Deficiency of Interleukin-1 Receptor Antagonist Promotes Neointimal Formation After Injury

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Background—The cytokine interleukin (IL)-1 is an important mediator of inflammation and cardiovascular disease. Activity of this cytokine is modulated endogenously via the IL-1 receptor antagonist (IL-1Ra). The role of IL-1Ra in neointima formation after injury, however, is poorly understood.

Methods and Results—Using IL-1Ra−deficient (IL-1Ra−/−) backcrossed 8 generations into the C57BL/6J background and wild-type (IL-1Ra+/+) mice, we investigated neointimal formation 3 weeks after femoral artery injury induced by an external vascular cuff model. Intima and media thicknesses were measured, and the intima/media ratio was calculated. The mean intimal thickness and the intima/media ratio of IL-1Ra−/− mice increased by 249% (31.8±2.9 μm [n=10] versus 9.1±0.7 μm [n=10]; P<0.0001) and 257% (2.5±0.2 versus 0.7±0.1; P<0.0001), respectively, compared with IL-1Ra+/+ mice. No significant differences were observed in the medial thickness. Control immunostaining for IL-1Ra in injured vessels localized IL-1β and the endogenous inhibitor in the endothelium and inflammatory cells of the adventitia in IL-1Ra−/− but not IL-1Ra−/− mice.

Conclusions—The absence of IL-1Ra promotes neointimal formation in mice after injury. These results suggest that endogenous IL-1Ra may suppress other occlusive vascular responses to injury, such as atherosclerosis and restenosis after angioplasty. (Circulation. 2003;108:516-518.)

Key Words: genes ■ inflammation ■ interleukins

Interleukin (IL)-1 is a proinflammatory cytokine thought to play an important role in inflammation and atherosclerosis.1–2 Activity of this interleukin is counterregulated by its endogenous inhibitor IL-1 receptor antagonist (IL-1Ra).1–2 A recent study investigating expression of IL-1 and IL-1Ra transcripts in the vascular wall after mechanistic insult suggested the possibility that IL-1Ra may attenuate the biological function of IL-1β in this setting.3 However, direct evidence implicating endogenous IL-1Ra in neointimal formation remained yet to be provided. The present study definitively tested the hypothesis that IL-1Ra deficiency promotes intimal hyperplasia after arterial injury by using IL-1Ra–deficient mice.

Methods

Animals

IL-1Ra−deficient (IL-1Ra−/−) mice were generated in our laboratory by replacing the exons encoding the secreted form with the neo gene, as previously described.4 Embryonic stem cells were aggregated with 2 (C57BL/6J × DBA/2)F1 mice at the 8-cell stage. In these mutant mice, all 4 isoforms of the IL-1Ra were destroyed. These mice were backcrossed to C57BL/6J strain mice for 8 generations. Next, heterozygous mice were intercrossed with each other to obtain homozygous mutant mice. The studies were carried out according to the protocols approved by the National Defense Medical College Board for Studies in Experimental Animals.

Femoral Artery Injury

Mice (8 weeks of age) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and the left femoral artery was dissected from its surroundings, as described previously.3 Vascular injury was inflicted by placing a nonocclusive polyethylene cuff (length 2 mm; internal diameter 0.56 mm; Becton Dickinson) around the femoral artery.

Tissue Preparation and Histology

Subsequent to tail-cuff systolic blood pressure measurement, the animals were euthanized by pentobarbital injection and the vascular tree perfused with 0.9% NaCl followed by 4% paraformaldehyde. After perfusion, the femoral artery was harvested, fixed overnight in 4% paraformaldehyde, embedded in OCT compounds (Tissue-Tek; Sakura Finetechnical Co, Tokyo, Japan), and sectioned (10-μm thickness). All samples were routinely stained with hematoxylin-eosin and Masson’s trichrome, as well as by antigen-specific immunohistochemistry. Smooth muscle cells (SMCs) were visualized with α-SMC actin staining (Boehringer Mannheim), and anti–IL-1Ra (Santa Cruz Biotechnology) and anti–IL-1β antibodies (Gen-
zyme) were used to detect the respective proteins. Proliferating cell nuclear antigen (PCNA) staining (Santa Cruz Biotechnology) was performed to examine vascular proliferation.

**Morphometry**

Ten equally spaced cross sections were used in all mice to quantify intimal lesions. The luminal circumference, the circumference of internal elastic lamina, and the circumference of external elastic lamina were measured by using the NIH Image 1.55 (public domain software). Mean diameter was calculated as circumference/π. Mean intimal thickness was determined as (internal elastic lamina diameter – luminal diameter)/2 and mean medial thickness was calculated as (external elastic lamina diameter – internal elastic lamina diameter)/2.

**Enzyme-Linked Immunosorbent Assay**

Serum levels of IL-1β were determined by a sandwich ELISA, as previously described.6

**Statistical Analysis**

The results are shown as mean±SEM. The two groups were compared using Student's t test or Student-Newman-Keuls's test with the 1-way analysis of variance. P<0.05 was regarded as a significant difference.

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**Results**

Phenotypic analysis of the generated mutant mice revealed that animals homozygous for IL-1Ra deficiency showed diminished gain in weight4,7 (body weight at 12 weeks of age: IL-1Ra+/+ mice = 25.2±0.7g [n=6] versus IL-1Ra−/− mice = 31.1±1.1g [n=6]; P<0.01). Systolic blood pressures, however, were similar between the groups (IL-1Ra+/+ mice = 83.3±2.7 mm Hg [n=6] versus IL-1Ra−/− mice = 86.7±4.3 mm Hg [n=6]).

To assess the role of IL-1Ra in mediating intimal hyperplasia, we investigated the effect of cuff-induced injury to the femoral arteries in both IL-1Ra+/+ and IL-1Ra−/− mice at 8 weeks of age. Mice in both groups were euthanized at 3 (n=6), 7 (n=6), 14 (n=6), or 21 (n=10) days after placement of the cuff. At 3 days, although inflammatory cells were visible in the adventitia, no neointima was noted (data not shown). At 7 days, a modest degree of neointimal formation was noted (data not shown), whereas at 21 days after injury, a significant degree of neointimal hyperplasia was observed (Figure, A). These intimal formations in both groups of mice consisted predominantly of α-SMC actin-positive cells (Fig-
ure, A, bottom panels). The mean intimal thickness and the intima/media ratio of IL-1Ra−/− mice increased by 249% (31.8±2.9 versus 9.1±0.7 μm; P<0.0001; n=10) and 257% (2.5±0.2 versus 0.7±0.1; P<0.0001) in comparison with the IL-1Ra+/+ mice. There was no significant difference in medial thickness (12.6±0.4 versus 12.5±0.5 μm).

To examine whether IL-1Ra deficiency affected cell proliferation, we next performed PCNA staining in the arteries of both groups of mice. In both groups of mice, PCNA-positive cells in the adventitia, media, and intima were observed within 3 to 7 days after cuff placement. We thereafter determined the PCNA index at 7 days. More nuclei of both intima and adventitia of IL-1Ra−/− mice stained positively for PCNA than those of IL-1Ra+/+ mice (Figure, B), and thus IL-1Ra−/− mice displayed a 110% (30.8±2.5 versus 14.6±1.4; P<0.001; n=6) and 862% (35.6±3.9 versus 3.6±0.7; P<0.001; n=6) increase in the PCNA index of the intima and adventitia.

Furthermore, we localized the IL-1Ra and IL-1β protein in the injured vessels. Immunohistochemical analysis in wild-type mice revealed that both the cytokine and its endogenous inhibitor predominantly localized in the endothelium and in inflammatory cells of the adventitia. The expression pattern of IL-1β did not differ between IL-1Ra+/+ and IL-1Ra−/− mice. Moreover, serum levels of IL-1β were comparable between IL-1Ra+/+ and IL-1Ra−/− mice at all time points tested (data not shown). As expected, the IL-1Ra protein was not detected in IL-1Ra−/− mice (Figure, C).

**Discussion**

Neointimal hyperplasia is characterized by SMC activation, migration, and proliferation and is associated with inflammatory mediators such as cytokines. IL-1β is a chemoattractant and mitogen for SMCs that is overexpressed at sites of active proliferation and migration of this cell type subsequent to injury. Furthermore, Rector et al previously demonstrated that IL-1 type I receptor gene–deficient mice tended to develop less neointima than wild-type mice, an observation attributed to diminished shear stress. In sum, these previous studies suggested that IL-1 might promote neointimal formation. However, whether IL-1Ra, the endogenous inhibitor of this central cytokine, could significantly suppress this response of the vasculature to injury remained uncertain. The present study demonstrated definitively that deficiency of endogenous IL-1Ra promotes neointimal formation, revealing a crucial role for this protein in hyperplastic responses of the vasculature. Of note, the differences in intimal thickening between IL-1Ra+/+ and IL-1Ra−/− mice were observed despite the comparable expression pattern of IL-1β in the arterial wall and similar serum levels of the cytokine.

Four isoforms of IL-1Ra are derived by alternative splicing from a single gene, yielding 1 secreted form and 3 intracellular proteins. All 4 isoforms can inhibit IL-1 activity. Of note, the IL-1Ra−/− mice generated for the present study lacked all 4 isoforms. Accordingly, IL-1Ra protein was not detected in these mice, even after injury. These results suggest that IL-1Ra protein prevents inflammation of both the intima and adventitia after cuff injury. Indeed, IL-1Ra−/− mice showed an increase in the PCNA index of the intima and adventitia after injury. Within the adventitia, proliferating monocytes and macrophages comprised the majority of PCNA-positive cells. Recent studies have shown that adventitial passive fibroblasts can become active myofibroblasts under conditions of adventitial inflammation. On the other hand, SMCs were the predominant proliferating cell type in the intima. IL-1 itself is mitogen for SMCs, and furthermore, a recent study showed that vascular intima formation after mechanical injury was found to consist mainly of inflammation-associated cells that originated from the bone marrow. As a result, IL-1Ra reduced the inflammation in both the intima and adventitia while also inhibiting neointimal formation to a greater extent than in IL-1Ra−/− mice.

The present study is the first to demonstrate that IL-1Ra plays an important role in the suppression of neointimal formation after injury in vivo, thus suggesting that IL-1Ra supplementation may represent a useful strategy to inhibit neointimal formation after angioplasty and atherosclerosis.

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**References**

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