Cilostazol, Not Aspirin, Prevents Stenosis of Bioresorbable Vascular Grafts in a Venous Model

Shuhei Tara, Hirotugu Kurobe, Juan de Dios Ruiz Rosado, Cameron A. Best, Toshihiro Shoji, Nathan Mahler, Tai Yi, Yong-Ung Lee, Tadahisa Sugiura, Narutoshi Hibino, Santiago Partida-Sanchez, Christopher K. Breuer, Toshiharu Shinoka

**Objective**—Despite successful translation of bioresorbable vascular grafts for the repair of congenital heart disease, stenosis remains the primary cause of graft failure. In this study, we investigated the efficacy of long-term treatment with the antiplatelet drugs, aspirin and cilostazol, in preventing stenosis and evaluated the effect of these drugs on the acute phase of inflammation and tissue remodeling.

**Approach and Results**—C57BL/6 mice were fed a drug-mixed diet of aspirin, cilostazol, or normal chow during the course of follow-up. Bioresorbable vascular grafts, composed of poly(glycolic acid) mesh sealed with poly(1-lactide-co-ε-caprolactone), were implanted as inferior vena cava interposition conduits and followed up for 2 weeks (n=10 per group) or 24 weeks (n=15 per group). Both aspirin and cilostazol suppressed platelet activation and attachment onto the grafts. On explant at 24 weeks, well-organized neotissue had developed, and cilostazol treatment resulted in 100% graft patency followed by the aspirin (67%) and no-treatment (60%) groups (P<0.05). Wall thickness and smooth muscle cell proliferation in the neotissue of the cilostazol group were decreased when compared with that of the no-treatment group at 24 weeks. In addition, cilostazol was shown to have an anti-inflammatory effect on neotissue at 2 weeks by regulating the recruitment and activation of monocytes.

**Conclusions**—Cilostazol prevents stenosis of bioresorbable vascular graft in a mouse inferior vena cava implantation model up to 24 weeks and is accompanied by reduction of smooth muscle cell proliferation and acute inflammation. (Arterioscler Thromb Vase Biol. 2015;35:2003-2010. DOI: 10.1161/ATVBAHA.115.306027.)

**Key Words:** antiplatelet drugs • constriction, pathologic • inflammation • mice • monocytes

Approximately 0.6% of live births are affected by moderate to severe forms of congenital heart disease, many of which require surgical intervention with various prosthetics to restore normal cardiac function. However, synthetic materials, such as polytetrafluoroethylene and polyethylene terephthalate, lack growth potential, and their use requires reoperation to up-size the conduit as the child grows. To address this challenge, novel tissue engineering techniques allow the implantation of bioresorbable vascular grafts that restore function and transform into biologically active blood vessels. A bioresorbable vascular graft is entirely reconstituted by host-derived cells over the course of its degradation via an inflammation-mediated process. This technique has been successfully applied in the clinical arena, and evidence has shown that this therapy is safe and effective in pediatric patients. The application of bioresorbable vascular grafts has several advantages, such as growth potential, favorable biocompatibility, and low risk of infection or rejection; however, the incidence of stenosis because of neotissue hyperplasia, which is thought to be related to excessive inflammation, platelet activation, and smooth muscle cell (SMC) proliferation, is nearly equivalent to that of polytetrafluoroethylene grafts currently used in the Fontan surgery. Therefore, the top priority in the development of second-generation bioresorbable vascular grafts is to safely reduce the incidence of stenosis.

Aspirin, a widely used antiplatelet drug, is routinely used as a therapeutic in our clinical trial to prevent platelet aggregation on the graft directly after implantation. Aside from its antiplatelet effects, aspirin has been shown to inhibit SMC migration and proliferation in blood vessels, to protect endothelial cells (ECs), and to suppress vascular inflammation. The phosphodiesterase 3 inhibitor cilostazol is another antiplatelet drug, which can reduce platelet aggregation and can improve peripheral vasodilation by increasing intracellular cAMP content.
Similar to aspirin, cilostazol has been reported to exert pleiotropic effects on SMCs, ECs, and vascular inflammation. 

Although previous findings support the potential of the antiplatelet drugs, aspirin and cilostazol, to suppress excessive neotissue formation during the process of vascular remodeling, the effect of these drugs on preventing the development of stenosis in bioresorbable vascular grafts is currently unknown. The purpose of this study was to clarify the impacts of long-term (24 weeks) administration of aspirin and cilostazol on neotissue hyperplasia causing stenosis after the implantation of bioresorbable vascular grafts as inferior vena cava (IVC) interposition conduits in a mouse model. Furthermore, our previous findings also suggest that the natural history of graft stenosis in the murine model begins within 2 weeks after implantation, and that this time point is critical to assess vascular inflammation and neotissue formation in implanted bioresorbable grafts. Thus, we also investigated the acute phase (2 weeks) effect of antiplatelet treatment with aspirin and cilostazol on the inflammation of and tissue remodeling processes in the bioresorbable vascular grafts.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Aspirin and Cilostazol Reduce Platelet Activation and Attachment Onto Bioresorbable Grafts In Vitro

To confirm the antiplatelet effects of aspirin and cilostazol administration in our mouse model, we examined the activation potential of platelets isolated from mice of each experimental group (aspirin, cilostazol, or no treatment) by evaluation of P-selectin and glycoprotein IIb expression on platelets with flow cytometry under both resting and thrombin-activated conditions. Platelet activation was suppressed in both aspirin and cilostazol groups (no treatment, 44.6%; aspirin, 11.3%; and cilostazol, 7.58%; Figure 1A). Furthermore, platelet attachment onto bioresorbable grafts after thrombin activation was reduced by both aspirin (P<0.05) and cilostazol (P<0.01; Figure 1B). These effects were not observed in resting, nonactivated conditions (Figure 1A and 1B). These results indicate that both aspirin and cilostazol reduced platelet function in our mouse model to the extent that they inhibited platelet attachment onto the bioresorbable graft.

Cilostazol Prevents Stenosis of Bioresorbable Vascular Grafts

A total of 75 mice received bioresorbable vascular grafts as IVC interposition conduits and were followed up for 2 weeks (n=10 per group) to evaluate the effects of aspirin and cilostazol on tissue remodeling and inflammation during the acute phase, or for 24 weeks (n=15 per group) to clarify the impact of long-term administration of these drugs on graft stenosis and neotissue formation. All mice survived during the course of observation.

Macroscopically, implanted grafts were still distinguishable from native IVC at the 2-week time point but were fully integrated with native IVC by 24 weeks (Figure 2A). Serial ultrasonographic imaging was performed on all mice that received grafts for 24 weeks, and graft patency was determined with color Doppler and pulse Doppler in the graft lumen. Graft patency of the cilostazol group was maintained from 2 to 24 weeks after implantation, whereas that of the aspirin group gradually decreased. The no-treatment group experienced the lowest patency rate at each time point (Figure 2B). There was a statistically significant difference in patency between the no-treatment and the cilostazol groups at 8 weeks (no treatment, 46.7% versus cilostazol, 93.3%; P=0.014; Figure 2B).

On explant, sufficient cell infiltration and cell growth were observed in all groups at the 24-week time point (Figure 3A). Cilostazol treatment resulted in 100% graft patency followed by the aspirin (67%) and the no-treatment groups (60%) at 24 weeks (Figure 3B). In addition, wall thickness was

**Figure 1.** Platelet function evaluated by activation potential and graft attachment. A. Activated platelets were defined by expression both of P-selectin and glycoprotein IIb (GpIIb) with flow cytometry. Both aspirin and cilostazol treatments suppressed platelet activation. B. Aspirin and cilostazol treatments reduced thrombin-activated platelet attachment to grafts. Data are shown as means±SD and evaluated by 1-way ANOVA followed by Tukey HSD. *P<0.05, **P<0.01. FITC indicates fluorescein isothiocyanate.
Cilostazol Suppresses SMC Proliferation

Endothelialization on the luminal surface is thought to be a crucial step in the development of well-organized neotissue of a bioresorbable vascular graft. To evaluate endothelialization of implanted bioresorbable grafts, immunostaining for the EC marker CD31 was used. EC coverage on the graft luminal surface was achieved in all groups by the 24-week time point (Figure 4A). For the quantitative comparison of endothelialization between groups at the 24-week time point, gene expression of platelet endothelial cell adhesion molecule-1 and endothelial nitric oxide synthase in explanted grafts was measured. No statistically significant differences between groups were observed (Figure 4B).

Abundant SMCs, which were defined by α-smooth muscle actin (α-SMA) immunolabeling, surrounded the endothelium at the 2-week time point, and the total number of SMCs decreased during the course of tissue remodeling in the aspirin and the control groups (Figure 4A). A significant decrease in the number of SMCs was observed in the neotissue of the cilostazol group when compared with that of the no-treatment group at the 24-week time point (no treatment, 481.5±127.3; cilostazol, 313.3±78.6 per mm²; P<0.05; Figure 4C). Interestingly, some α-SMA-positive cells were coincident with CD31 at the 2-week time point, and these double-positive cells decreased at the 24-week time point (Figure 4A).

Aspirin and Cilostazol Do Not Affect Extracellular Matrix Deposition in Neotissue

Extracellular matrix (ECM) is the primary determinant of the biomechanical properties of a neovessel. Consequently, we evaluated ECM components including collagen and elastin by histology. Masson’s trichrome and Alcian Blue staining showed a gradual increase in collagen deposition. Hart’s staining showed no elastin deposition during neotissue formation (Figure I in the online-only Data Supplement). However, no differences were identified among groups (Figure II in the online-only Data Supplement), suggesting that both aspirin and cilostazol do not affect the development of a robust ECM in the neotissue of bioresorbable vascular grafts.

To quantify collagen deposition in the grafts and to confirm graft polymer degradation at the 2- and the 24-week time points, the proportion of thin and thick collagen fibers was measured using Picrosirius red staining visualized with polarized light microscopy. On the basis of previous reports,

Figure 2. Serial monitoring of implanted grafts. A, Macroscopic assessment showed an integration of the implanted graft with native vein. Yellow bars indicate implanted grafts. B, Ultrasound evaluation demonstrated statistically significant differences in graft patency between no-treatment and cilostazol groups at 8 weeks, when evaluated by Fisher exact probability test with Bonferroni–Holm correction (P<0.016 was considered statistically significant). †P=0.014.

significantly less in the cilostazol group at the 24-week time point (no treatment, 633.2±250.3 μm; aspirin, 454.4±330.2 μm; cilostazol, 202.5±50.9 μm; P<0.001; Figure 3C).

Figure 3. Comparison of morphometric analysis of explanted grafts at 24 weeks after implantation. A, Representative hematoxylin-eosin staining images from each group. B, Cilostazol treatment resulted in 100% graft patency followed by aspirin (67%) and no-treatment groups (60%). Data were evaluated by Fisher exact probability test. C, Wall thickness was significantly lower in the cilostazol group than in the no-treatment and aspirin groups. For comparisons among multiple groups, data were evaluated by nonparametric Kruskal–Wallis test. A post hoc Mann–Whitney test was performed to detect significant difference between groups with Bonferroni–Holm correction (P<0.016 was considered statistically significant). *P<0.05, ***P<0.001.
we correlated orange and yellow (thick fibers) with collagen type I, green (thin fibers) with collagen type III, and attributed white regions to remaining scaffold fibers or suture material. Although scaffold fibers were present at 2 weeks, all scaffold material had been completely resorbed at 24 weeks. Collagen type I deposition increased over the time course of neotissue formation (Figure IIIA in the online-only Data Supplement). In addition, no differences in the distribution of collagen type I or type III at any time point were observed among experimental groups (Figure IIIB in the online-only Data Supplement).

Vascular basement membranes are a specialized form of ECM and are important structural and functional components of a blood vessel. A main component of vascular basement membrane is collagen type IV, and collagen IV deposition has been demonstrated in similar mouse models. To this end, we performed immunofluorescent staining for collagen type IV at the 24-week time point and demonstrated deposition of luminal collagen type IV in all experimental groups (Figure IV in the online-only Data Supplement).

Cilostazol Regulates Monocyte Recruitment and Activation in the Acute Phase of Tissue Remodeling

Monocyte- and macrophage-mediated inflammation is understood to play a crucial role in the formation of both vascular neotissue and the development of stenosis in a bioresorbable vascular graft. Immunohistochemical F4/80 staining showed a decrease in infiltration of monocytes/macrophages in the grafts from the cilostazol group when compared with that from the no-treatment group at the 2-week time point (P<0.05; Figure 5A). Flow cytometric analysis revealed that recruited monocytes (CD115+ F4/80+low) are present in the graft explants 2 weeks after implantation. Fewer absolute numbers of inflammatory lymphocyte antigen 6C positive high (Ly6C+high) monocytes were observed in the aspirin and cilostazol groups than in the no-treatment group, but the difference between groups was only significant at P=0.19 (Figure 5B). On the contrary, no discernible macrophage populations (CD115+ F4/80+high Ly6C+low) were identified in any 2-week graft explants with our staining methods and gating strategies for flow cytometric analysis (Figure VA in the online-only Data Supplement).

Figure 4. Endothelialization and smooth muscle cell proliferation at 2- and 24-week time points. A, CD31-positive endothelial cells covered the luminal surface in each group at the 2-week time point. CD31-positive cells were also identified in the neotissue, and these cells were coincident with α-smooth muscle actin (SMA). At the 24-week time point, favorable endothelialization was achieved in all groups, and double-positive cells for CD31 and α-SMA had decreased. Arrows indicate double-positive CD31 and α-SMA cells. B, There was no statistically significant difference in gene expression of platelet endothelial cell adhesion molecule (PECAM)-1 and endothelial nitric oxide synthase (eNOS) between groups. C, α-SMA-positive cells in the cilostazol group decreased significantly in comparison with the no-treatment group at the 24-week time point. Data are shown as means±SD and evaluated by 1-way ANOVA followed by Tukey HSD. *P<0.05.

Because inducible nitric oxide synthase (iNOS) is a marker for classical activation of inflammatory cells, we determined iNOS expression in neotissue of the grafts by immunohistochemistry. Fewer activated inflammatory cells in the neotissue of the cilostazol group were found when compared with that of the either aspirin or no-treatment groups, and statistical significance in the number of iNOS-positive cells was detected between the cilostazol and the no-treatment groups at the 2-week time point (P<0.01; Figure 5C). Negative and positive controls for iNOS staining are shown in Figure VI in the online-only Data Supplement. Because our gating strategy for flow cytometric analysis indicated that monocytes accounted for most of the infiltrating leukocytes (CD45-positive cells) in the 2-week graft explants (Figure VA in the online-only Data Supplement), we think that the majority of iNOS-positive cells at the 2-week time point are activated monocytes.

To confirm the functional effect of aspirin and cilostazol in the activation of monocytes in vitro, we induced classical activation of bone marrow monocytes (Ly6C+high and Ly6C+low) by stimulation with lipopolysaccharide and interferon-gamma (IFN-γ) after incubation with aspirin, cilostazol, or vehicle control and determined iNOS expression by flow cytometry. Lipopolysaccharide /IFN-γ stimulation increased the number of iNOS-positive cells in both Ly6C+high and Ly6C+low monocytes. Interestingly, only cilostazol prevented iNOS expression in both monocyte subsets after lipopolysaccharide /IFN-γ stimulation in a dose-dependent manner (Figure 6A–6D).

Discussion

The primary finding of our study was that cilostazol treatment, in contrast to aspirin, achieved 100% patency of bioresorbable vascular grafts up to 24 weeks with favorable neotissue formation. Bone marrow–derived mononuclear cells (BM-MNCs) have the potential to reduce the incidence of stenosis when seeded onto grafts before implantation. However, previous clinical data demonstrate that 16% of patients who have received bioresorbable vascular grafts with BM-MNC seeding still developed stenosis requiring angioplasty, even when aspirin is used postoperatively. In the present study, we implanted BM-MNC–free (unseeded) bioresorbable vascular grafts in a mouse IVC interposition graft model. We have previously demonstrated that unseeded grafts result in a higher incidence...
of stenosis than seeded grafts, and we selected the unseeded graft model for the current study to focus on the impact of antiplatelet drugs in preventing the graft stenosis and demonstrated the efficacy of cilostazol in this application. We suggest that combined therapy of BM-MNC seeding and systemic cilostazol treatment, instead of aspirin, has a potential to reduce the incidence of stenosis after the implantation of biodegradable vascular grafts. However, seeding BM-MNCs onto the grafts may affect the response of the graft to these antiplatelet drugs. Additional studies to evaluate the combined effect of
BM-MNC seeding and antiplatelet treatment are, therefore, required before clinical translation can be advocated.

In the present study, both aspirin and cilostazol treatments reduced platelet activation and attachment on the grafts in vitro. Because activated platelets release several growth factors, such as transforming growth factor-beta and platelet-derived growth factor, which promote SMC recruitment and proliferation leading to neointimal hyperplasia, prevention of platelet activation and aggregation on the graft surface was expected to attenuate stenosis. Ultrasound assessment demonstrated that both aspirin and cilostazol had a similar effect on the development of stenosis at early time points (≤8 weeks), indicating that the antiplatelet effect of these drugs may work to prevent stenosis because of thrombosis during the acute phase. Cilostazol treatment sustained this high patency rate throughout our observation period. On the contrary, the patency rate of the aspirin group decreased gradually, supporting similar results in the literature in which oral administration of aspirin has been shown ineffective in amelioration of neointimal lesions in a mouse vein graft model.23 Interestingly, clinical findings demonstrated that cilostazol successfully prevented neointimal hyperplasia after implantation of arterial stents23,24 even when compared with aspirin.25 On the basis of these findings, we propose that multiple effects of cilostazol, including anti-SMC proliferation, EC protection, and anti-inflammation, in addition to its antiplatelet effect, may have worked in concert to prevent neointimal hyperplasia throughout the time course of its formation in our bioresorbable vascular graft.

Wall thickness is an important metric by which to evaluate neotissue formation and the development of stenosis in a bioresorbable vascular graft. Size mismatch is routinely used in clinical application of vascular grafts in the pediatric population to minimize reoperation to up-size the conduit because of somatic overgrowth. Over time, the scaffold materials degrade, and the graft wall is expected to remodel into a neovessel that closely resembles native IVC. We demonstrated that wall thickness 24 weeks after implantation was least in the cilostazol group, with a reduction in the number of α-SMA–positive SMCs in the developing neotissue although there was no difference in graft material absorption, endothelialization, and ECM deposition between groups at this time point. Although vascular SMCs are essential for the functional integrity of the neovessel, excessive proliferation of SMCs leads to neointimal hyperplasia followed by graft stenosis and occlusion. Cilostazol is understood to inhibit the proliferation of SMCs directly by increased intracellular cAMP.11,26 SMCs are complex cells capable of existing in heterogeneous populations and switching phenotypes on a variety of stimuli (ie, contractile to synthetic).27 The synthetic dedifferentiated phenotype of SMCs, which have characteristics of migration, proliferation, and ECM synthesis in the vascular wall, promotes neointimal hyperplasia and can be identified by the expression of α-SMA, which is detectable early in the developing vasculature.28 On the contrary, differentiated SMCs have a contractile apparatus with less potential for proliferation and are distinguished by the expression of differentiated SMC markers, such as smooth muscle-myosin heavy chain.28 In this study, much fewer smooth muscle-myosin heavy chain–positive cells were observed in the neotissue of implanted grafts (Figure VII in the online-only Data Supplement) than α-SMA–positive cells (Figure 4A), indicating that synthetic SMCs account for most of the SMCs in the neotissue of the bioresorbable grafts during the remodeling process in our model. Breakdown products from bioresorbable poly(glycolic acid), which was used in the present study, may have induced dedifferentiation of SMCs to the synthetic phenotype. Furthermore, the combination of scaffold geometry, biochemical, and mechanical stimulation are thought to affect SMC phenotypes. Cilostazol may exert its effect on these scaffold characteristics to suppress dedifferentiation or proliferation of SMCs.

During the acute phase (2 weeks after the graft implantation) of tissue remodeling, we demonstrated that cilostazol regulates Ly6C<sup>high</sup> monocyte recruitment to implanted grafts and decreased the number of iNOS–positive activated monocytes in neotissue. Ly6C<sup>high</sup> monocytes have been recognized to play a crucial role in inflammation, yet little is known about the role of Ly6C<sup>low</sup> monocytes in this process. Our data do not identify the function of Ly6C<sup>low</sup> monocytes in the inflammatory process of tissue remodeling. However, we consider Ly6C<sup>low</sup> monocytes to still have inflammatory properties because Ly6C<sup>low</sup> monocytes are thought to be derived from Ly6C<sup>high</sup> monocytes,29 and these cells still express Ly6C on their surface. Recent findings suggest that Ly6C<sup>low</sup> monocytes initiate an early immune response and differentiate into macrophages.30 We could not determine which monocyte subset is most crucial to the development of graft stenosis in the present study; however, we report that only cilostazol prevents activation of both Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes after inflammatory lipopolysaccharide /IFN-γ stimulation. This observation may highlight one mechanism by which cilostazol prevents stenosis of bioresorbable vascular grafts because inflammatory stimulation is known to switch the phenotype of contractile SMCs to synthetic SMCs.31

Cilostazol increases intracellular content of cAMP, which is a second messenger, used for intracellular signal transduction in many biological processes. Interestingly, in the present study, cilostazol prevented iNOS expression in bone marrow monocytes (Figure 6C and 6D), but not in bone marrow–derived macrophages (Figure VIII in the online-only Data Supplement). These results indicate that increased CAMP may affect iNOS expression only in monocytes, but not in macrophages, although precise role of CAMP in iNOS expression in these cells is still unknown. Indeed, CAMP activity was recently shown to be cell specific with regards to iNOS expression.32–34

We acknowledge some limitations in the present study. First, the precise mechanism of cilostazol’s activity during neotissue formation remains to be fully elucidated because we did not investigate every possible effect of cilostazol on the development of graft stenosis. Second, we routinely use anti-coagulation drugs in our clinical trial to prevent acute thrombosis because anti-coagulation drugs, rather than antiplatelet drugs, are more effective in preventing venous thrombosis.35 Thrombosis is another possible mechanism of stenosis in bioresorbable vascular grafts although clinical data indicate that graft occlusion is primarily because of a hyperplastic intima.5 To verify this assumption, additional studies using
anticoagulation drugs are required. Third, in the present study, we could not detect fully mature macrophages in the neotissue of implanted grafts at the 2-week time point by flow cytometric analysis, in contrast to our previous findings in which abundant F4/80+ macrophages were identified by immunohistochemical staining at this time point. The different results for macrophage presence in the graft between the current study and other reports published by our group may be explained by differences in the sensitivity and specificity of the experimental methods used to identify macrophages because tissue infiltrating monocytes also express the F4/80 antigen (albeit at lower levels than resident macrophages). The flow cytometric analysis used in this study more precisely distinguishes among these 2 cell populations. Fourth, an analysis of protein levels would be more appropriate than that of gene expression for the quantitative assessment of endothelialization. However, we could not prepare samples for a protein assay in this study because of a limited amount of tissue from each explant. Fifth, the optimal wall thickness after tissue remodeling of bioresorbable vascular grafts has not been established. Because in clinical application, the conduit is used for much larger vessels than that of the mouse abdominal IVC, the thick wall observed in the no-treatment group of the present study (≥600 μm) may be tolerated.

In conclusion, our work demonstrates that cilostazol prevents stenosis of bioresorbable vascular grafts for 24 weeks in mouse IVC implantation model when compared with aspirin and no-treatment groups. Cilostazol treatment effectively suppresses SMC proliferation and reduces acute phase (2 weeks) inflammation mediated mainly by monocyte infiltration and activation, and we suggest that these effects may consequently attenuate neotissue hyperplasia—causing stenosis.

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Disclosures
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References
14. Inoue T, Uchida T, Sakuma M, Imoto Y, Ozeki Y, Ozaki Y, Hikichi Y, Nide K. Cilostazol inhibits leukocyte integrin Mac-1, leading to a poten-
Bioreabsorbable vascular grafts offer the potential of a synthetic conduit that ultimately transforms into a neovessel capable of growth throughout the lifespan of the host patient. However, neointima hyperplasia leading to stenosis is the primary cause of graft failure in a clinical trial, evaluating these grafts in the treatment of congenital heart disease. Aspirin is used in this application and is expected to prevent platelet aggregation on the graft, which could promote smooth muscle cell proliferation causing neointima hyperplasia. However, we report here that aspirin failed to prevent the development of neointima hyperplasia in a mouse inferior vena cava implantation model. On the contrary, cilostazol (a related antiplatelet drug) was shown to prevent graft stenosis up to 24 weeks and to reduce acute inflammation mediated by monocyte recruitment and activation. These findings provide further strategies of antiplatelet therapy after implantation of bioreabsorbable vascular grafts in the clinical setting.
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MATERIALS AND METHODS

Bioresorbable vascular grafts
All bioresorbable vascular grafts, constructed from a nonwoven poly(glycolic acid) fiber mesh coated with a 50:50 copolymer sealant solution of poly(L-lactide-co-ε-caprolactone) as previously described,¹ were provided by Gunze Ltd. Each graft was 3.00 mm in length with an inner diameter of 1.06 mm.

Platelet isolation and flow cytometric analysis
8-10 weeks old mice were fed a drug-mixed diet of aspirin (0.1% of diet), cilostazol (0.3% of diet), or normal chow (no treatment control) three days before platelet harvesting. Approximately 0.5 ml of whole blood was obtained from a mouse by intracardiac puncture by a collection syringe preloaded with 100μl of citrate-dextrose solution (C3821, Sigma). Platelet rich plasma was prepared by centrifugation (120 g for 8 min x 2), and platelet number was counted with an ABX Micros 60 hematology analyzer (Horiba). After centrifugation (740 g for 10 min), the supernatant was removed from the platelet rich plasma, and the isolated platelet pellet was diluted with PBS to obtain a concentration of 0.2 x 10⁶ cells/μl. Platelet suspensions obtained by this method were utilized to evaluate platelet activation with flow cytometric analysis and to quantify platelet attachment on the grafts to compare platelet function between groups. To activate platelets, thrombin (T4648, Sigma) was added to the platelet suspension to achieve 0.1 U/ml concentration, and incubated for 5 min at room temperature.

For flow cytometric analysis, anti-CD62P-FITC, RB40.34 (BD Biosciences) and anti-CD41-PE, MWReg30 (BD Biosciences) was added to the platelet solution and incubated for 15 min at room temperature. Incubation was terminated with a dilution of cold 1% PFA-PBS. Stained and fixed platelets were stored at 4°C in the dark for 30 min. Flow cytometric analysis was performed on a BD LSR II cytometer (BD Bioscience). Analysis of 10,000 events for each sample was performed. Compensation and data analysis was performed with FlowJo software (Tree Star). Activated platelets were defined as double positive for P-selectin and glycoprotein IIb.²

In vitro assessment of platelet attachment on grafts
Platelets were isolated from whole blood collected from mice of each group. Bioresorbable vascular grafts were incubated with a platelet suspension (0.2 x 10⁶ cells/μl) for 60 min at 37°C with or without thrombin activation (0.1 U/ml). To lyse attached platelets, grafts were soaked in 2 % Triton X-100 (93427, Sigma) for 60 min at room temperature, and an LDH assay was performed for quantitative platelet analysis according to the manufacturer’s instructions (MK401, Takara Bio).³ Absorbance was measured at 490 nm using a SpectraMax M5 plate reader (Molecular Devices), and a standard curve was applied to obtain the total number of platelets attached to each graft.

Animal model
All animals received humane care in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Nationwide Children’s Hospital approved the use of animals and all procedures described in this study. C57BL/6 mice were purchased from Jackson Laboratories and used for all experiments in the present study.

Drug-mixed diet
A drug-mixed diet of aspirin (0.1% of diet), cilostazol (0.3% of diet), or normal chow (no treatment control) were fed to mice of each group three days before graft implantation through the end of follow-up.⁴ All diets were provided by Otsuka pharmaceutical Co., Ltd.
Surgical implantation of bioresorbable vascular grafts

Bioresorbable vascular grafts were implanted in 8-10 weeks old female mice (n=25 for each group) as inferior vena cava interposition grafts using standard microsurgical techniques as previously described. No medications except for the drugs under investigation were administrated at any point during this study. Mice were sacrificed either 2 weeks (n=10 per group) or 24 weeks (n=15 per group) after implantation, and grafts were explanted for analysis.

Serial monitoring of implanted grafts by ultrasound

Ultrasonography (Vevo Visualsonics 2100; Visualsonics) was employed to monitor the patency of implanted grafts at 8, 16, and 24 weeks after implantation. Mice were anesthetized with 1.5% inhaled isoflurane during ultrasound measurements.

Histology and immunohistochemistry

Explanted grafts were fixed in 4% para-formaldehyde, embedded in paraffin, sliced (5 μm thick sections), and stained with Hematoxylin and Eosin (HE), Masson’s Trichrome, Alcian Blue, and Hart’s. Collagen deposition was assessed with Picrosirius red staining and images were obtained with polarized light microscopy. The proportion of collagen type I (orange to yellow) and collagen type III (green) in neotissue at the 2- and 24-week time points was measured with Image J software (NIH).

Identification of smooth muscle cells (SMCs), monocytes/macrophages, and activated inflammatory cells was accomplished by immunohistochemical staining of paraffin-embedded explant sections with anti-α-smooth muscle actin (α-SMA), 1A4 (1:500, DAKO), anti-F4/80, Cl:A3-1 (1:1000, AbD Serotec), and anti-inducible nitric oxide synthase (iNOS) (1:20, ab3523, Abcam), respectively. Primary antibody binding was detected using species appropriate biotinylated IgG (Vector), and this was followed by the binding of streptavidin-horse radish peroxidase (Vector) and color development with 3,3-diaminobenzidine (Vector). Light field images were obtained with a Zeiss Axio Imager.A2 microscope (Carl Zeiss).

SMCs identified by α-SMA expression, F4/80 positive monocytes/macrophages, and activated inflammatory cells identified by iNOS positive staining were quantified by manual counting. One representative section from each explant was stained and imaged. Low magnification (5x) images were divided into nine sections (3x3). Four of these regions (upper middle, center, lower right, and lower left) were selected to obtain high magnification (20x) images, the area of which was 0.12 mm². All positively stained nuclei were counted from high magnification images. Averages from these four regions represented the number of positive cells in each section.

Immunofluorescent staining for CD31 as a marker of endothelial cells, α-SMA and smooth muscle–myosin heavy chain (SM-MHC) as markers of SMCs, and collagen type IV was performed using anti-CD31 antibody (1:50, ab28364, Abcam), anti-α-SMA, 1A4 (1:500, DAKO) anti-SM-MHC antibody, 1G12 (1:400, Abcam), and anti-collagen IV (1:500, ab6586, Abcam) followed by Alexa Fluor 647 anti-mouse IgG secondary antibody (1:300, ab150115, Invitrogen) or Alexa Fluor 488 anti-rabbit IgG secondary antibody (1:300, ab150077, Invitrogen), respectively. Fluorescence images were obtained with an Olympus IX51 microscope (Olympus).

Morphometric analysis

Outer and luminal perimeters of graft explants were manually measured from HE staining with Image J software (NIH) to obtain luminal diameter and wall thickness measurements. A patent graft was defined as having a luminal diameter greater than 0.53 mm, equivalent to 50% of the inner diameter of the graft at the time of implantation.
RNA extraction and reverse transcriptase-quantitative polymerase chain reaction

Total RNA was extracted from frozen samples and purified using the RNeasy mini kit (74104, Qiagen). Reverse transcription was performed using the High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems) to obtain complementary DNA for polymerase chain reaction (PCR) assay. All reagents and instrumentation for quantitative PCR were obtained from Applied Biosystems. Reverse transcriptase-quantitative PCR was performed with a Step One Plus Real-Time PCR System using the TaqMan Universal PCR Master Mix Kit per the manufacturer’s instructions. Reference numbers for primers are: PECAM-1 (Mm01242584_m1), eNOS (Mm00446968_m1), and HPRT (Mm00446968_m1).

The quantitative PCR data was analyzed using the comparative threshold cycle method and normalized to the expression of HPRT as an endogenous reference. Results are reported as relative values (ΔΔCT) to those of the no treatment control group.

Tissue digestion to obtain cells from implanted grafts

The grafts harvested from each group at the 2-week time point were minced into small pieces with scissors and digested in a HBSS solution containing 1 mg/ml collagenase type IV, 0.2 mg/ml DNase I, 200 U/ml hyaluronidase, and 1 mg/ml bovine serum albumin/fraction V (invitrogen). Cells were filtered (40 μm cell filter, BD Bioscience) to remove remaining graft debris. After counting the number of cells obtained from each graft using Trypan blue exclusion with a manual hemocytometer, cells were stained for flow cytometry.

Monocyte isolation from bone marrow cells and inflammatory stimulation

Bone marrow cell suspensions were isolated by flushing femurs and tibias of 8-10 weeks old C57BL/6 mice with complete RPMI1640 (+10% FCS, +1% Pen/Strep). Cell suspensions were diluted 5:1 with Hank’s balanced salt solution, overlaid on Ficoll solution (Histopaque 1083, density 1.083 g/ml, 10831, Sigma) and centrifuged for 45 min at 500 g, at room temperature. The mononuclear cell interface was collected and washed two times in HBSS. Cells were incubated with a mixture of antibody MicroBeads according to the manufacturer’s protocol (130-100-629, Miltenyi Biotec). The cells were then run through a LD-negative selection column. The negative fraction was collected (monocytes), and cultured in complete RPMI1640. Cultures of purified monocytes were stimulated overnight with lipopolysaccharide (100 ng/ml, L2630, Sigma) and interferon gamma (10 ng/ml, 575302, BioLegend) in absence or presence of aspirin (3.0μM, 10μM and 30μM) (A5376, Sigma) or cilostazol (10μM, 50μM and 100μM) (C0737, Sigma).

Flow cytometric analysis of macrophage lineage cells

Harvested cells from grafts or bone marrow were incubated in saturated doses of anti-mouse Fc receptor antibody in 100uL PBS 0.5% BSA 0.02% NaN3 (FACS buffer) for 15 min on ice. After washing, 1-3 x 10⁶ cells were stained in FACS buffer for 15 min 4 °C with various fluorescent mAb combinations and further collected on a LSR II cytofluorometer (BD Bioscience). Cells were gated according to size and scatter to eliminate debris. Blue-fluorescent reactive dye, L23105 (Life Technologies) was used to discriminate dead cells. The following antibodies were used for extracellular staining: anti -CD11b-Alexa Fluor 700, M1/70 (BioLegend); anti -CD115-PE-Cy7, AFS98 (eBiosciences); anti-F4/80-Brilliant violet 605, BM8 (BioLegend); anti Ly6G-PER-CP, 1A8 (BioLegend); anti-Ly6C-PE-Cy7, HK1.4 (BioLegend); anti-Ly-6C-eFluor 450, HK1.4 (eBioscience); anti-F4/80- PE-Cy7, BM8 (eBioscience); anti- CD45- Brilliant Violet 605, 30-F11 (BioLegend) and anti-F4/80- FITC, BM8 (BioLegend).

For intracellular staining, cells were washed with FACS buffer and stained according to the protocol of the cytofix/cytoperm kit (554714, BD Biosciences) and stained using anti-iNOS-PE, CXNFT (eBiosciences). Compensation and data analysis was performed using FlowJo software (Tree Star).
Monocytes were defined as CD45+ CD11b+ CD115+ F4/80low and Ly6C+high or Ly6C+low, and macrophages were defined CD45+ CD11b+ CD115+ F4/80+high Ly6C+low, as represented in the gating strategy shown in Figure VA,B in the online-only Data Supplement.

Statistical analysis
Numeric values are listed as mean±standard deviation. The number of experiments is shown in each case. For comparisons among multiple groups, data of continuous variables with normal distribution were evaluated by one-way ANOVA followed by Tukey HSD. A nonparametric Kruskal-Wallis test was used in instances when data had a non-normal distribution. A post hoc Mann-Whitney test was performed to detect significant differences between groups with Bonferroni-Holm correction for multiple comparisons, when the Kruskal-Wallis test was significant. Fisher’s exact probability test was used for dichotomous variables of multiple groups with Bonferroni-Holm correction. P values less than or equal to 0.05 were considered statistically significant.

References
Supplemental Figure I. Histological assessment of both patent and occluded bioresorbable grafts at the 2- and 24-week time points. Extracellular matrix components including collagen and elastin were evaluated by standard histologic staining. HE staining (a, b, g, h, m, n, s, t) revealed dense cellular infiltration within the graft and occluded neotissue at 24 weeks. Masson’s Trichrome (c, i, o, u) and Alcian Blue (d, j, p, v) staining showed a gradual increase in collagen deposition over the time course of implantation and elastin deposition was not observed with Hart’s staining at the 2- and 24-week time points (e, k, q, w). Immunohistochemical staining for α-SMA (f, l, r, x) identified abundant SMCs in the neotissue of occluded grafts at the 2-week time point, which decreased by the 24-week time point.
Supplemental Figure II. Histological comparison of extracellular matrix deposition between bioresorbable grafts in each group and native inferior vena cava at the 24-week time point. Although grafts were thicker than native IVC, ECM deposition and organization in the grafts were similar to native IVC. There was no observable difference in ECM deposition between groups.
Supplemental Figure III. Graft degradation and collagen deposition in the graft at 2 weeks and 24 weeks after implantation. A, Representative polarized light images of Picosirius red staining. At the 2-week time point, poly(glycolic acid) fibers still existed in the graft layer, but by 24 weeks, all graft fibers had completely degraded. B, No differences between experimental groups in the distribution of collagen type I or type III at any time point were observed. Color range, orange–yellow: thick fibers correlating to collagen type I, green: thin fibers correlating to collagen type III, and white: graft fiber or suture material. Data are shown as mean±standard deviation, and evaluated by one-way ANOVA followed by Tukey HSD.
Supplemental Figure IV. Immunofluorescent staining for collagen type IV at the 24-week time point. Expression of collagen type IV was observed on the luminal surface of all groups.
Supplemental Figure V. Gating strategy to identify Ly6C{superscript+high} and Ly6C{superscript+low} monocytes from A, graft explants at 2-week time point and B, isolated bone marrow monocytes.
Supplemental Figure VI. Negative and positive control for iNOS staining. A, Negative control for iNOS staining was obtained from no treatment graft at the 2-week time point. Anti-iNOS antibody was excluded from the primary antibody reaction and sections were incubated with diluent only. B, Spleen of C57BL/6 mouse was utilized for a positive control of iNOS staining. Completely equivalent methods and antibody concentrations as shown in Figure 5C were utilized.
Supplemental Figure VII. Endothelialization and contractile smooth muscle cells at 2 and 24 weeks. Favorable endothelialization was achieved in all groups by the 24-week time point. Contractile SMCs were identified as smooth muscle–myosin heavy chain positive cells. Relatively few contractile smooth muscle cells were observed in all groups at each time point.
Supplemental Figure VIII

Supplemental Figure VIII. Flow cytometric analysis to evaluate the anti-inflammatory effect of cilostazol on bone marrow derived macrophage. Cilostazol did not prevent iNOS expression of macrophages cultured in different concentrations of LPS/IFN-γ. Data are shown as mean±standard deviation.