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

Hakuoh Konishi, MD; Youichi Katoh, MD, PhD; Norihide Takaya, MD; Yuji Kashiwakura, MD; Seigo Itoh, MD, PhD; Chisei Ra, MD, PhD; Hiroyuki Daida, MD, PhD

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 (for neointimal area, 16.635±1406 versus 31.483±2309 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, 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. It is well established that they fail to respond to collagen. We produced a transluminal balloon injury model with 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), 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. Platelets from FcRγ-deficient (FcRγ−/−) mice lack GP VI; hence, they fail to respond to collagen. 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 reproducible neointimal hyperplasia. This model also provides an

Received September 11, 2001; revision received January 2, 2002; accepted January 7, 2002.
From the Department of Cardiology (H.K., Y. Katoh, N.T., Y. Kashiwakura, S.I., H.D.) and the Allergy Research Center (H.K., N.T.), Juntendo University School of Medicine, Tokyo, and the Department of Molecular Cell Immunology and Allergology (C.R.), Advanced Medical Research Center, Nihon University School of Medicine, Tokyo, Japan.
Correspondence to Youichi Katoh, MD, PhD, Department of Cardiology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail katoyo@med.juntendo.ac.jp
© 2002 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.02.105256
alternative to the unavailable GP VI–knockout (K/O) mouse, which has not yet been established.

**Methods**

**Animals**

FcRγ−/− mice were generated by a homologous recombination method, as previously described.6 FcRγ−/− (n=16) and wild-type (n=16) mice of the same age (8 to 12 weeks) and from the same genetic background were used in the present study.

**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 (Meiva Co), postfixed, and stained with tannin acid, in addition to uranyl acetate and lead citrate, to detect very thin filaments.

**Histology and Immunohistochemistry**

Within 7 days 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.10 The embedded tissues were then stained with hematoxylin-eosin (H&E) for overall morphology or by elastica van Gieson’s (EV) staining to depict the internal elastic lamina (IEL) and the external elastic lamina (EEL). Two sections of each artery were immunohistochemically stained. Vascular smooth muscle cells (VSMCs) were identified by staining with a mouse monoclonal antibody specific for α-smooth muscle actin (clone 1A4, Sigma). Polymorphonuclear leukocytes (PMNs) were identified by anti-mouse neutrophils (clone 7/4, Serotec).

**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 FcRγ−/− mice and control mice. Means were considered significantly different at a value of P<0.05.

**Results**

Transmission Electron Microscopy Showed Decreased Aggregation of Thrombocytes in FcRγ−/− Mice

Two hours after guidewire-induced endothelial denudation of femoral arteries of wild-type and FcRγ−/− mice, a layer of platelets that was several platelets thick appeared along the surface of the damaged vessels in the wild-type mice, with a few leukocytes attached to the platelets. In the FcRγ−/− mice, the platelet layer appeared to be composed of no more than a diffusely spread monolayer of platelets, with few leukocytes attached to the platelets (Figure 1A).

Reduced Neutrophils and VSMCs Were Observed in FcRγ−/− Mice 7 Days After Injury

Seven days after injury, neointimal formation was observed. The neointimal cells and perivascular area in the wild-type mice contained PMNs (Figure 1B). In contrast, few cells were detected in the neointimal and perivascular area of the FcRγ−/− mice (Figure 1B). To discriminate between neointimal cells 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. FcRγ−/− mice had few PMNs in the neointimal and perivascular area. The neointima in wild-type and FcRγ−/− mice contained few VSMCs positive for α-actin antibody by immunohistochemical staining. In FcRγ−/− mice, neointimal cells and neutrophils were both reduced, particularly the latter.

Neointimal Formation Was Suppressed in FcRγ−/− 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 FcRγ−/− mice than in the wild-type mice (for neointimal area, 16 635±1406 versus 31 483±2309 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 FcRγ−/− mice. With a model of transluminal endothelial injury designed to simulate the vascular injury induced by percutaneous coronary interventions in humans, we observed that FcRγ−/− mice were protected from developing intimal hyperplasia. The NI/M ratio was reduced by 46% in FcRγ−/− mice compared with wild-type mice. Two hours after guidewire-induced endothelial denudation of the femoral arteries of FcRγ−/− 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 FcRγ−/− 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 the FcRγ/−− mice.

Damage to the integrity of the vessel wall results in exposure of the subendothelial extracellular matrix, which then triggers the adhesion and aggregation of platelets. The consequence of this process is the formation of a thrombus that leads to subacute coronary occlusion or restenosis in diseased vessels after PTCA. In humans, there is growing 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, FcγRIII, and FcεRI.14 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 play only a minor role in atherosclerotic plaque formation, suggesting that impaired cellular and humoral immunity do not affect fibrous plaque formation or lesion size in mice fed a high-fat diet.13 Further studies using K/O mice with selective defects in FcRγ and GP VI will help to distinguish the relative contributions of these molecules in the generation of intimal hyperplasia after vascular injury. FcRγ may be a more effective target than GP VI itself for therapeutic use.

Leukocyte activation with platelet adherence, the release of inflammatory mediators, and other inflammatory reactions are triggered after PTCA14 and are believed to play an important role in restenosis. Very recently, Smyth et al15 reported that β3-integrin–deficient mice, a strain that lacks αIβIIIγ (GP IIb/IIIa) and αVβ3 integrin, demonstrated decreased platelet deposition but no protection from intimal hyperplasia after balloon injury. In contrast, the present study showed that FcRγ−/− mice were protected from the development of intimal hyperplasia after balloon injury. The major difference between β3-integrin–deficient mice and FcRγ−/− mice in the vascular response after balloon injury was the amount of leukocyte recruitment. Because the roles of several receptor pairs implicated in platelet-leukocyte interactions remain less clear in FcRγ−/− mice, further experiments should be conducted to investigate the influence of FcRγ−/− on the P-selectin expression16 of endothelial cells and platelets. In unpublished studies, we have confirmed that the P-selectin expression level on the surface of platelets from platelet-rich plasma from left ventricles increases 2 hours after femoral arterial injury in FcRγ−/− and wild-type mice, but we did not find any significant difference in the activating level of P-selectin between the 2 strains (data not shown). Nevertheless, the fact that FcRγ deficiency has been shown to result not only in decreased platelet deposition but also in protection from the development of intimal hyperplasia suggests that collagen-induced platelet activation and the subsequent leukocyte recruitment may play a pivotal role in the initiation and development of intimal hyperplasia.

Taken together, our results provide strong evidence that FcRγ plays a pivotal role in the generation of neointimal hyperplasia after balloon injury in mice, probably through collagen-induced activation of platelets and leukocyte recruitment. We used FcRγ−/− mice as an alternative to the unavailable GP VI K/O mice, because the correct expression and function of mouse GP VI are strictly dependent on the presence of the FcRγ subunit. These observations, combined with reports that human GP VI is also physically and functionally associated with FcRγ,17 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.18 Because the antagonists of GP IIb/IIIa have been shown to be efficient in preventing acute ischemic complications of percutaneous coronary interventions,19 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


Platelets Activated by Collagen Through Immunoreceptor Tyrosine-Based Activation Motif Play Pivotal Role in Initiation and Generation of Neointimal Hyperplasia After Vascular Injury
Hakuoh Konishi, Youichi Katoh, Norihide Takaya, Yuji Kashiwakura, Seigo Itoh, Chisei Ra and Hiroyuki Daida

_Circulation_. 2002;105:912-916
doi: 10.1161/hc0802.105256

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/8/912

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/