Original Article

Locally Applied Cilostazol Suppresses Neointimal Hyperplasia and Medial Thickening in a Vein Graft Model

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Background: Pathological changes in vein grafts begin immediately after arterial circulation is applied to the grafts. Chemical mediator stimulation and mechanical strain induce neointimal hyperplasia and medial thickening of the vein grafts, resulting in their failure. We investigated the inhibitory effect of locally applied cilostazol, an inhibitor of cyclic adenosine monophosphate phosphodiesterase III, on neointimal hyperplasia and medial thickening of the grafts.
Methods and Results: We established a distal anastomotic stricture model of femoral vein-abdominal aorta interposition grafting in rats. In this model, neointimal hyperplasia was observed not only at the distal anastomotic sites, but also in the graft body at postoperative day 14 and was markedly progressed at day 28. A strong expression of tenascin-C was found in the media and neointima of the graft body. In the grafts around which cilostazol was administered locally using Pluronic gel, neointimal hyperplasia was significantly suppressed compared with control grafts treated with the gel alone, with the mean neointimal cross-sectional area reduced by 87.1% for the graft body and by 78.9% for the distal anastomotic sites and mean medial cross-sectional area of the graft body reduced by 54.2% at day 28 versus the control. Cilostazol treatment decreased cell proliferation and the number of tenascin-C–producing cells seen by in situ hybridization, but the expression of tenascin-C protein was not suppressed.
Conclusion: We concluded that a single perivascular application of cilostazol inhibits neointimal hyperplasia and medial thickening of vein grafts in a rat model. (Ann Thorac Cardiovasc Surg 2007; 13: 322–330)

Key words: cilostazol, neointima, smooth muscle cell, vein graft

Introduction

Autologous vein grafts are widely used as a bypass conduit for coronary artery bypass grafting (CABG).[1)] It has been reported that rapid medial and intimal thickening resulting from smooth muscle cell (SMC) migration and proliferation and the synthesis of extracellular matrix (ECM) occur in vein grafts within 4 weeks after grafting.[2)] Furthermore, anastomotic problems such as surgical trauma, stricture, and the caliber difference between the coronary artery and the grafted vein accelerate neointimal hyperplasia.

Recently, several experimental studies have demonstrated that locally applied drugs can apparently reduce neointimal hyperplasia in balloon-injured arteries[3–5)] or grafted veins.[6,7)] In fact, local drug application is an attractive and feasible therapy in bypass surgery with few side effects. We previously demonstrated that a local administration of cilostazol, a specific inhibitor of cyclic

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adenosine monophosphate (cAMP) phosphodiesterase III, suppressed neointimal hyperplasia in a free artery graft model.[8] The pharmacological effects of cilostazol are considered to inhibit platelet aggregation and SMC proliferation and to induce vasodilatation.[9–11] Furthermore, cilostazol suppresses the tenascin-C (TN-C) mRNA expression induced by platelet-derived growth factor (PDGF)-BB.[8] TN-C, an ECM glycoprotein, blocks the adhesion of SMCs to fibronectin[12] and mediates SMC migration.[13] Several studies have suggested that TN-C may play a crucial role in the remodeling of cardiovascular tissue by affecting cell activity.[14–17] Recently, using TN-C–deficient mice we demonstrated that TN-C is essential for the progression of neointimal hyperplasia after aortotomy.[18]

In the present study we investigated whether a single local administration of cilostazol is capable of suppressing neointimal hyperplasia and medial thickening in implanted vein grafts, and we also evaluated its effects on SMC proliferation and TN-C expression in grafted veins.

Materials and Methods

Rat anastomotic stricture model of vein graft stenosis

Male inbred Wister-Kyoto rats (mean body weight: 320±59 g) were used. All studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Mie University Animal Experiment and Care Committee. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and atropine (0.1 mg). Using a sterile technique, we harvested a 1.0-cm segment of right femoral vein, and the aorta was then exposed below the renal artery through an abdominal incision. After an injection of 100 U/kg of heparin into the right lumbar vein, the abdominal aorta was clamped between the renal arteries and the bifurcation of the aorta, then dissected. The vein segment was transplanted into the abdominal aorta in an end-to-end fashion. Anastomosis was performed by interrupted 10-0 nylon sutures. Distal anastomotic stricture was experimentally produced by ligating the distal anastomosis with a 22-gauge catheter (outer diameter: 0.7 mm) using 1-0 silk thread and then removing it (Fig. 1). In the cilostazol-treated group, rats with the anastomotic stricture received a topical application of 20 mg of cilostazol (Otsuka Pharmaceutical Co., Tokushima, Japan) dissolved in 200 μL of dimethyl sulfoxide containing 25% Pluronic gel around the interposed graft, as previously described.[8] Rats in the vehicle-treated group received the dimethyl sulfoxide-Pluronic gel without cilostazol.

Graft harvesting

Vein grafts were harvested under anesthesia at 7, 14, and 28 days after surgery (5 to 7 rats per group each time). The rats were perfused with 100 mL of heparin-prepared saline followed by 100 mL of 10% neutral-buffered formalin. The grafts were carefully exposed and extracted, including the proximal and distal normal aorta, postfixed overnight at 4°C in the same fixative, and then embedded in paraffin. For histological analysis of the graft body, the following three portions were selected: the center of the graft and the midportions between each anastomotic site and the center of the graft. Five cross sections (4 μm thick) were cut from each portion. Four cross sections were selected from the distal anastomotic sites and stained with elastica van Gieson to demarcate the internal elastic lamina. At each section, the cross-sectional areas of the neointima and the media were calculated using an image analysis system (Adobe Photoshop version 5.0J and NIH Image version 1.61, Macintosh) as previously described.[3]

Immunohistochemical analysis

To identify the cellular source of the neointima, immunohistochemical staining for α-smooth muscle actin (SMA) was performed using peroxidase-conjugated anti–α-SMA antibody (DakoCytomation, Kyoto, Japan). The sections were treated with diaminobenzidine/H_2O_2 solution and lightly counterstained with hematoxylin. Furthermore, to assess the differentiation and proliferation of SMCs, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed using mouse monoclonal antibody against PCNA (PC10, DakoCytomation). The sections were then incubated with peroxidase-conjugated antimouse immunoglobulin G (1:200, MBL, Nagoya, Japan). The PCNA labeling index was determined by dividing the number of PCNA-positive nuclei by the total number of nuclei in the media from each section.

To identify monocytes and macrophages, immunohistochemical staining for CD68 was performed using mouse monoclonal antirat CD68 antibody (10 μg/mL, Serotec, Oxford, UK), followed by the immunoperoxidase procedure.

Immunohistochemical staining for TN-C was performed according to the method previously reported.[19]

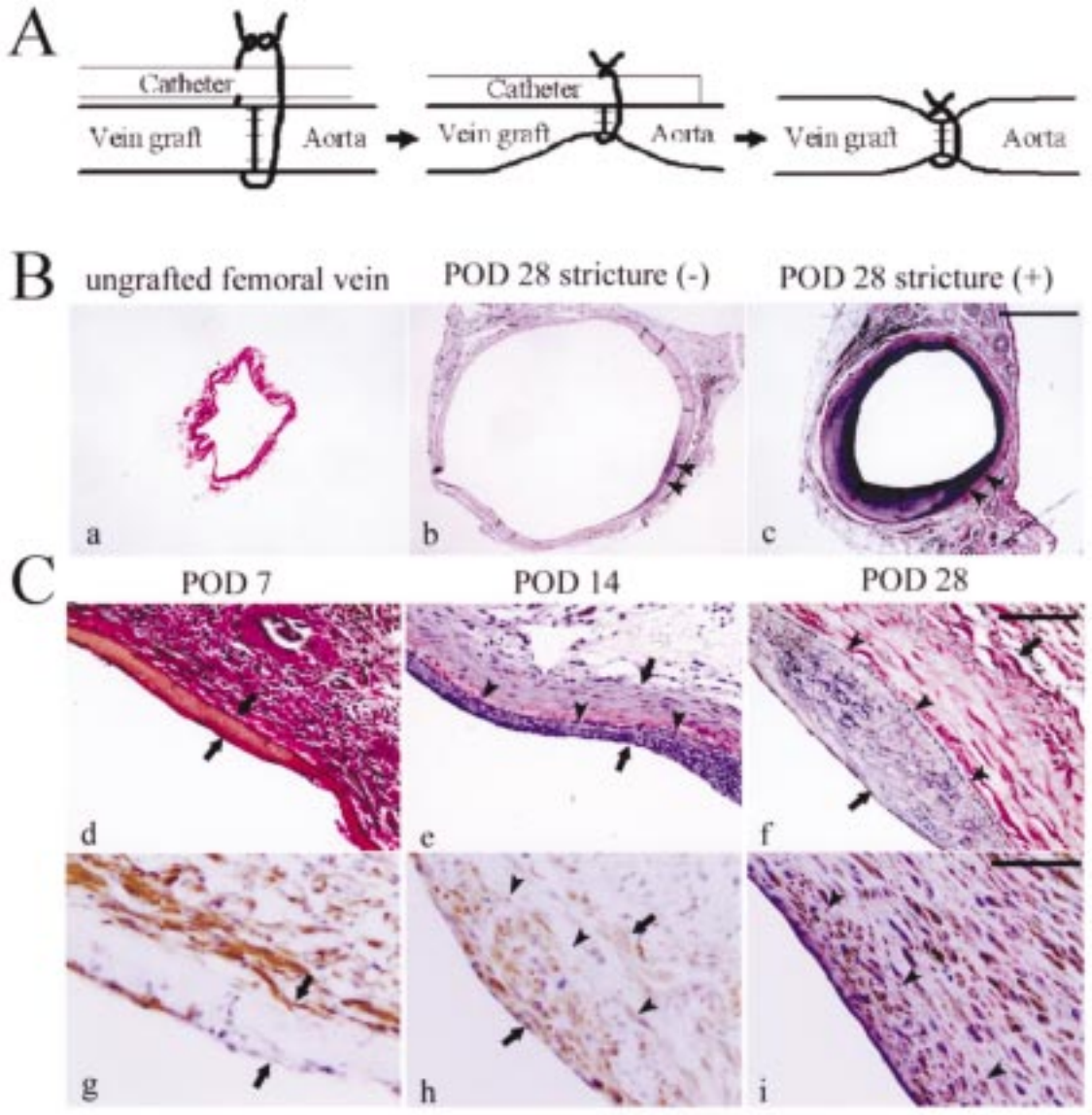

Fig. 1. Neointimal hyperplasia and medial thickening of vein grafts.
A: Diagram of the distal anastomotic stricture model. **B:** Effect of distal anastomotic stricture on neointimal hyperplasia of vein grafts. **a–c:** Elastica van Gieson-stained cross sections at postoperative day (POD) 28. **a:** Ungrafted femoral vein. **b:** Grafted vein without anastomotic stricture. **c:** Grafted vein with distal anastomotic stricture. Marked neointimal hyperplasia was seen in grafted veins with distal anastomotic stricture. Arrowheads indicate internal elastic lamina. Scale bar in **c,** 500 μm. **C:** Neointimal hyperplasia and medial thickening of vein grafts with distal anastomotic stricture. **d–f:** Elastica van Gieson-stained cross sections. **g–i:** Immunostaining for α-SMA. **d, g:** POD 7; **e, h:** POD 14; **f, i:** POD 28. Neointimal hyperplasia was seen in vein grafts at POD 14 and was markedly progressed at POD 28. Medial thickening was seen in vein grafts at PODs 14 and 28. α-SMA–positive cells were absent in the media but abundant in the periadventitia at POD 7. At PODs 14 and 28, α-SMA–positive cells were seen in the neointima and media, but not in the periadventitia. Arrowheads indicate internal elastic lamina. Arrows indicate whole graft area. Scale bar in **f,** 100 μm for **d–f**. Scale bar in **i,** 50 μm for **g–i**. POD, postoperative day.

In brief, the sections were first incubated with mouse monoclonal antirat f antibody (1 μg/mL, IBL, Takasaki, Japan) and then with peroxidase-conjugated antimouse immunoglobulin G (1:400, MBL, Nagoya, Japan). The sections were treated with diaminobenzidine/H_2O_2 solution and counterstained with hematoxylin.

In situ hybridization

TN-C mRNA expression was detected by in situ hybridization using the method previously reported.[20] Rat vein grafts were treated with proteinase K for 15 min and hybridization signals were visualized using alkaline phosphatase-conjugated antidigoxigenin antibody and incubation in nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl phosphate solution.

Measurements of tissue and plasma concentrations of cilostazol

The rats were sacrificed at 14 and 28 days (5 to 7 rats each time) after a local administration of cilostazol to measure plasma and tissue concentrations of the drug. Vein grafts were harvested and washed in cold phosphate-buffered saline to eliminate any possible drug contamination on the surface. The vein grafts and plasma were stored at –80°C until the measurements of cilostazol concentrations, which were done by high-performance liquid chromatography according to the method previously reported.[4]

Statistical analysis

Values were expressed as the mean ± standard deviation (SD). A statistical analysis between groups was performed by analysis of variance. When a statistically significant overall effect was observed, individual data were compared using the Mann-Whitney test or the Bonferroni correction. Differences between the groups were considered significant at $P<0.05$.

Results

Neointimal and medial hyperplasia in rat vein grafts

The femoral vein was interposed into the abdominal aorta with no experimental anastomotic stricture. However, a small amount of neointimal hyperplasia was observed in grafted veins at postoperative day 28 (Fig. 1). Distal anastomotic stricture of the vein graft yielded severe neointimal hyperplasia in the entire graft at postoperative day 14, followed by marked progression at day 28 (Fig. 1). Medial thickening also progressed until postoperative day 14, but there was no significant difference in the mean medial cross-sectional area of the graft body from day 14 to day 28. Immunohistochemical staining revealed many α-SMA–positive cells in the periadventitia at day 7, but none in the media. At days 14 and 28 these cells were observed in the neointima and media, but not in the periadventitia (Fig. 1). Immunostaining also revealed CD68-positive cells, either monocytes or macrophages, in the neointima, media, and periadventitia of the implanted vein grafts at postoperative day 7. These cells had almost disappeared from the vein grafts by day 28 (Fig. 2). Although three portions of the graft body (the center of the graft and the midportions between each anastomotic site and the center of the graft) were evaluated, no histological differences were found among these portions.

Cilostazol-suppressed neointimal and medial hyperplasia in rat vein grafts

Mean neointimal cross-sectional areas at day 28 were significantly smaller in the cilostazol-treated group than in the vehicle-treated group (graft body: 0.025±0.032 vs. 0.194±0.149 mm^2, $P<0.05$; distal anastomotic sites: 0.040±0.016 vs. 0.190±0.140 mm^2, $P<0.05$; Figs. 3 and 4). The rate of reduction compared with the vehicle-treated group was 87.1% for the graft body and 78.9% for the distal anastomotic sites.

Mean medial cross-sectional areas of the graft body at days 14 and 28 were also significantly smaller in the cilostazol-treated group than in the vehicle-treated group (0.241±0.061 vs. 0.448±0.175 mm^2 at day 14, $P<0.05$; 0.243±0.079 vs. 0.530±0.228 mm^2 at day 28, $P<0.05$; Fig. 4). The rate of reduction compared with the vehicle-treated group was 46.2% for day 14 and 54.2% for day 28. PCNA indices in the media at days 14 and 28 were also significantly lower in the cilostazol-treated group than in the vehicle-treated group (25.0±5.7% vs. 43.3±7.2% at day 14, $P<0.001$; 21.0±6.8% vs. 45.0±9.4% at day 28, $P<0.001$; Fig. 4). Thus treatment with cilostazol markedly suppressed neointimal hyperplasia and medial thickening at both the distal anastomotic sites and the graft body at day 28 in comparison with vehicle-treated grafts.

Expression of TN-C in rat vein grafts

In the vehicle-treated group, immunostaining of TN-C was seen in the media and periadventitia at postoperative day 7. At days 14 and 28, intense TN-C staining was detected in the neointima and media of the graft body (Fig. 5). In the cilostazol-treated group, TN-C expression similar to that observed in the vehicle-treated group was seen in the neointima and media (Fig. 5).

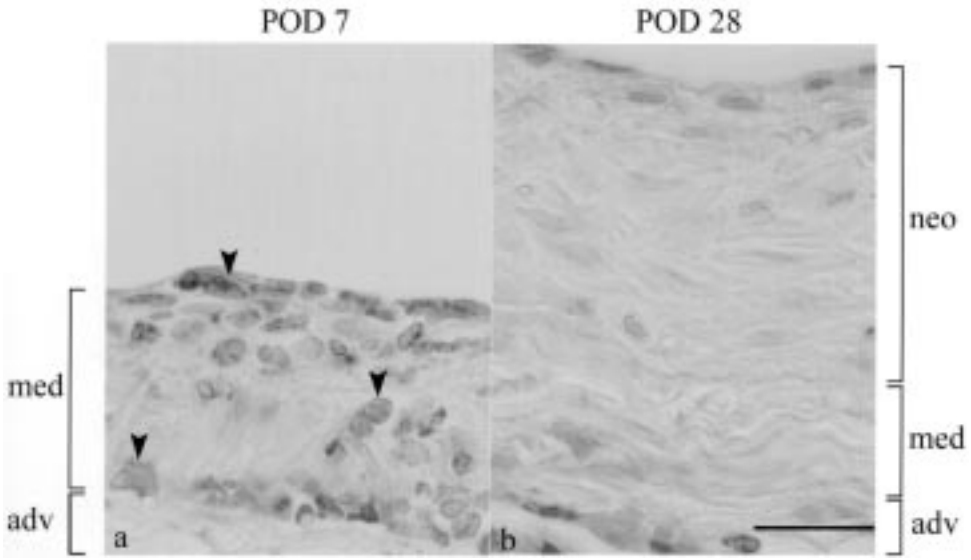

Fig. 2. Immunostaining for CD68 of vein grafts.
a: POD 7; **b:** POD 28. CD68-positive cells were seen in the neointima, media, and periadventitia at POD 7. At POD 28 there were almost no CD68-positive cells in the vein grafts. Arrowheads indicate CD68-positive cells. Scale bar in **b,** 20 μm for **a, b**. POD, postoperative day; Adv, adventitia; med, media; neo, neointima.

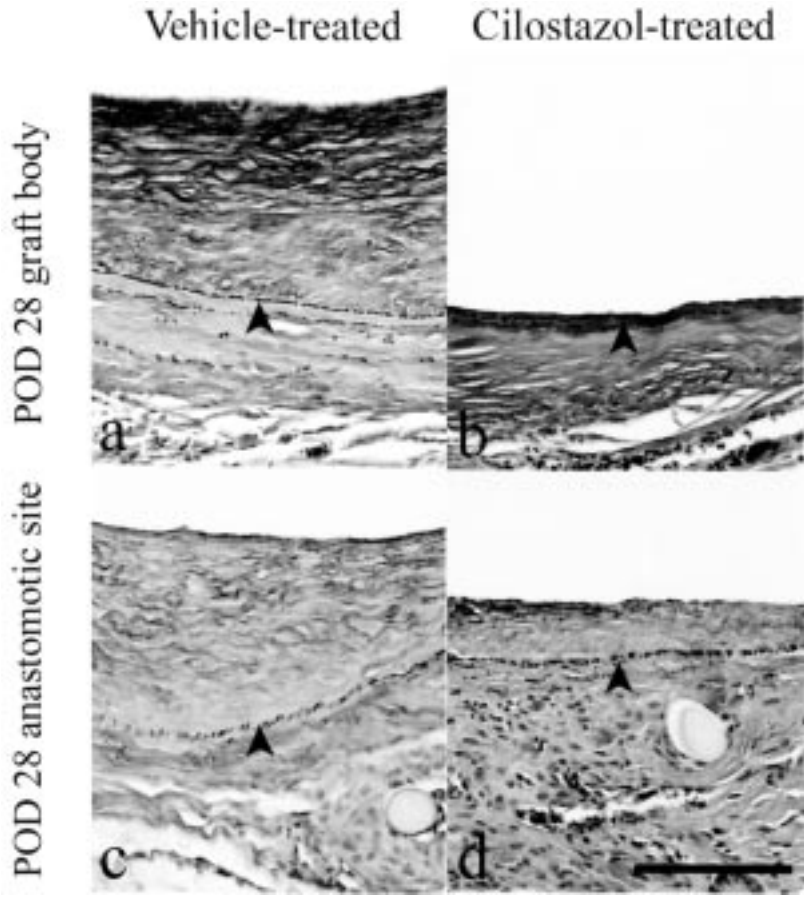

Fig. 3. Elastica van Gieson-stained cross sections of cilostazol-treated vein grafts at POD 28.
a, c: Vehicle-treated grafts. **b, d:** Cilostazol-treated grafts. **a, b:** Graft body. **c, d:** Distal anastomotic site. Neointimal hyperplasia and medial thickening were markedly inhibited in the cilostazol-treated grafts. Arrowheads indicate internal elastic lamina. Scale bar in **d,** 100 μm for **a–d**. POD, postoperative day.

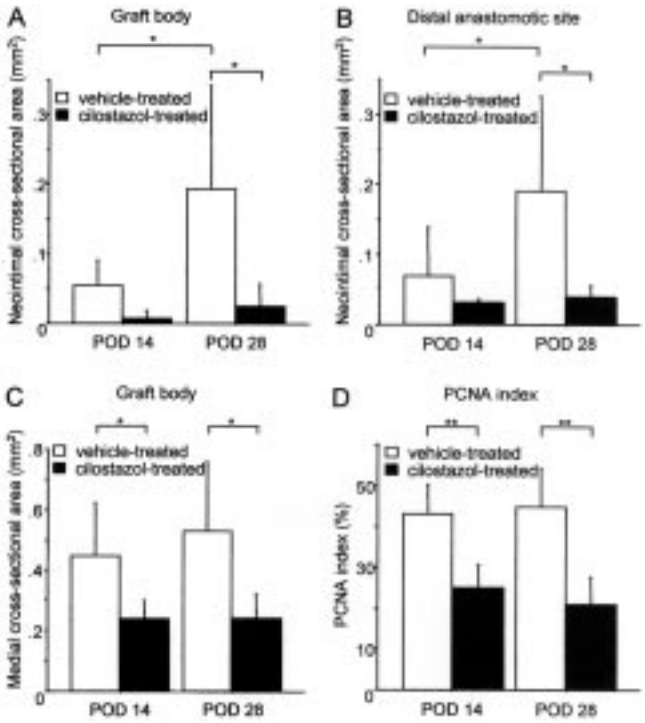

Fig. 4. Effect of cilostazol in inhibiting neointimal hyperplasia and medial thickening in vein grafts.
A: Neointimal cross-sectional area of graft body. **B:** Neointimal cross-sectional area of distal anastomotic sites. **C:** Medial cross-sectional area of graft body. **D:** PCNA index. Four sections per animal were selected, and data are the mean ± SD of the neointimal cross-sectional area, medial cross-sectional area, and PCNA index for 5 animals per group each time. **A, B:** Significant differences in neointimal cross-sectional areas between PODs 14 and 28 in vehicle-treated group (*P<0.05). Significant differences in neointimal cross-sectional areas between vehicle-treated and cilostazol-treated groups at POD 28 (*P<0.05). **C:** Significant differences in medial cross-sectional area between vehicle-treated and cilostazol-treated groups at PODs 14 and 28 (*P<0.05). **D:** Significant differences in PCNA index between vehicle-treated and cilostazol-treated groups at PODs 14 and 28 (**P<0.001). POD, postoperative day.

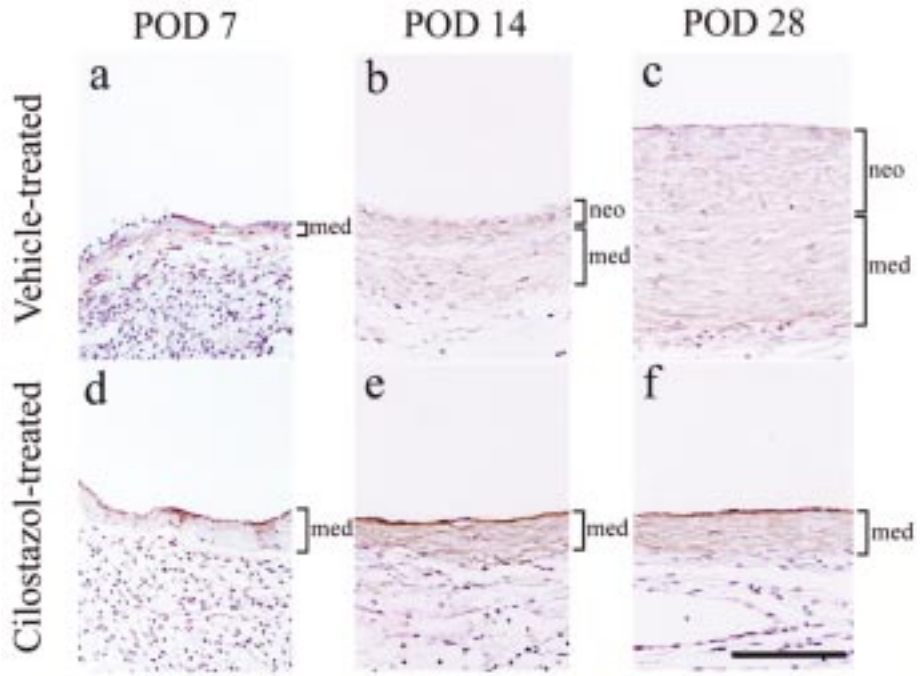

Fig. 5. Immunostaining for TN-C of vein grafts in vehicle-treated group (**a–c**) and cilostazol-treated group (**d–f**).
a, d: POD 7; **b, e:** POD 14; **c, f:** POD 28. In the vehicle-treated group, the TN-C expression was seen in the media and periadventitia at POD 7 and was localized in the progressed neointima and media at PODs 14 and 28. In the cilostazol-treated group, TN-C expression similar to that observed in the vehicle-treated group was seen in the neointima and media. Scale bar in **f:** 100 μm for **a–f**. POD: postoperative day. Med, media; neo, neointima.

In situ hybridization revealed TN-C mRNA-positive cells in the periadventitia at postoperative day 7 in the vehicle-treated group. A great many TN-C–expressing cells were observed in both the media and the neointima at day 14, with the number decreasing at day 28 (Fig. 6). In the cilostazol-treated group, very few TN-C mRNA–positive cells were detected in the periadventitia at day 7, and though they were found in the media and neointima at days 14 and 28, the amount was less than those in the vehicle-treated group (Fig. 6). These results demonstrated that a local administration of cilostazol clearly decreases the number of TN-C–expressing cells.

Tissue and plasma concentrations of cilostazol

The plasma concentration of cilostazol was below the detectable level (0.01 μg/mL) in all samples. In contrast, the mean tissue concentration of cilostazol in the vein graft was 182.05 μg/g tissue at 14 days after topical application. However, the tissue concentration decreased to 0.77 μg/g tissue at day 28.

Discussion

In the present study we established a distal anastomotic stricture vein graft model by interposing the femoral vein into the abdominal aorta in rats. Artificial stenoses at distal anastomotic sites induced a rapid progression of neointimal hyperplasia at both the graft body and the anastomotic sites. Distal strictures of vein grafts presumably produce blood turbulence and/or shear stress in grafts, which could stimulate SMC proliferation and migration by inducing certain factors. Immunohistochemical examination revealed that although α-SMA–positive cells were not seen in large numbers in the media at postoperative day 7, they reappeared in the neointima and media at days 14 and 28. Moreover, although accumulations of CD68-positive cells, either monocytes or macrophages, were predominantly observed in the media and adventitia of the implanted vein grafts at postoperative day 7, they had almost disappeared by day 28. These findings indicate that SMCs in the media of the vein grafts were once eradicated by necrosis or apoptosis at day 7, and α-SMA–positive cells were then recruited and proliferated in the newly formed vascular walls, followed by neointimal formation and medial thickening, as previously reported.[21]

Cilostazol is a specific inhibitor of cAMP phosphodiesterase III that is clinically used for the treatment of peripheral arterial occlusive disease by oral delivery.[22] Cilostazol inhibits PDGF-induced SMC proliferation.[10] It was reported that the mean intimal cross-sectional area of grafted veins in dogs was reduced to 16.7% of the control value at postoperative day 28 by a twice-daily oral administration of cilostazol at 30 mg/kg.[23] We recently demonstrated that a perivascular application of cilostazol using Pluronic gel maintained a high concentration of the drug at the site and dramatically suppressed neointimal hyperplasia in a rat-free artery graft model.[8] Pluronic gel has been shown to be an effective carrier for drugs, and it has no effect on neointimal hyperplasia.[4,6,7] In the present study we examined the effectiveness of cilostazol applied locally to implanted vein grafts in suppressing neointimal hyperplasia in our rat model. A high tissue concentration of cilostazol in the vein grafts was seen in the early stages after surgery, as was previously seen in balloon-injured rat carotid arteries[4] and rat-free artery grafts.[8] At day 14, the mean neointimal cross-sectional area showed a tendency to decrease by a topical application of cilostazol to the implanted vein graft in comparison with the vehicle-treated control, and at day 28 it was decreased to less than one-sixth in the graft body and to almost one-fifth at the distal anastomotic stricture sites. Neointimal hyperplasia in the native aorta just distal to the anastomotic stricture sites was also inhibited by cilostazol treatment (data not shown). Furthermore, the mean medial cross-sectional area of the graft body in the cilostazol-treated group was decreased to almost half at day 14 and to less than half at day 28 in comparison with the vehicle-treated control. PCNA indices in the media of the graft body in the cilostazol-treated group were also decreased to about half those in the vehicle-treated control. These results indicate that the inhibitory effects of topical application of cilostazol on neointimal hyperplasia and medial thickening in vein grafts are demonstrated within the first 28 days after surgery, with a high concentration of the drug at the application site, and that these inhibitory effects might be partly due to the cilostazol's suppression of SMC proliferation.

TN-C is implicated in the remodeling of pathological tissue by modulating cell adhesion, growth, and migration.[12,13] In vascular lesions, using TN-C–deficient mice we previously demonstrated that TN-C is a crucial molecule in the neointimal hyperplasia of arterial sutured sites.[18] We also previously demonstrated that locally applied cilostazol markedly suppressed TN-C expression and neointimal hyperplasia in rat-free artery grafts.[8] PDGF and angiotensin II upregulate the synthesis of TN-C in cultured SMCs.[12,24] The increased expression of TN-C mRNA induced by PDGF-BB in cultured SMCs is completely inhibited by the addition of cilostazol.[8] In the

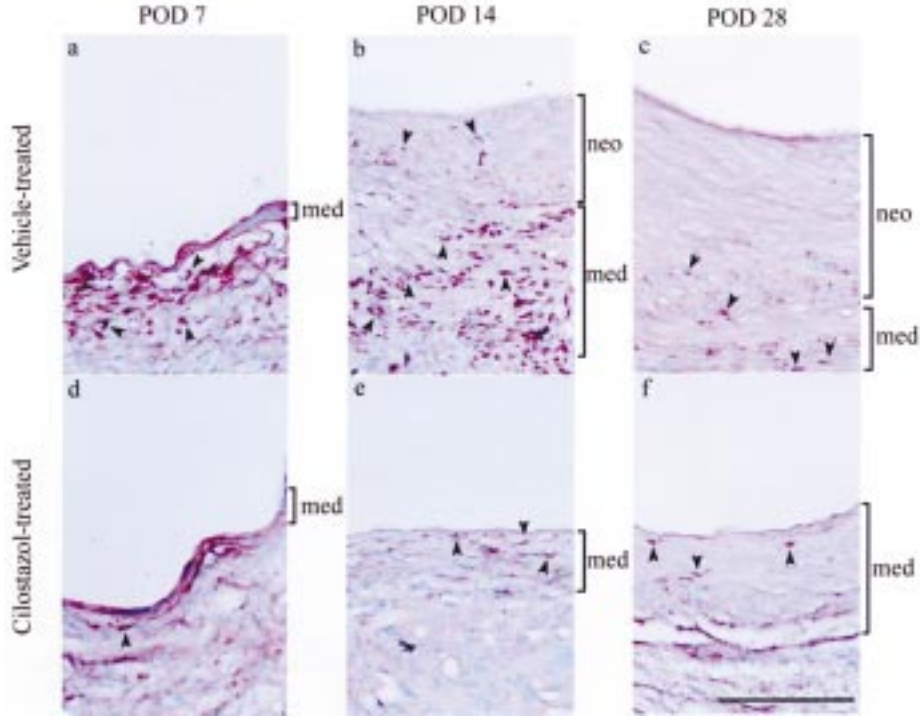

Fig. 6. Localization of TN-C by in situ hybridization in vehicle-treated group (**a–c**) and cilostazol-treated group (**d–f**).
a, d: POD 7; **b, e:** POD 14; **c, f:** POD 28. In the vehicle-treated group, TN-C mRNA–positive cells were detected in the periadventitia at POD 7 and were predominantly seen in the media and neointima at POD 14, but the numbers in the media and neointima decreased at POD 28. In the cilostazol-treated group, the numbers of TN-C mRNA-positive cells detected in each area were less than those in the vehicle-treated group. Arrowheads indicate TN-C mRNA–positive cells. Scale bar in **f,** 100 μm for **a–f**. POD, postoperative day; Med, media; neo, neointima.

present study, the number of TN-C–producing cells seen by in situ hybridization was also decreased by cilostazol treatment, though no inhibitory effect was apparent at the protein level. In tissue repair after myocardial infarction, TN-C promotes the migration and α-SMA expression of cardiac fibroblasts, resulting in an enhancement of myofibroblast recruitment to the injured tissue.[25] Cilostazol treatment might inhibit the recruitment of SMC precursors to the newly formed vascular walls, being associated with TN-C down-regulation.

An unavoidable problem with vein grafts implanted in the arterial circulation is the exposure to different vascular environments, especially mechanical stress, which suddenly stimulate the vascular wall. Mechanical stress per se directly regulates TN-C synthesis in stretched fibroblasts.[26,27] The signaling pathways believed to be involved in the response to mechanical stress are the following three routes: the mitogen-activated protein (MAP) kinase pathway, the transcriptional factor NF-κB pathway, and the pathway via protein kinase C.[26] Cilostazol has the pharmacological effect of increasing the cytoplasmic level of cAMP, thus preventing activation of the MAP kinase pathway at the upstream.[8] Thus cilostazol may inhibit only a part of the signal pathways for TN-C synthesis triggered by mechanical stress in vein grafts.

It has been reported that there are two phases in the processes contributing to neointimal hyperplasia and medial thickening, an initial phase (within 4 weeks) of rapid SMC proliferation and migration and ECM synthesis, and a late phase of slower SMC proliferation.[2] It has also been proposed that the suppression of neointimal hyperplasia and medial thickening within 4 weeks after grafting might be an efficient strategy for preventing vein graft disease.[28] Therefore in the present study we assessed changes in neointimal hyperplasia, medial thickening, and TN-C expression during the 4 weeks after grafting. In the future it will be necessary to histopathologically examine long-term changes in neointimal hyperplasia and medial thickening to properly evaluate the efficacy of topical application of cilostazol for the prevention of vein graft failure.

In conclusion, the topical application of cilostazol could inhibit neointimal hyperplasia and medial thickening in vein grafts as well as in arterial grafts, suggesting the importance of TN-C down-regulation in preventing graft restenosis.

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