Imatinib Mesilate Inhibits Neointimal Hyperplasia via Growth Inhibition of Vascular Smooth Muscle Cells in a Rat Model of Balloon Injury

YASHIRO MAKIYAMA,1 KEN TOBA,2 KIMINORI KATO,1 SATORU HIRONO,1 TAKUYA OZAWA,1 TAKASHI SAIGAWA,1 SHIRO MINAGAWA,1 MANABU ISODA,3 FUYUKI ASAMI,3 NOBORU IKARASHI,1 MASATO ODA,1 MASATO MORIYAMA,2 MASUTAKA HIGASHIMURA,2 TOSHIKI KITAJIMA,2 KEITA OTAKI1 and YOSHIFUSA AIZAWA1

1Division of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
2Division of Hematology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
3Division of Cardiovascular Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Restenosis is a major problem in percutaneous catheter intervention (PCI) for coronary artery stenosis in patients with acute myocardial infarction. Coronary restenosis arises from intimal hyperplasia, i.e., hyperplasia of the vascular smooth muscle cells (SMCs) caused by endothelial cell (EC) damage due to PCI. Drug eluting stent (DES), a novel stent coated with a cell-growth inhibitor, such as rapamycin, has been utilized to block SMC proliferation, but DES also blocks EC repair and thus requires the administration of anti-platelets for a long time to prevent thrombus formation after PCI. Moreover, insufficient prevention of platelet aggregation sometimes induces restenosis after PCI. One of the signal transduction inhibitors, imatinib mesilate, blocks tyrosine kinase activity of platelet-derived growth factor receptor (PDGFR), and therefore it may block the development of neointima through growth inhibition of SMCs without the obstructive effect on EC-repair. We therefore studied the effects of imatinib on neointimal hyperplasia in a balloon injury model of rat carotid arteries. Rats were orally administered with imatinib for 14 days after balloon injury, and sacrificed to analyze the neointimal formation. Intimal hyperplasia was inhibited by imatinib in a dose-dependent manner. Therefore imatinib presumably obstructed the growth of SMCs via interception on growth-signaling of PDGFR. The administration of imatinib after coronary stenting or the use of an imatinib-eluting stent may further reduce the risk of restenosis in patients. ——— coronary intervention; intimal hyperplasia; restenosis; imatinib mesilate; coxsackievirus and adenovirus receptor.

© 2008 Tohoku University Medical Press
Therapeutics of acute myocardial infarction (AMI) has become increasingly more sophisticated in the last two decades. Applications of percutaneous catheter intervention (PCI) including plain old balloon angioplasty (POBA) and stenting have improved the prognosis. However, restenosis arising from intimal hyperplasia is a major problem in PCI. A drug-eluting stent (DES) has partially solved this problem, however it requires long-term administration of anti-platelet drugs because it blocks the growth of smooth muscle cells (SMCs) as well as vascular endothelial cells (ECs) that results in a delay or absence of EC-repair. Imatinib mesilate blocks the growth of SMCs, but not ECs, in vitro via tyrosine kinase inhibition of PDGFR (Rocha et al. 2007). We therefore studied the effects of imatinib in vivo.

Vascular endothelial cells (ECs) and smooth muscle cells (SMCs) constitutionally express the coxsackievirus and adenovirus receptor (CAR), and this results in the accumulation of adenovirus vector in injured vessels (Rekhter et al. 1998). CAR is one of the adhesion molecules of the Ig-superfamily, and its extracellular domain is homologous to the vascular cell adhesion molecule (VCAM) and the intercellular adhesion molecule (ICAM) (Bergelson et al. 1997). CAR is a homophilic receptor/ligand (Honda et al. 2000), and may function as a sensor of cell-cell interaction to control the healing process of vascular injury (Nasuno et al. 2004).

Smooth muscle cells grow in the presence of cytokines as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and so forth (Reidy 1992). Platelets adhere to injured vessels and secrete PDGF, and therefore it is considered to work as a key cytokine to initiate intimal hyperplasia after coronary intervention. One of the signal transduction inhibitors, imatinib mesilate (STI571), blocks the tyrosine kinase activities of \( abl \) and \( bcr/abl \), and has been utilized to treat patients with chronic myelogenous leukemia (CML) (Druker et al. 1996). It also blocks the tyrosine kinase activity of \( c-kit \), and is effective in treating gastrointestinal stromal tumors (GIST) (Tuveson et al. 2001). The third target molecule of imatinib mesilate is the PDGFR receptor (PDGFR) (Carroll et al. 1997), and so this drug may block the growth of smooth muscle cells activated by PDGF (Mylläriemi et al. 1999). We studied the relationship of cell confluence and cell growth in cultured SMCs in vitro. We also studied the effect of imatinib mesilate on neointimal hyperplasia induced by balloon injury in rat carotid arteries to simulate the treatment of coronary restenosis.

**METHODS**

**Culture of vascular SMCs**

Rat aortic vascular SMCs (second passage derived from the tunica intima and tunica media of healthy, fibrous plaque-free rat aorta) were purchased from Cell Applications, San Diego, CA. The purchased cell suspension was directly utilized for analysis without expansion pre-culture. The cells were washed and suspended in a smooth muscle cell proliferation medium (SMC P-STIM, Becton Dickinson, San Jose, CA: MCDB131 medium supplemented with 5% fetal bovine serum, 5 ng/ml of human recombinant insulin, 10 ng/ml of human recombinant EGF, 2 ng/ml of human recombinant bFGF, 100 U/mL penicillin G sodium, and 100 mg/mL of streptomycin sulfate). Cells (1 × 10^4) in 0.7 ml medium were seeded in a collagen type-1 coated culture slide (Biocoat, Becton Dickinson: 8 wells/slide, 0.69 cm^2/well), and incubated at 37°C in 5% CO₂ in humidified air for 4 days to obtain confluently grown SMCs. The confluent cell-sheet in culture was scratched with a needle to reproduce loss of confluence in the cells, and harvested for staining 12 hours after scratching. Culture slides were harvested, washed with PBS, fixed with ice-cold Zamboni solution for 30 min, and stained for CAR and proliferating cell nuclear antigen (PCNA). CAR was stained by serial incubation with rabbit anti-CAR (Nasuno et al. 2004), biotinylated goat anti-rabbit Ig (Nichirei, Tokyo, Japan) and FITC-conjugated streptavidin (Vector, Burlingame, CA). Cell nuclei were stained with DAPI.

**Balloon injury model**

Adult male Sprague-Dawley rats (430-500 g) were obtained from Charles River Japan Inc. (Yokohama, Japan). All procedures were performed under sterile conditions with the approval of the Institutional Animal Therapeutics of acute myocardial infarction (AMI) has become increasingly more sophisticated in the last two decades. Applications of percutaneous catheter intervention (PCI) including plain old balloon angioplasty (POBA) and stenting have improved the prognosis. However, restenosis arising from intimal hyperplasia is a major problem in PCI. A drug-eluting stent (DES) has partially solved this problem, however it requires long-term administration of anti-platelet drugs because it blocks the growth of smooth muscle cells (SMCs) as well as vascular endothelial cells (ECs) that results in a delay or absence of EC-repair. Imatinib mesilate blocks the growth of SMCs, but not ECs, in vitro via tyrosine kinase inhibition of PDGFR (Rocha et al. 2007). We therefore studied the effects of imatinib in vivo.

Vascular endothelial cells (ECs) and smooth muscle cells (SMCs) constitutionally express the coxsackievirus and adenovirus receptor (CAR), and this results in the accumulation of adenovirus vector in injured vessels (Rekhter et al. 1998). CAR is one of the adhesion molecules of the Ig-superfamily, and its extracellular domain is homologous to the vascular cell adhesion molecule (VCAM) and the intercellular adhesion molecule (ICAM) (Bergelson et al. 1997). CAR is a homophilic receptor/ligand (Honda et al. 2000), and may function as a sensor of cell-cell interaction to control the healing process of vascular injury (Nasuno et al. 2004).

Smooth muscle cells grow in the presence of cytokines as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and so forth (Reidy 1992). Platelets adhere to injured vessels and secrete PDGF, and therefore it is considered to work as a key cytokine to initiate intimal hyperplasia after coronary intervention. One of the signal transduction inhibitors, imatinib mesilate (STI571), blocks the tyrosine kinase activities of \( abl \) and \( bcr/abl \), and has been utilized to treat patients with chronic myelogenous leukemia (CML) (Druker et al. 1996). It also blocks the tyrosine kinase activity of \( c-kit \), and is effective in treating gastrointestinal stromal tumors (GIST) (Tuveson et al. 2001). The third target molecule of imatinib mesilate is the PDGFR receptor (PDGFR) (Carroll et al. 1997), and so this drug may block the growth of smooth muscle cells activated by PDGF (Mylläriemi et al. 1999). We studied the relationship of cell confluence and cell growth in cultured SMCs in vitro. We also studied the effect of imatinib mesilate on neointimal hyperplasia induced by balloon injury in rat carotid arteries to simulate the treatment of coronary restenosis.

**METHODS**

**Culture of vascular SMCs**

Rat aortic vascular SMCs (second passage derived from the tunica intima and tunica media of healthy, fibrous plaque-free rat aorta) were purchased from Cell Applications, San Diego, CA. The purchased cell suspension was directly utilized for analysis without expansion pre-culture. The cells were washed and suspended in a smooth muscle cell proliferation medium (SMC P-STIM, Becton Dickinson, San Jose, CA: MCDB131 medium supplemented with 5% fetal bovine serum, 5 ng/ml of human recombinant insulin, 10 ng/ml of human recombinant EGF, 2 ng/ml of human recombinant bFGF, 100 U/mL penicillin G sodium, and 100 mg/mL of streptomycin sulfate). Cells (1 × 10^4) in 0.7 ml medium were seeded in a collagen type-1 coated culture slide (Biocoat, Becton Dickinson: 8 wells/slide, 0.69 cm^2/well), and incubated at 37°C in 5% CO₂ in humidified air for 4 days to obtain confluently grown SMCs. The confluent cell-sheet in culture was scratched with a needle to reproduce loss of confluence in the cells, and harvested for staining 12 hours after scratching. Culture slides were harvested, washed with PBS, fixed with ice-cold Zamboni solution for 30 min, and stained for CAR and proliferating cell nuclear antigen (PCNA). CAR was stained by serial incubation with rabbit anti-CAR (Nasuno et al. 2004), biotinylated goat anti-rabbit Ig (Nichirei, Tokyo, Japan) and FITC-conjugated streptavidin (Vector, Burlingame, CA). Cell nuclei were stained with DAPI.

**Balloon injury model**

Adult male Sprague-Dawley rats (430-500 g) were obtained from Charles River Japan Inc. (Yokohama, Japan). All procedures were performed under sterile conditions with the approval of the Institutional Animal
Care and Use Committee in compliance with procedures and methods outlined by the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23; National Institutes of Health, Bethesda, MD). The Animal Study Committee in our institution also approved the experiment projects.

Rats were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). Carotid arteries were denuded of the endothelium by the introduction of a 2F Fogarty balloon embolectomy catheter (Baxter Healthcare, Santa Ana, CA) into the left common carotid artery through the external carotid. The balloon was inflated to distend the common carotid artery and then withdrawn to the external carotid artery. This procedure was repeated three times. After removal of the catheter, the external carotid artery was ligated and the wound was closed.

The rats were daily administered with 0, 6, 12.5, 25, or 50 mg/kg of STI571 (kindly provided by Novartis Pharma) dissolved in water and dispensed once a day for 14 days, and were sacrificed by administration of a lethal dose of pentobarbital sodium on day 14 of treatment, and the injured left carotid arteries were isolated and excised. Untreated carotid arteries in the control rats were also studied for comparison.

**Tissue preparation and staining**

After excision and washing of the carotid, the samples were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), rapidly frozen in liquid nitrogen and stored at −80°C. A series of specimens was stained with hematoxylin-eosin for digital imaging, and others were immunohistochemically stained as follows. Serial frozen sections (10 μm thick) were applied to APS-coated slides (Matsunami Glass, Osaka, Japan), fixed in cold Zamboni liquid for 30 min, and then stained with rabbit polyclonal antibodies against factor VIII-related antigen (FVIIIIRAg; Zymed, San Francisco, CA), and a mouse monoclonal antibody against alpha smooth muscle actin (αSMA, Sigma, St. Louis, MO). The rabbit antibody was detected with biotinylated goat anti-rabbit Ig (Nichirei, Tokyo, Japan) and FITC-conjugated streptavidin (Vector, Burlingame, CA), and the mouse monoclonal antibody was detected with a TRITC-conjugated goat anti-mouse secondary antibody (Vector). Sections were also stained with control rabbit IgG to estimate background fluorescence.

**Image processing**

The histology by HE-staining was digitized using a microscope (magnification ×100, Olympus BX60, Olympus, Tokyo, Japan) and video system (Olympus TV System DP50, Olympus). Digital RGB pictures of the tissues samples were acquired, and analyzed using MacSCOPE software (Mitani, Fukui, Japan) as described previously (Ozawa et al. 2006). The I/M ratio (the area ratio of intima and media) and percent stenosis (the area of intima divided by the whole area inside the internal elastic membrane) were calculated as the representative of intimal hyperplasia and arterial stenosis, respectively. Briefly, the borders of media, intima, and endothelium were digitally traced upon a new layer of the RGB pictures, the trace layers were then extracted, and the areas measured by pixel² were counted using the software.

Fluorescence was observed with a microscope (Olympus IX71), confocal laser scan unit (Olympus FV500), using Fluoview software (Olympus). Fluorescent probes were excited with a multi-argon laser (488 nm) and Green He-Ne laser (543 nm). The excited probes were detected with barrier filter sets BA505/525 and BA5601F, and assigned the color of green (FITC), red (TRITC) and blue (DAPI). Two-color images were obtained by overlaying images acquired from individual channels for each sample.

**Statistics**

The numerical data was expressed by mean and standard deviations (SD). The values were compared among groups by one-way ANOVA followed by the Bonferroni multiple comparison test, and the difference was considered to be significant when the p-value was less than 0.05.

**RESULTS**

**Cultured SMC**

The expressions of CAR and PCNA in the cultured SMCs are shown in Fig. 1. CAR is a homophilic cell adhesion molecule, i.e., the ligand for CAR is CAR itself expressed in another cell (Honda et al. 2000), and presumably functions as a sensor of cell-cell interaction in the cardiovascular system (Ito et al. 2000). PCNA is expressed in growing cells. CAR together with PCNA was abundantly expressed in the neointima of the rat carotid artery, and declined to a basal level after the regeneration of EC (Nasuno et al. 2004). In the present study, the growing SMCs in the cul-
ture highly expressed CAR from days 1 to 3. The expression of CAR decreased in the confluent grown cells, and when a part of the confluent cell-sheet was scratched to reproduce a loss of cell-cell contact, PCNA expression (red, panel E) and CAR increase (panels E and F) reappeared on the scratched edge of the cell sheet. Thus CAR may monitor cell continuity to regulate cell growth in SMCs.

Balloon injury model

Some of the rats died due to the operation within a day. Others survived, and administration of imatinib mesilate didn’t produce adverse effects such as weight loss, decline in activity, etc. Seven untreated rats and 32 operated rats (5, 4, 7, 8, and 8 rats in the groups of 0, 6, 12.5, 25, and 50 mg/kg/day oral administration of imatinib mesilate) were observed to have an effect of imatinib mesilate on the formation of intimal hyperplasia. Intimal hyperplasia and arterial stenosis following balloon injury were inhibited by the oral administration of imatinib mesilate given for 14 days (Figs. 2 and 3). The I/M ratio was almost normalized at 12.5 mg/kg of imatinib mesilate, and arterial stenosis was blocked at 25 mg/kg (Fig. 4). The dosage was within the therapeutic range used...
Imatinib Inhibits Neointimal Hyperplasia

To treat chronic myelogenous leukemia (400 to 800 mg/body), and it would be expected to prevent restenosis after coronary intervention in humans. Moreover, contrasting with the treatment of CML, short-term administration of the drug: for several weeks, would seem to be effective in inhibiting SMC proliferation until the endothelium recovers at the injured site of the artery.

**DISCUSSION**

In a previous study of rat carotid balloon injury experiments (Nasuno et al. 2004), we observed neointimal hyperplasia and a strong expression of proliferating cell nuclear antigen (PCNA) in the newly observed SMCs in the neointima on day 7. The endothelium regenerated and SMC proliferation terminated by day 14. Therefore, 14 days administration of imatinib mesilate was expected to be sufficient to prevent neointimal hyperplasia in this model, and 4 weeks administration might be enough for clinical application in percutaneous catheter intervention (PCI).

Vascular SMCs are presumed to migrate from the media to the intima through the internal elastic membrane in the presence of PDGF after balloon injury, and the cells proliferate in the presence of bFGF secreted by SMCs themselves until the endothelium recovers (Reidy 1992). The proliferation of SMCs as well as ECs is affected by bFGF, so that the inhibition of the FGF-receptor may retard the recovery of the endothelium. In contrast, inhibition of PDGFR blocks the initiation as well as the progression of intimal hyperplasia in the absence of EC repair-blockage.

Some clinical trials of anti-hyperplasic drugs including ACE-blocker, ARB, CD41 (GPIIb/IIIa)-inhibitor, statins, and Tranilast, were disappointing. These general, but not SMC specific, drugs do not have sufficient power to intervene with the growth signal via the PDGF/PDGFR system.

**Fig. 2.** *In vivo* effects of imatinib mesilate on carotid intimal hyperplasia developed after balloon injury. Rat carotid arteries were stained with H.E. 14 days after balloon injury. The rats were orally administered with water (no imatinib mesilate: panel A) or 50 mg/kg of imatinib mesilate (panel B) for 14 days. The development of intimal hyperplasia was blocked by the imatinib mesilate administration. The intact carotid is also shown for comparison (panel C). Magnification: × 20.
Fig. 3. Immunohistochemistry of injured carotid arteries. Rat carotid arteries were stained with anti-CAR (coxsackievirus and adenovirus receptor) (panel A: green, 7 days after balloon injury) or anti-VIIIIRAg (factor VIII-related antigen) (panels B, C, D: green, endothelium and thrombus, 14 days after injury). Smooth muscle cells were also stained with anti-αSMA (alpha smooth muscle actin, red). The proliferating SMCs highly expressed CAR 7 days after injury. The development of intimal hyperplasia and thrombus (panel B, administered with water) were inhibited by the administration of imatinib mesilate (panel C, 50 mg/kg for 14 days). An enlarged photo of panel C is shown in panel D (nuclei: DAPI, blue). A monolayer of repaired endothelium is seen (VIIIIRAg, green). Magnification: panel A, × 400, panels B, C, × 100, and panel D, × 250).
PCI for coronary artery stenosis is progressing, and the employment of drug a eluting stent (DES) coated with polymer containing rapamycin or paclitaxel has decreased restenosis after PCI (Marx et al. 2001; Moses et al. 2003). However, the eluting drugs inhibit the growth of SMCs as well as ECs. The stent would then remain uncovered by EC, and the exposure of the naked stent to blood requires the administration of anti-platelets to prevent thrombus formation as a result (Kereiakes et al. 2004; McFadden et al. 2004), which may then lead to other problems such as bleeding or a polymer allergy (Virmani et al. 2004).

In the present study, oral administration of imatinib mesilate prevented intimal hyperplasia possibly as a result of inhibition on SMC migration and growth, but the inhibition of endothelium recovery was not evident. Therefore, further clinical studies are necessary to estimate the preventive effect of the drug on intimal hyperplasia after PCI. Administration of imatinib mesilate after PCI may be preferred to DES especially when patients have complications with hematological problems or allergies. Coronary stenosis in small arteries less than 2.5 mm, winding arteries (Schunkert et al. 1999), or arterial stenosis in limbs (Costanza et al. 2004) would be treated with PCI followed by imatinib mesilate administration. In fact, the placement of a stent is not suitable for an elbow-shaped bending artery in the inguinal or popliteal region, and interventional therapy has not been established for class II lesions in the femoral-popliteal or infrapopliteal regions because of the high rate of restenosis (Hirsch et al. 2006). Administration of imatinib mesilate may be an alternative in such cases.

**CONCLUSION**

Imatinib mesilate inhibited the proliferation of vascular SMCs in the injured artery presumably via blocking of PDGF-signaling. Oral administration of imatinib mesilate or the use of a drug eluting stent coated with imatinib-containing polymer may prevent restenosis after PCI without impaired EC-restoration.

**Acknowledgment**

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (H17-18, No. 17590714, and H18-19, No. 18590806). This work was also supported in part by a Japanese Ministry of Health, Labor, and Welfare Grant “Research on Regulatory Science of Pharmaceutical and Medical Devices (H18-020).”

**References**


Carroll, M., Ohno-Jones, S., Tamura, S., Buchdunger, E., Zimmermann, J., Lydon, N.B., Gilliland, D.G. & Druker, B.J. (1997) CGP 57148, a tyrosine kinase inhibitor, inhib-
its the growth of cells expressing BCR-ABL, TEL-ABL, and TEL-PDGFR fusion proteins. Blood, 90, 4947-4952.


