Platelets Activated by Collagen Through Immunoreceptor Tyrosine-Based Activation Motif Play Pivotal Role in Initiation and Generation of Neointimal Hyperplasia After Vascular Injury

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Background—Platelet adhesion on components of the extracellular matrix and platelet activation by those components are crucial for the arrest of posttraumatic bleeding, but they can also harm tissue by occluding diseased vessels. Recent studies have shown that the activation of platelets by collagen is mediated through the same pathway used by immune receptors, with an immunoreceptor tyrosine-based activation motif on the Fc receptor γ chain (FcRγ) playing a pivotal role.

Methods and Results—We examined the role of collagen-stimulated platelets in the development of injury-induced neointimal formation by using mice deficient in FcRγ. The left femoral arteries of 8- to 12-week-old FcRγ-deficient mice (n=16) and C57BL/6 (wild-type) mice (n=16) were injured by a straight spring wire (0.35-mm diameter). Segments of the injured and uninjured femoral arteries were excised at 7 days and 28 days after the vascular injury. Arterial segments were examined by immunohistochemistry and electron microscopy. Two hours after injury, electron microscopy showed marked decreases in platelet adhesion and neutrophil attachment to the vascular wall surface in FcRγ-knockout mice compared with wild-type mice. At 7 days after injury, staining with anti-neutrophil antibody showed fewer neutrophils in FcRγ-knockout mice than in wild-type mice. Computer-aided morphometry performed to measure the neointimal area, intima/media ratio, and stenotic area at 28 days after injury showed a significantly smaller ratio and area in FcRγ-knockout mice than in wild-type mice. At 28 days after injury, staining with anti-neutrophil antibody showed fewer neutrophils in FcRγ-knockout mice than in wild-type mice. Computer-aided morphometry performed to measure the neointimal area, intima/media ratio, and stenotic area at 28 days after injury showed a significantly smaller ratio and area in FcRγ-knockout mice than in wild-type mice (for neointimal area, 16.635±1.406 versus 31.483±2.309 μm², respectively; for intima/media ratio, 1.25±0.40 versus 2.68±0.04, respectively; and for stenotic area, 26.8±2.1% versus 49.3±4.1%, respectively).

Conclusions—These results demonstrate that FcRγ may play important roles in the initiation and generation of neointimal hyperplasia after balloon injury through the activation of platelets by collagen. (Circulation. 2002;105:912-916.)

Key Words: platelets ■ restenosis ■ immune system ■ collagen

Despite the advent of new devices, the principal factor limiting the long-term benefit of coronary angioplasty is still restenosis. Because intimal hyperplasia contributes to restenosis after vascular injury,1 it is particularly important to understand the molecular mechanisms involved in its development. In the earliest phase after vascular damage, platelets adhere to subendothelial collagen, thereby leading to platelet degranulation and aggregation.2 It is well established that platelet deposition and the subsequent leukocyte recruitment occur soon after vascular injury.3 However, there has been no clarification on the exact role of collagen-induced platelet activation and how it contributes to the subsequent smooth muscle cell proliferation and migration that result in intimal hyperplasia.

The purpose of the present study was to characterize the contribution of collagen-induced platelet activation to the lesion formation after vascular injury. Using mice deficient in the Fc receptor γ chain (FcRγ),4 we evaluated the role of collagen-stimulated platelets in the development of injury-induced neointimal formation. FcRγ constitutes an integral part of several Fc receptors and is coexpressed with glycoprotein (GP) VI, forming a platelet collagen receptor.5 Platelets from FcRγ-deficient (FcRγ−/−) mice lack GP VI; hence, they fail to respond to collagen.6 We produced a transluminal mechanical injury of the femoral artery from an exposed muscular branch in FcRγ−/− mice. This procedure can induce the rapid onset of medial cell apoptosis followed by reproduceable neointimal hyperplasia. This model also provides an
alternative to the unavailable GP VI–knockout (K/O) mouse, which has not yet been established.

Methods

Animals

FcRγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma

Arterial Injury

Transluminal mechanical injury of the femoral artery was induced directly to the neointima by using a method developed by Sata et al.8 Briefly, the animals were anesthetized with sodium pentobarbital (50 g/kg body wt IP), and the left or right femoral artery was exposed by blunt dissection. More than 5 mm of a straight spring wire (0.35 mm in diameter) was carefully inserted into the femoral artery toward the iliac artery from the exposed muscular branch artery. After the wire was left in place for 1 minute to denude and dilate the artery, the wire was removed, and the proximal portion of the muscular branch artery was secured by stitching with silk suture loops. The flow of the femoral artery remained and dilated the injured artery.

Transmission Electron Microscopy

The injured femoral arteries were examined after 2 hours for ultrastructural analysis as previously described.9 Briefly, each mouse was perfusion-fixed in 2% (vol/vol) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), and dissected arterial segments were postfixed for 16 hours in the same fixative. The tissue fragments were sliced at a thickness of ~200 μm by using a Vibratome (Meiwa Co), postfixed, and stained with tannin acid, in 5 to 10 minutes under physiological pressure via the left ventricle.10

Histology and Immunohistochemistry

Within 7 and 28 days after injury, the femoral arteries were fixed by perfusion in 20% phosphate-buffered formalin (pH 7.0) for 5 to 10 minutes under physiological pressure via the left ventricle.9 The embedded tissues were then stained with hematoxylin-eosin (H&E) for overall morphology or by elastica van Gieson (EV) for elastic filaments. We determined from each artery were examined, and the analysis system was computerized morphometry (KS400 software). Two sections obtained from each artery were examined.

Morphometry

Morphometric analysis was carried out on femoral arteries harvested 28 days after injury. All animals were perfusion-fixed under physiological pressure. Arterial specimens were blindly analyzed by computerized morphometry (KS400 software). Two sections obtained from each artery were examined, and the analysis system was used to measure areas enclosed by the EEL, IEL, and vessel lumen. The intima-media ratio and percentage of luminal narrowing were calculated.

Statistical Analysis

The Student t test was used to compare the values between FcRγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma Newly Formed Neointima

FcRγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma and Activation Motif in Arterial Injury

Platelets were retrieved from the femoral arteries of wild-type mice, and the platelet layer appeared to be composed of no more than a diffusely spread monolayer of platelets, with few leukocytes attached to the platelets.

Reduced Neutrophils and VSMCs Were Observed in FcRγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma Mice 7 Days After Injury

Seven days after injury, neointimal formation was observed. The neointimal cells and perivascular area in the wild-type mouse contained PMNs (Figure 1B). In contrast, few cells were detected in the neointimal and perivascular area of the FcRγγγγγγγγγγγγγγγγγγγγγγγγgamma mice (Figure 1B). To discriminate between neointimal and perivascular cells, we performed immunohistochemical staining with anti-neutrophil antibody. Anti-neutrophil antibody–stained cells appear in Figure 1B as cells with brown staining on the surface. FcRγγγγγγγγγγγγγγγγγγγγγγγγgamma mice had few PMNs in the neointimal and perivascular area. The neointima in wild-type and FcRγγγγγgamma mice contained few VSMCs positive for anti–α-actin antibody by immunohistochemical staining. In FcRγγγγγgamma mice, neointimal cells and neutrophils were both reduced, particularly the latter.

Neointimal Formation Was Suppressed in FcRγγγγγγγγγγγgamma Mice Within 28 Days After Injury

Twenty-eight days after injury, neointimal formation and luminal stenosis were observed. α-Actin staining confirmed that VSMCs were the main cellular component of intimal proliferative lesions. Anti-neutrophil antibody–stained cells were absent in the neointima and media. We stained the vessels with EV to calculate the area. The neointimal area, degree of stenosis, neointima/media (Ni/M) ratio, and degree of intimal hyperplasia (defined as any proliferative lesion within the IEL circumference) were calculated by KS400 software. The neointimal area, Ni/M ratio, and percent stenosis were significantly smaller in the FcRγγγγγγγgamma mice than in the wild-type mice for neointimal area, 16.635 ± 4.1% versus 31.483 ± 23.09 μm²; respectively; for the Ni/M ratio, 1.25 ± 0.40 versus 2.68 ± 0.04, respectively; and for percent stenosis, 26.8 ± 2.1% versus 49.3 ± 4.1%, respectively (Figure 2).

Discussion

In the present study, we investigated the specific role of collagen-stimulated platelet activation in injury-induced neointimal formation by using FcRγγγγγγγgamma mice. With a model of transluminal endothelial injury designed to simulate the vascular injury induced by percutaneous coronary interventions in humans, we observed that FcRγγγγγgamma mice were protected from developing intimal hyperplasia. The Ni/M ratio was reduced by 46% in FcRγγγγγgamma mice compared with wild-type mice. Two hours after guidewire-induced endothelial denudation of the femoral arteries of FcRγγγγγgamma mice, the platelet layer observed by electron microscopy appeared to be restricted to no more than a monolayer of platelets, many of which were less extensively spread along the surface, with few platelet-platelet interactions. In contrast to the results found for wild-type mice, there was a striking decrease in leukocyte attachment to the platelets of FcRγγγgamma mice.
Clearly, FcRγ is involved in the processes leading to cell proliferation associated with the neointimal formation that occurs after balloon injury. These data also strongly suggest the early involvement of inflammatory cells in mediating this effect, inasmuch as leukocyte accumulation was observed in the developing neointima of wild-type mouse femoral artery sections at 7 days after ligation, whereas very few leukocytes were observed in FcRγ−/− mice. A few platelets were attached to subendothelial cells, but few leukocytes were attached. The endothelial cells were denuded, and subendothelial basal laminas appeared (g through i). k indicates ×1000. B, Cross sections of mouse femoral arteries 7 days after transluminal wire injury. H&E staining of arterial segment of wild-type (WT) mouse revealed neointimal formation. Immunohistochemistry using anti-neutrophil antibody shows neutrophils in neointima and adventitia in wild-type mouse (original magnification ×20). There were also a few neutrophils in K/O arteries (original magnification ×20), but these were much scarcer than those found in wild-type mouse arteries. C, H&E and EV staining of arterial segment from wild-type and FcRγ−/− mice 28 days after injury. Neointimal formation was suppressed in FcRγ−/− mice compared with wild-type mice. Anti–SMA-positive cells were scarcer in FcRγ−/− mice than in wild-type mice. There were few anti-neutrophil antibody–stained cells in wild-type and FcRγ−/− mice 28 days after injury.

Evidence that GP VI is the major collagen receptor for platelet activation. Although genetically altered mice have considerable advantages for investigating the roles of specific molecules, a GP VI K/O strain of mice has not yet been established. Recent studies have shown that activation of platelets by collagen is mediated through the same pathway used by immune receptors, with an immunoreceptor tyrosine-based activation motif on the FcRγ playing a pivotal role. Moreover, the correct expression and function of mouse GP VI are strictly dependent on the presence of the FcRγ subunit. It has also been reported that platelets from FcRγ−/− mice lack GP VI and fail to respond to collagen. Thus, this model provides an alternative to the unavailable GP VI K/O mouse and helps to clarify the exact role of the collagen-
induced platelet activation in the generation of intimal hyperplasia after vascular injury.

FcRγ/− mice exhibited functionally impaired antibody-mediated responses and were shown not to express FcγRI, FcγRII, and FcεRI. We cannot completely rule out the possibility that FcRγ deficiency influences the cellular and humoral immunity and that such an influence is involved in this model. However, the exact role of antibody-mediated immune responses after balloon injury has not yet been elucidated. Furthermore, the previous reports using Rag-1 and apolipoprotein E K/O mice showed that T and B cells are triggered after PTCA and are believed to play an important role in restenosis. Very recently, Smyth et al reported that human GP VI is also physically and functionally associated with FcRγ subunit. These observations, combined with reports that human GP VI is also physically and functionally associated with FcRγ, suggest that GP VI and FcRγ are important therapeutic targets for cardiovascular disorders. Furthermore, it has recently been reported that stimulation of GP VI can shift GP IIb/IIIa from a low-affinity to a high-affinity state. Because the antagonists of GP IIb/IIIa have been shown to be efficient in preventing acute ischemic complications of percutaneous coronary interventions, our data are of potential therapeutic importance, raising the possibility that the addition of an antagonist to FcRγ and/or GP VI may provide additional protection against intimal hyperplasia and clinical restenosis.

References

Figure 2. Twenty-eight days after injury, FcRγ−/− mice, compared with wild-type mice, had significantly reduced neointimal area, Ni/M ratio, and stenosis (for neointimal area, 16 635±1406 versus 31 483±2309 μm², respectively; for Ni/M ratio, 1.25±0.40 versus 2.68±0.04, respectively; and for stenosis, 26.8±2.1% versus 49.3±4.1%, respectively).


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