Thrombosis and Neointima Formation in Vein Grafts Are Inhibited by Locally Applied Aspirin Through Endothelial Protection

Evelyn Torsney, Ursula Mayr, Yiping Zou, W. Douglas Thompson, Yanhua Hu, Qingbo Xu

Abstract—Vein graft failure within the first month after bypass surgery is largely because of thrombosis. However, systemic study of thrombus formation in vein grafts is still lacking, and few effective techniques are available to prevent this event. Herein, we analyzed the kinetics of thrombosis and tested the effectiveness of locally applied aspirin on prevention of the disease in a mouse model. En face analysis of vein grafts revealed that 67±12% and 54±17% of the surface areas were covered by microthrombi at 1 and 3 days, respectively. Thrombus generation was also identified by labeling of platelets and fibrin, which occurred in 35 grafts examined at 1 and 3 days and 1, 2, 4, and 8 weeks. In a fifth of grafts, the thrombus occluded the vessel lumen by ≥1/4. Furthermore, a significant loss of endothelial cells was evidenced by β-gal staining for vein grafts in transgenic mice expressing LacZ gene controlled by TIE2-endothelial specific gene promoter. Following thrombosis, neointimal lesions were significantly increased by 4-fold 2 weeks after the operation. When vein grafts were treated locally with aspirin in pluronic gel-127, the thrombus area was significantly reduced (P<0.005) at 1, 4, and 8 weeks. Interestingly, neointimal lesions were markedly reduced in the local, but not oral, aspirin-treated group at 4 and 8 weeks by 50% to 70% (P<0.005). The mechanism of reduced lesions by locally applied aspirin involved the protection of vein graft endothelium. Thus, we provide strong evidence that thrombus formation occurs before the development of neointimal lesions in vein grafts and that local aspirin treatment successfully reduces vein graft arteriosclerosis through endothelial protection, resulting in reduction of thrombosis. (Circ Res. 2004; 94:1466-1473.)

Key Words: thrombosis • arteriosclerosis • aspirin • endothelium • neointima

Abstract

A utologous vein grafts remain the only surgical alternative for many types of vascular reconstruction, but these often fail because of arteriosclerosis.1-3 The pathogenesis of arteriosclerosis is characterized by neointimal proliferation, which can lead to oblitative stenosis. The patency rate of grafts is variable, with ≈15% of grafts becoming occluded during the first year.1-4 Although it is believed that thrombus occlusion accounts for failure in early grafts,5 systemic study of thrombus formation in vein grafts is still lacking, and few effective techniques are able to prevent this event from leading to subsequent graft failure.

We demonstrated that the earliest cellular event in vein grafts is cell death, ie, apoptosis and necrosis.6 Others have shown that an extensive loss of cells was observed in the intima at the early stage of vein grafts.7 Although surgical process is a cause for the endothelial damage of vein grafts, it is believed that mechanical stress plays a major role,8 because the grafted veins are subjected to increased biomechanical forces in the form of stretch stress due to changes in blood pressure. On damaged endothelial cells, platelets aggregate and bind to each other through fibrin and von Willebrand factor to form thrombi.9 Thrombosis can rapidly occur when platelets adhere to collagen that is exposed after endothelial loss.10 Activated platelets release factors, such as platelet-derived growth factor, which promote smooth muscle cell recruitment.11 Subsequently, the fibrinolytic pathway is activated to break down thrombus when it is no longer required. However, the detailed process of thrombus formation in vein grafts in vivo is unknown.

We have established a mouse model of vein graft arteriosclerosis in which significant neointimal proliferation occurs within 2 weeks.12 This model has been proven to be a useful tool in studying the pathogenesis of the disease, as well as therapeutic intervention for vein graft arteriosclerosis.13,14 Because aspirin is a widely used clinical drug that blocks platelet activation and aggregation,15 inhibits smooth muscle cell proliferation and endothelial NF-κB activation, and protects endothelial cells from oxidant-induced cell damage,16 we wondered whether aspirin treatment could be sufficient to inhibit thrombus formation and subsequent
arteriosclerosis. Using this model, we have analyzed the kinetics of thrombus formation and tested the effects of aspirin on vein graft arteriosclerosis. We demonstrate that thrombus formation is a significant event in the early stage after vein grafting. We provide evidence that locally, but not orally, applied aspirin significantly inhibits arteriosclerotic lesions in vein grafts because of the protection of endothelial cells after grafting.

Materials and Methods

Mice

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Wild-type and transgenic TIE2-LacZ mice, expressing β-galactosidase (gal) under the control of the endothelial-specific protein TIE2 promoter, were purchased from The Jackson Laboratory (Bar Harbor, Me). β-Gal activity of cells from mice is mainly localized in the nucleus. Three genotypes of LacZ (−/−, +/−, and +/+ mice) were identified using The Jackson Laboratory’s PCR protocol (primers: 5′-ATC TCT TGC ATG GTG AGG TC-3′ and 5′-CGT GCC CTG ATT CAT TCC-3′). The mice were maintained on ad libitum.

Vein Graft Procedure and Aspirin Treatment

The vein graft procedure was similar to that described previously. Briefly, 3-month-old mice were anesthetized, and a vein segment (vena cava) was grafted between the 2 ends of the carotid artery. Immediately after vessel grafting, 50 μL of aspirin (100 μmol/L) in 20% pluronic-127 gel (pH 7.2) was applied to the adventitia. On contact with the tissues, the solutions gelled immediately, generating a translucent layer that enveloped the grafted vessel segment. Because it was difficult to measure aspirin release in situ, an experiment was designed to indirectly test its pharmacokinetics and bioavailability. When trypan blue–pluronic gel mixture was applied to the adventitia of vein grafts, the blue color became weaker at 1 day and disappeared at 3 days after grafting. These results suggest that aspirin–pluronic gel could be maintained for the time between 1 and 3 days.

After vein bypass grafting and local pluronic gel treatment, another group of mice received aspirin (30 mg/kg daily) in their drinking water for a week. This dose was selected based on the reports by other groups showing its ability to suppress platelet cyclooxygenase activity, as measured by serum thromboxane B2 (TXB2) levels. Blood samples were collected at the end of the treatment. Serum TXB2 levels were measured by using a kit from Cayman Chemical Co.

Histology

Mice were anesthetized and perfused with 0.9% NaCl solution through cardiac puncture in the left ventricle and subsequently perfusion-fixed with 4% phosphate-buffered formaldehyde (pH 7.2) for 2 and 5 minutes, respectively, as described previously. The grafts were harvested at 1, 3, and 7 days and 2, 4, and 8 weeks, postoperatively. The grafts were processed by routine histology and bioavailability. When trypan blue–pluronic gel mixture was applied to the adventitia of vein grafts, the blue color became weaker at 1 day and disappeared at 3 days after grafting. These results suggest that aspirin–pluronic gel could be maintained for the time between 1 and 3 days.

En Face Preparation and X-Gal Staining

Mice were anesthetized and perfused with 0.9% NaCl solution and subsequently perfusion-fixed with 2% formaldehyde and 0.2% glutaraldehyde (pH 7.2) for 2 and 10 minutes, respectively. The procedure for en face preparation is similar to that described elsewhere. In short, vein segments were harvested, and the samples were fixed with 2% formaldehyde and 0.2% glutaraldehyde at 4°C for 24 hours. Each vessel segment (≈5×5 mm2) was prepared free from the adventitia and cut open. Vessel segments were mounted with the endothelium facing up on a glass slide (2.6×7.5 cm). Total areas and areas covered by thrombosis on the surface of vein grafts were measured respectively, using software MO version 3 (Zeiss GmbH). The percentage of thrombus covered areas was calculated against total surface areas of vein grafts.

The procedure for X-gal staining was similar to that described previously. Briefly, vein segments were incubated at 37°C for 18 hours in PBS supplemented with 1 mg/mL X-Gal (Sigma). Vessel segments were rinsed with 3% DMSO in PBS and mounted with the endothelium facing up on a glass slide (2.6×7.5 cm). Positive stained (blue) cells were enumerated under the microscope.

Histochemical Staining

The Modified Martius Scarlet Blue histochemical stain was used to detect fibrin. An adapted method is as follows: tissue sections were taken to water, stained in 5% Iron Alum for 5 minutes, washed in water and stained in Mayers Hematoxylin for 5 to 7 minutes. The sections were then rinsed in water and differentiated in acid alcohol and washed in water and rinsed in 95% ethanol. The sections were then stained in Martius Yellow for 3 minutes, washed in water, and incubated with Brilliant Crystal Scarlet stain for 3 minutes. After being washed in water, the sections were differentiated in phosphotungstic acid for 3 minutes, washed in water again, and stained in Aniline Blue solution for 3 minutes. Finally, the sections were dehydrated and mounted. With this method, fibrin stains red, nuclei stain blue/black, collagen is blue, and red blood cells are yellow.

Immunohistochemical Staining

Immunohistochemical staining with a polyclonal goat anti-human CD42c (Santa Cruz) antibody was used to detect platelets and a polyclonal rabbit anti-mouse fibrin antibody to detect fibrin. The peroxidase-ABC detection method was used for both antibodies. Before incubation with primary antibody, antigen retrieval was performed: chromotropsin digestion (250 mg chymotropsin and 250 mg CaCl2 in 500 mL of PBS for 30 minutes) was required for CD42c and pressure cooking (microwavable pressure cooker containing 1L Tris buffer heated unsealed for 20 minutes at full power, then sealed to reach pressure for 7 minutes) for fibrin. Biotinylated rabbit anti-goat and biotinylated swine anti-rabbit secondary antibodies were used for platelets and fibrin, respectively.

Quantification of Thrombus and Lesion Formation

Using Axiovision software, the area of thrombus in a given image was highlighted and quantified (μm2) automatically. Each vessel was photographed at low power (magnification ×50), so that the whole of the vessel lumen was visible. It was then possible to zoom in on specific areas to highlight the large and small thrombi to be measured. The procedure used for lesion quantification was the same as previously described. Briefly, we determined the thickness of the vessel wall by measuring 4 regions of a section along a cross, recorded in microns (means ±SD). We obtained 5 cross sections by selecting the first of every 3 sections from each animal.

Statistical Analysis

Statistical analysis of thrombus formation was performed using the 2-tailed Student t test. For the analysis of neointimal lesion size, the Mann–Whitney U test was used. For both analyses, P<0.05 was considered significant.

Results

Kinetics of Thrombosis

En face analysis of thrombus formation was visualized at the early stage, ie, 1, 3, and 7 days after grafting (Figure 1). Thrombus was visible on the surface of the vessel wall and was a brown color because of the presence of red blood cells. As shown in the graph of Figure 1, thrombus formation appeared maximal between 1 and 3 days and decreased thereafter. The size of these thrombi was between 50 and
300 μm in diameter, which covered more than half the area of the surface in 1- and 3-day grafts.

Thrombus formation was further analyzed in vein graft tissue sections at 1 and 3 days and 1, 2, 4, and 8 weeks following grafting (Figure 2). Thrombus, visible as aggregates of platelets, fibrin, and red blood cells, was present in all samples within 24 hours (Figure 2a) and was also present in vein grafts at 3 days and at 1 and 2 weeks (Figure 2b through 2d), but less in 4- and 8-week grafts (Figure 2e and 2f). In a fifth of grafts, the thrombus occluded the vessel lumen by 1/4 or more (Figure 2g), and 2 vessels were completely occluded (Figure 2h).

To quantify thrombus formation, immunohistochemical staining with an anti-platelet antibody was performed. Positive staining for platelets was observed in all sections of vein grafts (Figure 3), implicating the formation of thrombi. During the first week, positive stained microthrombi cover the lining of the vessel, and larger thrombi are also present (Figure 3a through 3c). The intensity of positive staining for micro- and larger thrombi was reduced from 1 week onwards (Figure 3d through 3f). The area of the vessel wall, composed of positively stained thrombus at each time point, was measured using computer-aided quantification. Maximal thrombus occurred between 1 and 3 days postsurgery, then reduced, progressively, at 1, 2, 4, and 8 weeks (Figure 3g).

As previously described, neointimal hyperplasia is significant at 2 weeks following grafting. Comparison of kinetics of thrombus formation and neointimal proliferation indi-
cate thrombus formation before neointimal hyperplasia. The mean neointimal lesion area increased from $2.1 \times 10^4 \text{ mm}^2$ at 1 week to $28 \times 10^4 \text{ mm}^2$ at 8 weeks. In contrast, mean thrombus area was largest at 1 day with $12 \times 10^4 \text{ mm}^2$ reduced to $5 \times 10^4 \text{ mm}^2$ at 8 weeks. When thrombus areas measured in the sections were calculated against total areas of the (neo) intima and media, 40% of the area of the vessel wall at 1 day was positively stained for thrombus. Thus, thrombus formation is consistent in all samples across the time course. It is significant to note that maximal thrombus formation precedes neointimal proliferation. Furthermore, fibrin is a component of thrombus and is produced from fibrinogen by the action of thrombin.9 Fibrin was detected using an anti-fibrin antibody and also using the Modified Martius Blue histochemical stain. Positive staining for fibrin was observed in sections of vein grafts with a variety of ages by both detection methods. From 1 day to 1 week, positively stained fibrin was present as microfibrils around the vessel lumen and in larger thrombi (data not shown). From 2 weeks onward, it was also deposited within the neointima (Data not shown).

**Endothelial Damage**

To further investigate the mechanisms of thrombosis formation in vein grafts, we studied the integrity of endothelium on the surface of the vessel wall. The vein graft procedure was performed on TIE2-LacZ mice, which express β-gal under the control of the endothelial-specific protein TIE2 promoter.17 The extent of endothelial damage was assessed in these mice from 1 day to 8 weeks. En face analysis of the vessel wall allowed identification of endothelial cells by their blue color following X-gal staining as shown in Figure 4. An even monolayer of β-gal+ cells was present in the freshly harvested vein segments from TIE2-LacZ mice (Figure 4a). The density of endothelial cells was decreased at 1 and 3 days, compared with the control (Figure 4b and 4c versus 4a), which increased in number from 1 to 4 weeks (Figure 4d and 4e). When the number of endothelial cells per field (×400) was quantified at each time point, more than 70% of endothelial cells were lost during the first 24 hours after grafting (Figure 4f). Although increased β-gal+ endothelial cells were seen in 3-day vein grafts, it did not reach the level of normal veins until 4 weeks, postoperatively. Therefore, endothelial damage could be the main cause for the thrombotic response and subsequent neointimal formation.

**Effects of Aspirin Treatment on Platelet Deposition and Neointimal Proliferation**

To determine the effects of aspirin on vein grafts, 2 pathways for administration of this drug were used, ie, orally and locally. Aspirin was solubilized in the drinking water given daily to the mice after vein grafting or was mixed with pluronic-127 gel and applied locally to envelope the vein graft. Local treatment with aspirin has reduced thrombus formation as detected by platelet immunostaining (Figure 5c), compared with untreated samples (Figure 5a) and samples treated with oral aspirin (Figure 5b). Fibrin deposition within the neointima was also reduced in locally treated aspirin grafts (Figure 5e and 5g), in comparison to time-matched untreated controls (Figure 5d and 5f). To statistically analyze vein graft thrombus, Figure 5h summarizes data of thrombus areas using anti-platelet staining measured microscopically. Local aspirin treatment significantly reduced thrombus formation in both 4- and 8-week grafts ($P<0.05$).

Furthermore, neointimal lesions of vein grafts were markedly inhibited in the locally treated aspirin group at 4 and 8 weeks (Figure 6b and 6e) relative to the untreated group (Figure 6a and 6d) and orally treated animals (Figure 6c and 6f). The thickness of the vessel wall was measured and statistically compared. No significant difference between untreated (4 weeks, n=8; 8 weeks n=6) and pluronic-127 gel-treated groups was found. Local aspirin treatment (4 weeks, n=8) reduced neointimal thickness up to 70% for 4-week grafts and 50% for 8-week grafts, compared with untreated controls (Figure 6g). Although oral administration of aspirin showed a trend toward reduction of arteriosclerotic lesions, no statistical significance was found compared with untreated controls. These results indicate that local aspirin...
treatment is more effective than that of oral administration in inhibition of neointimal development in the mouse model.

**Aspirin Protects Endothelial Cells**

To investigate the mechanisms of aspirin-reduced neointimal formation, we studied thrombus formation and endothelial integrity at the early stage after vein grafting. Data shown in Figure 7A indicate that local aspirin application had no effects on serum TXB2 levels. Oral administration of aspirin markedly reduced the TXB2 levels in blood, implicating the effectiveness of aspirin in vein graft models. However, the surface areas of vein grafts covered by microthrombi were significantly reduced in local aspirin-treated animals, compared with untreated or pluronic gel-treated groups (Figure 7B). Again, oral aspirin treatment showed some reductions, but did not reach a significant level (Figure 7B). These results confirm the effect of local application of aspirin on thrombus formation.

Because thrombus formation is largely dependent on the integrity of endothelium, and because aspirin has an ability to protect endothelial cells from damage in vitro,16 we determined the number of endothelial cells in vein grafts using TIE2-LacZ transgenic mice. Interestingly, local treatment with aspirin showed a better preservation of vein graft endothelium at day 1 and 3, whereas oral administration of aspirin had no effect (Figure 8A). Furthermore, the number of endothelial cells in vein grafts was counted and statistically compared. Local aspirin treatment (n=7) resulted in increased endothelial cells up to 2-fold for 1- and 3-day grafts compared with untreated controls and pluronic gel-treated group (Figure 8B). No significant difference between untreated (n=5) and oral aspirin–treated groups was found. These results indicate that only local aspirin treatment is effective for the preservation or protection of vein graft endothelium.

**Discussion**

Vein grafts are widely used for treatment of severe atherosclerosis. Following grafting, atherosclerotic lesions can later develop with characteristic neointimal proliferation.7 Vein graft failure within the first month is largely because of thrombosis.5,26,27 Medical treatment to alter the coagulatory response to vein grafting could improve the prognosis for these patients.26,28,29 In the present study, we demonstrated that thrombus formation evoked by endothelial damage contributes to neointima development in a murine model. Locally applied aspirin preserved vein graft endothelium, resulting in significantly reduced thrombus formation and neointimal proliferation. Such treatment might also be applicable for bypass patients, based on the following reasons. First, no side effects in the mouse model have been observed, because little, if any, drug is released into the blood. Second, aspirin...
is a smaller molecule that should easily penetrate human vessel walls. Third, it is technically easy to use for local treatment of vein segments of bypass patients without prolonging surgery times. Finally, locally applied aspirin may have similar effects on the protection of vein graft endothelium and inhibition of thrombosis and subsequent atherosclerosis in humans, as seen in mice. Therefore, locally applied aspirin might be effective for treatment of bypass patients.

Endothelial cell loss occurs following grafting because of physical denudement and apoptosis of remaining damaged cells.6,30,31 In the present study, we found a marked loss of the endothelium in vein grafts within 3 days after surgery, which is not yet fully replaced by replicating endothelial cells. This indicates that certain surface areas of the vein graft lack a monolayer of endothelial cells for an initial period of time. Such exposure of the subendothelial matrix proteins to blood can be a risk for thrombus formation. Supporting this issue is the fact that about two thirds of the surface in vein grafts are covered by microthrombi at 24 hours and reduced thereafter. Thus, our findings of the presence of denuded endothelium, followed by massive microthrombus formation in vein grafts can explain why ≈10% of saphenous vein grafts in patients occlude in the first week.2,3,27

Thrombosis occurs when platelets adhere to collagen exposed after endothelial damage.10 In our murine vein graft model, microthrombi covered the surface of the vessel in the early stages, but fewer smaller thrombi were present from 4 weeks onward. Subsequently, we observed that blood-derived cells covered and infiltrated into the microthrombi of vein grafts within a week. These microthrombi were embedded between the media and regenerated endothelial and/or infiltrated blood cells. In this process, platelets within the microthrombi can release factors, such as platelet-derived growth factor, which promotes smooth muscle cell recruitment.11 Fibrin acts as a matrix for cell migration, such as for myofibroblasts and smooth muscle cells during the repair process.32,33 However, we should point out that our data did not provide direct evidence supporting the role of thrombosis in the development of neointimal lesions.

Aspirin has a range of pharmacological effects including endothelial protective, anti-inflammatory, analgesic, and antiplatelet effects.15 The anti-platelet effects involve reducing prostaglandin synthesis by inhibiting COX enzymes, which results in reduced thromboxane synthesis.34 Early studies of human aortocoronary-artery bypass patients indicated that aspirin treatment through oral administration treatment reduced graft occlusion.28,29 However, oral administration of aspirin has been shown to have no influence on neointimal
lesions in a primate model of vein bypass grafting and a mouse model of atherogenesis in apoE-deficient mice. The present data indicate that aspirin through the gastrointestinal pathway significantly reduces TXB2 concentrations in blood followed by a trend of reduction in thrombus and neointimal lesion formation, although it was not statistically significant. The different results for orally administered aspirin between mice and humans may be explained by different sensitivities to the drug or by the fact that the number of animals per group used was smaller than that of patients, and, hence, could not reach statistical significance. We may also point out that this study was not intended to compare oral and locally applied aspirin therapy for the long-term protection of the vascular bed. Moreover, an oral dosage of aspirin similar to that administered to humans results in ~70% and 90% reduction of blood TXB2 concentrations at 3 and 7 days, respectively, in mice. The degree of aspirin-reduced TXB2 levels in our study is comparable to the results observed in apoE-deficient mice by others, suggesting that the sensitivity of COX enzyme to aspirin in patients is higher than that of mice. On the other hand, locally applied aspirin has had marked effects on both thrombus and neointimal lesions, although a small number of animals were included in the group. It suggests that aspirin applied locally to vein grafts in mice is much more effective than when orally administered.

Previously, we demonstrated elevated mechanical stress results in vascular cell apoptosis in vein grafts in mice, in which PKCδ-p38 MAPK pathway-mediated oxidative damage is crucial for signal initiation. These damaged or dead endothelial cells were replaced by circulating progenitor cells. Grosser and Schroder found that aspirin can activate endothelial nitric oxide synthase to enhance NO production in cultured endothelial cells, thus, resulting in protection from oxidative damage. It is conceivable that locally applied aspirin can directly penetrate into the vessel wall (2 to 3 layers of cells), where it influences the balance between NO and other free radical generation within the vascular cells, and endothelial protection is achieved. In addition, sodium salicylates have a number of platelet-independent actions, including inhibition of NF-κB activation, adhesion molecule expression in endothelial cells, and vascular smooth muscle cell proliferation. In the present study, it is also possible for locally applied aspirin to be metabolized to salicylate, which exerts its role in reduction of neointimal lesions in vein grafts through inhibiting local inflammatory responses and smooth muscle proliferation.

In summary, thrombus formation due to endothelial damage is involved in the pathogenesis of vein graft arteriosclerosis. Aspirin applied locally to the graft resulted in endothelial protection and possibly anti-inflammatory effects, which lead to reduced thrombosis and neointimal proliferation. There is potential for aspirin to be used in a similar way to treat human vein graft arteriosclerosis and provide a much needed means of improving graft patency.

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