI571 only or FITC-NP, markedly attenuated expression of PDGF-R protein and phosphorylation of PDGF-R kinase, phosphorylation of ERK1/2, and c-Abl TK, at 7 days after grafting (Figure 4A through 4C).

Figure 4. Effects of STI571-NP on the expression of phospho-PDGF-R, PDGF-R-β, phospho-ERK 1/2, MAP kinase (ERK 1/2), c-Abl TK, and actin, 7 days after grafting. A, Immunoblots are representative of 5 individual vein grafts from each group, showing identical results. B, Densitometric analysis of PDGF-R-β and phosphorylation expression (n=5 each). Data are expressed as percent change from PBS group (100%). *P<0.05 vs PBS-treated group. **P<0.01 vs PBS-treated group. C, Densitometric analysis of phospho-ERK 1/2 and c-Abl TK expression (n=5 each). Data are expressed as percent change from PBS group (100%). *P<0.05 vs PBS-treated group.

getting drugs in excised veins. This NP system is bioabsorbable polymer with a long history of safe use in medical applications. Therefore, this system may represent a novel NP-mediated drug delivery system to prevent vein graft failure.

We showed that the NP was endocytosed rapidly by VSMCs and was retained stably in the intracellular space. Impressive ex vivo delivery of NP into human veins suggests that this NP-mediated drug delivery system can be applied to clinical settings for humans. An important finding was that long-term retention of NP in neointima and medial cells of vein grafts was detected until day 28. After cellular uptake of NP, NP slowly releases encapsulated drugs or genes into the cytoplasm as PLGA is hydrolyzed, resulting in an intracellular drug delivery. The bio-absorption time of PLGA in the body can be controlled by changing material make-up of PLGA, thus the function of the intracellular drug delivery system can be modified. Therefore, (1) this NP-mediated drug delivery system works as an excellent ex vivo delivery system for the excised vein; and (2) this system provides an effective means of delivering drugs or genes that target intracellular proteins involved in the pathogenesis of vein graft neointima formation.

Discussion

We demonstrated for the first time that PEG-PLGA NP is an excellent system for intracellular delivery of molecular tar-
We selected STI571 because this compound is known to target the PDGF-R TK (see Introduction). NP-incorporated with STI571 attenuated the proliferation of human VSMCs in vitro and the formation of vein graft neointima formation in vivo, both of which are known to be associated with the inhibition of the target molecules of STI571 (PDGF-R TK, c-Abl TK) and downstream signal of PDGF-R (ERK). NP-incorporated with STI571 did not affect endothelial regeneration process after vein grafting. In preliminary experiments, tissue concentrations of STI571 were measured immediately after and 6 hours after incubation of excised veins with STI571-NP by HPLC system, which showed under the limit of detection (1 ng/mL). Although the precise intracellular concentration and distribution of STI571 is unclear, our present data (Figures 2 and 4) provide evidence that (1) STI571-incorporated NP may block PDGF-R signaling possibly via slow release of STI-571 into the cytoplasm as NP is hydrolyzed; and (2) PDGF-R signaling blockade by NP-incorporated with STI571 is a means for treating vein graft neointima formation in vivo.

Inflammatory-proliferative changes have been shown to play a central role in the pathogenesis of vein graft neointima formation. In early stages, the neointima lesion has an inflammatory nature characterized by mononuclear cell infiltration, followed by VSMC proliferation.19 We recently reported that blockade of monocyte chemoattractant protein-1 (MCP-1) by adenovirus-mediated ex vivo transfer of 7ND gene to autologous vein grafts suppressed neointima formation in dogs.18 We also have demonstrated that MCP-1 plays a central role in neointima formation following arterial mechanical injury.20,21,22 In a previous study,18 we showed that blockade of MCP-1 attenuated both inflammation (monocyte infiltration) and proliferation (appearance of proliferating VSMC) in vein grafts. In contrast, data from this study show that NP-mediated delivery of STI571 reduced PDGF-induced proliferation but not inflammation, suggesting that (1) PDGF-mediated proliferative changes might be located downstream of inflammatory changes, or (2) the mechanism of action of STI571-mediated inhibition of proliferation might be distinct from that of anti-MCP-1-mediated attenuation of proliferation and inflammation. If STI571 and anti-MCP-1 treatment exert their effects through different pathways, it would be interesting to examine whether combined blockade of PDGF and MCP-1 would have additive inhibitory effects on vein graft failure.

Expression of PDGF is known to be low in normal blood vessels, but mechanical forces stimulate SMC expression and release of PDGF, and induce PDGF-R phosphorylation (activation).23 We show here that PDGF and the phosphorylation levels of its receptor were up-regulated in vein grafts. STI571-incorporated NP did not affect increased PDGF expression, but it did suppress the protein expression of PDGF-R, PDGF-R kinase, and c-Abl TK in vivo. This could suggest the presence of a positive-feedback loop that, in the absence of STI571, potentiates PDGF-mediated proliferation in vein grafts. It is also possible that reduced PDGF-producing cells (PCNA-positive cells) in the vein graft or blockade of multiple intracellular kinases might have contributed to the beneficial effects of STI571-incorporated NP on vein graft neointima formation in vivo.

One limitation in the present study is that only single dose of STI571-NP was examined. It is practically difficult to obtain the dose–response relationship of this NP system in small animals, because the dose–response relation of STI571 and polymer needs to be examined. For translation of our present findings into clinical medicine, further studies are therefore needed to define a dose-response relation in large animal models.

In conclusion, blockade of PDGF signaling by STI571-incorporated NP-inhibited proliferation of VSMCs in vitro and suppressed vein graft neointima formation in vivo. This NP-mediated drug delivery system provides an innovative and clinically feasible therapeutic strategy for preventing vein graft failure.

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**Disclosures**

Dr Egashira holds a patent on the results reported in the present study. The other authors report no conflicts.

**References**


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Materials and Methods

Preparation of PEG-PLGA NP

STI571 (a PDGF-R tyrosine kinase inhibitor, Novartis) was purchased from pharmacy. A poly-(ethylene glycol)-block-lactide/glycolide copolymer (PEG-PLGA) with an average molecular weight of 22,900 and a PLGA copolymer ratio of lactide to glycolide of 75:25 (Absorbable Polymers International, USA) was used as a wall material for the NP. Fluorescein-isothiocyanate (FITC, Dojindo laboratories, Kumamoto, Japan) and coumarin-6 (MP Biomedicals) were used as fluorescent markers of the NP.

The fluorescence markers and the STI571-encapsulated PEG-PLGA NP was prepared using an emulsion solvent diffusion method, as previously reported.¹,² These three ingredients to be encapsulated in PEG-PLGA were prepared separately. Both 0.1 g of FITC and 0.001 g of coumarin-6 as fluorescence marker were dissolved in a 10 ml ethanol solution respectively and then, mixed with a 20 ml acetone solution containing 2.0 g of PEG-PLGA. Also 0.3 g of STI571 was dissolved in a 10 ml methanol solution and mixed a 20 ml acetone solution containing 2.0 g of PEG-PLGA. Each one of the solutions was dropped into 50 ml purified water at 40 °C with a speed of 400 rpm using the propeller-type agitator with four blades.

After evaporating the organic solvent for about 2 hours under reduced pressure at 40 °C, the prepared suspension of the fluorescence markers or the STI571-encapsulated PEG-PLGA NP was filtrated using a membrane filter having 32 µm in average pore size for removing agglomerates of the NP.
We prepared a poly-(ethylene glycol)-block-lactide/glycolide copolymer (PEG-PLGA) using emulsion solvent diffusion method. The encapsulated agents are entrapped into the core of PEG-PLGA polymer matrix as shown below.

**Advantages of PLGA NP-based drug delivery system (DDS) include:**
- Matrix polymer (both PEG and PLGA) is bioabsorbable.
- NP can incorporate water-soluble drugs/oligonucleotides/DNAs.
- NP can cross cell membrane via endocytosis (efficiency of cellular uptake: 90% or more), and deliver the encapsulated agents into the cytoplasm.
- Incorporated drugs are slowly released from NP with hydrolysis of PLGA.
The mean particle size was analyzed by light scattering method (Microtrack UPA150; Nikkiso, Tokyo, Japan). A sample of nanoparticulate suspension in distilled water was used for particle size analysis. An average diameter of PEG-PLGA NP was 44 nm with a narrow size distribution (see Figure in the next page). The FITC-, coumarin-6 and STI571-encapsulated PEG-PLGA NP contained 5 % (w/w) FITC, 3 % (w/w) coumarin-6, and 13 % (w/w) STI571, respectively.
**Cellular uptake and intracellular distribution of NP in vitro**

Rat aortic smooth muscle cells (SMCs) were obtained from a commercial source (Toyobo) and then cultured in DMEM (Sigma) supplemented with 10 % FBS (Equitech-Bio, Inc.) except where otherwise indicated. Each cells were used between passages 4 to 8. SMCs were seeded on chambered cover glasses and incubated at 37 °C/5 % CO₂ environment until cells were subconfluent. The growth medium was replaced with the coumarin-6- encapsulated PEG-PLGA NP suspension medium (0.5 mg/ml) and then further incubated for 1 hour. At the end of experiment, the cells were washed three times with PBS to eliminate excess NP which were not incorporated into the cells. Then, the cells were fixed with 1 % formaldehyde/PBS buffer and nuclear was counterstained with propidium iodide (PI). Cellular uptake of coumarin-6- encapsulated PEG-PLGA NP was evaluated by fluorescence microscopy. The images were digitized and analyzed with Adobe Photoshop and Scion Image Software. The total number of fluorescence positive cells in each field and the number of total cells was counted. Cellular uptake percentage was assessed by the percentage of fluorescence positive cells per total cells in each field. In part of experiment, the internal properties of NP was examined by transmission electron microscopy (H-7000E, Hitachi, Tokyo, Japan). To confirm NP is uptaken via endocytosis pathway, the chambered cover glasses seeded SMCs were treated with chlorpromazine hydrochloride (CPZ) (Sigma) at 50 μM for 30 minutes and then, with the coumarin-6-encapsulated PEG-PLGA NP-suspension medium (0.05 mg/ml) and then further incubated for 30 minutes. SMCs were fixed and counterstained with PI for fluorescence microscopic study.

**Measurement of in vitro FITC release kinetics from NP**

To measure FITC release kinetics, FITC-NP (n = 8) was immersed in Tris-EDTA buffer, and the released FITC from NP was measured.

**Cell proliferation, migration, cytotoxicity and TUNEL assay**
Human coronary artery SMCs (Lonza. Inc. Walkersville, MD, USA) were cultured, and placed into 48-well culture plates. The cells were stimulated by the addition of PDGF at 10 ng/mL (Sigma, Tokyo, Japan). Either various concentration of STI571 (0.1, 1, 10 μM), STI571-encapsulated PEG-PLGA NP (PEG-PLGA at 0.05 mg/ml containing STI571 at 10 μM), or vehicle alone was added to the wells, and four days later, the cells were fixed with methanol and stained with Diff-Quick staining solution (Sysmex corporation, Kobe, Japan) and a single observer counted the number of cells/plate.

Migration of rat aortic SMCs was assessed with a Boyden chamber type cell migration assay kit housing a collagen-precoated polycarbonate membrane with 8.0-μm pores (Chemicon international Inc.), as we previously described. Lower chambers were filled with solvent or human PDGF at 10 ng/mL. Then cells (1 × 10^5 cells/mL) were placed on the upper side of the membrane and allowed to migrate through the pores. STI571 (0.1, 1, 10 μM), STI571-encapsulated PEG-PLGA NP (PEG-PLGA at 0.05 mg/ml containing STI571 at 10 μM), or vehicle alone was added to the upper chamber. After 4 hours of incubation, the number of cells that migrated to the lower surface of the membrane was counted per x 200 high-power fields.

The cytotoxicity of PEG-PLGA NP on human coronary artery SMCs was determined using a MTS assay (Cell Counting Kit-8, Dojindo Inc.). The cells were grown in 96-well microtiter plates for 24 hours, and then they were treated with different concentrations of FITC-incorporated PEG-PLGA NP suspension medium with 10 % FBS. The plates were incubated for 48 hours and then the medium was replaced with 200 μl of fresh medium. Next, 2 mg/ml MTS solution was added and the plates were incubated again for 4 hours at 37°C/5%CO₂. Finally, absorbance was measured at 490 nM using microplate reader. Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with PEG-PLGA NP and for control non-treated cells.
Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (in situ apoptosis detection kit, Takara).

**Experimental animal models**

All *in vivo* experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, according to the Guidelines of the American Physiologic Society.

Male Japanese white rabbits (KBT Oriental, Tokyo) weighting 2.5 to 3.0 kg, were fed a high cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks before the operation. Animals were anesthetized with intramuscularly injection with ketamine hydrochloride (50 mg/kg) and xyradine (10 mg/kg), and a midline neck incision was made to expose the left jugular vein and the left common carotid artery. An approximately 3-cm segment of the jugular vein was dissected free using a non-touch technique, and all side branches were ligated with 4-0 silk ties. The vein segments were gently flushed to remove residual blood, and placed in a buffer alone (*n* = 11) or in a solution containing either FITC-encapsulated PEG-PLGA NP at 0.5 mg/ml (*n* = 11), STI571-encapsulated PEG-PLGA NP at 0.5 mg/ml containing STI571 at 100 μM (*n* = 11), or and STI571 alone at 100 μM (*n* = 11) for 30 minutes at room temperature. The treated vein segments were interposed into ipsilateral carotid arteries in an end-to-side fashion with 7-0 polypropylene monofilament sutures after intravenous heparin at 1000 IU. The animals were maintained on the same high cholesterol diet throughout experimental period.

All animals received aspirin at 20 mg/day until euthanasia from 3 days before the graft procedure. After venous blood samples were taken, animals were killed with a lethal dose of anesthesia on days 7 (*n* =5 each) and 28 (*n* =6 each). The vein grafts were harvested, flushed with saline and divided into two parts at the center of the segment. The proximal part was used for histopathological and immunohistochemical study. The distal part was snap-frozen.
in liquid nitrogen and stored at -70 °C used for Western blotting analyses.

**Ex vivo NP delivery in human vein**

Segments of internal thoracic vein were obtained from patients undergoing coronary arterial bypass surgery. Ethical permission was obtained from the ethical committee of Kyushu University. The vein segments were incubated *ex vivo* with FITC- encapsulated PEG-PLGA NP at 0.5mg/ml for 30 minutes at room temperature. Cellular uptake of FITC was evaluated by fluorescence microscopy.

**Histopathological and Immunohistochemical Study**

Tissue sections from the grafts were prepared and either stained with Elastica Van Gieson. The neointimal area, the area within the internal elastic lamina (IEL), and the lumen area were measured by computerized morphometry, which was carried out by a single observer who was blinded to the experimental protocol. The sections were also subjected to immunohistochemical staining using with either non-immune mouse IgG (Dako) as a control, or with antibodies against PDGF-B (PGF-007, Mochida), macrophage (RAM-11, 1:100), proliferating cells (PCNA, 1:50), endothelial cells (CD31, 1:50, all from DAKO, Tokyo, Japan), and FITC (American Researcharch Product, Belmont, MA, 1:1000). Following avidin-biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. To quantify proliferating cells, at least four representative images were collected, and the percentage of PCNA-positive cells per total cells in each picture was calculated; the average of the four pictures was reported for each animal. The percentage of total areas of positive cells with PDGF-B, macrophage, and FITC in each section was measured. Endothelial cell coverage was also quantified followed by the formula: 100 x (the length of CD31-positive layer / the length of luminal surface in cross-sections). All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analyzed using Adobe Photoshop CS and Scion Image.
Western blot analysis

Protein was extracted from cultured vascular SMCs or frozen vein graft tissues. Samples were homogenized in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 50 mM NaCl, 30 mM sodium phosphate, 50 mM NaF, 1 % aprotinin, 0.5 % pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 5 mM leupeptin and phosphatase inhibitor cocktail (Pierce, Rockford, IL). Cell lysates (20 µg) were separated on 7.5 % polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore Co. Hercules, Ca). Protein expression was analyzed using antibodies against human PDGF-R-β (0.1 mg/ml), phospho-PDGFR-β (0.5 mg/ml), ERK 1/2 (0.5 µg/ml, all from R&D Systems Inc., MN), phosphotyrosine (0.5 µg/ml, clone 4G10, Upstate), phosho-p44/42 MAPK (1:2000, Cell Signaling), c-Abl TK (0.1 µg/ml, Santa Cruz Biotechnology Inc.), or actin (Sigma). Immune complexes were visualized with horseradish peroxidase–conjugated secondary antibodies. Bounded antibodies were detected by chemiluminescence with the use of an ECL detection system (Amersham Biosciences) and quantified by densitometry.

Cytotoxicity and TUNEL assays

The cytotoxicity of PEG-PLGA NP on human coronary artery SMCs was determined using a MTS assay (Cell Counting Kit-8, Dojindo Inc., #343-07626). The cells were grown in 96-well microtiter plates for 24 hours, and then they were treated with different concentrations of FITC-encapsulated PEG-PLGA nanoparticles NP suspension medium with 10 % FBS. The plates were incubated for 48 hours and then the medium was replaced with 200 µl of fresh medium. Next, 2 mg/ml MTS solution was added and the plates were incubated again for 4 hours at 37°C/5%CO₂. Finally, absorbance was measured at 490 nM using an enzyme-linked immunosorbent assay microplate reader. Cell viability
was expressed as the ratio between the amount of formazan determined for cells treated with PEG-PLGA nanoparticles NP and for control non-treated cells.

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (in situ apoptosis detection kit, Takara).

**Statistical Analysis**

Data are expressed as the mean ± 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.
References


**Figure I.** Effects of an endocytosis inhibitor chlorpromazine hydrochloride (CPZ) on NP-mediated intracellular incorporation of fluorescence

Fluorescence microscopic pictures of rat aortic SMCs incubated with coumarin-6--encapsulated PEG-PLGA NP (0.05 mg/ml) for 30 minutes in the presence and absence of chlorpromazine hydrochloride (CPZ) at 50 μM. The Nuclei were counterstained with propidium iodide (PI). Scale bar = 50 μm
Figure II. Efficacy of NP-mediated drug delivery system to vein grafts

A, Fluorescence micrographs of cross-sections of excised rabbit jugular vein and human internal thoracic vein after *ex vivo* incubation with PBS or FITC-NP for 30 minutes. FITC fluorescence is green. Nuclei are stained red. M: media, A: adventitia. Scale bar = 100 µm

B, Micrographs of cross-sections of excised rabbit jugular vein stained with FITC antibody immediately after *ex vivo* incubation with FITC-NP, and at 7 and 28 days after implantation. The nuclei are counterstained with hematoxylin. M: media, IM: intima + media complex, A: adventitia. White line shows external elastic lamina. Scale bar = 100 µm
**Figure III.** Effects of NP-based STI571 delivery on inflammatory and proliferative changes, and endothelial regeneration

**A.** Pictures of cross-sections of vein grafts 7 days after implantation, stained to detect monocytes/macrophages (RAM-11), proliferating cells (PCNA), and endothelial cells (CD31). IM: intima + media complex; A: adventitia. White line shows external elastic lamina. Bar = 50 µm

**B.** Summary data on inflammation

**C.** Summary data on the appearance of PCNA-positive cells

**D.** Summary data on endothelial regeneration. Endothelial cell coverage was quantified by the following formula: 100 × (the length of CD31-positive layer / the length of luminal surface in cross-sections). **P < 0.01** versus PBS-treated group, NS; Not significant.
**Endothelial regeneration**

[Bar chart showing percentage of endothelial regeneration for different groups: PBS, FITC, ST1571 NP only, and ST1571 NP. The chart shows a decrease in percentage from Day 7 to Day 28 with ST1571 NP having a significant effect.]
Figure IV. Effects of STI571-NP on PDGF expression

A. Photomicrographs of cross-sections of vein immunohistochemically stained for PDGF from normal vein and vein graft tissues 7 days after grafting. Lower photomicrographs of cross sections is magnified in box of upper image. Scale bar in low- and high-powered field = 100 and 50 µm

B. Percentage of PDGF-positive cells in PBS, FITC-NP, STI571 only, and STI571-NP groups (n = 5 each). There was no treatment effect. Data are mean ± SEM. NS; Not significant.