Interleukin-10 Inhibits Intimal Hyperplasia After Angioplasty or Stent Implantation in Hypercholesterolemic Rabbits

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Background—Intimal hyperplasia after stent implantation is the main cause of in-stent restenosis. Activated monocytes play a key role in intimal growth. The anti-inflammatory cytokine interleukin-10 (IL-10) is a potent monocyte deactivator, endogenously produced in the atherosclerotic plaque. We tested the hypothesis that exogenous IL-10 may limit postangioplasty intimal hyperplasia after balloon angioplasty or stenting.

Methods and Results—Hypercholesterolemic rabbits were treated with recombinant human IL-10 (rhuIL-10) for 3 days after balloon angioplasty or 28 days after stent implantation. High IL-10 serum levels and intense deactivation of circulating monocytic cells, assessed by inhibition of IL-1β release by lipopolysaccharide-stimulated whole blood, were detected for at least 8 hours after rhuIL-10 intravenous injection (ELISA). Morphometric analyses, performed 28 days after injury, indicated that rhuIL-10 reduced intimal growth by ~50% after balloon angioplasty or stenting, resulting in more preserved lumen in stented arteries. Moreover, rhuIL-10 reduced macrophage infiltration by 67% and proliferative activity by 81% in the intima and the media. No toxic effect was detected except minor changes in blood cell count.

Conclusions—The anti-inflammatory cytokine rhuIL-10 reduces postinjury intimal hyperplasia. The potent attenuation of in-stent intimal growth by rhuIL-10 and its favorable toxicity profile suggest that rhuIL-10 may be useful in the prevention of in-stent restenosis. (Circulation. 2000;101:908-916.)

Key Words: interleukins • stents • restenosis

Intimal hyperplasia is a universal response of the arterial wall to balloon injury1 and the main mechanism of restenosis after stent implantation,2 the technique used for the majority of percutaneous coronary interventions.3 Although stents mitigate the risk of restenosis in selected coronary artery lesions, in-stent restenosis is still a frequent and often intractable clinical problem.4 Therefore there is a need for efficient treatment to reduce in-stent intimal hyperplasia.

Inflammatory cells play a key role in postinjury intimal hyperplasia. In the days after balloon angioplasty5 or stenting,6 monocytes are recruited at the injury site, where they become activated macrophages. The magnitude of macrophage infiltration in stented lesions is correlated to subsequent intimal growth.7 Moreover, in patients with coronary artery disease, the presence of activated circulating monocytes before angioplasty predicts more severe late luminal loss.8 Altogether, these data suggest that deactivation of circulating monocytes may limit postinjury intimal hyperplasia and in-stent restenosis.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine with a powerful inhibitory effect on monocytes.9-11 We studied the effect of recombinant human IL-10 (rhuIL-10) on intimal growth, after angioplasty or stent implantation, in hypercholesterolemic rabbits.

Methods

Pharmacokinetics of rhuIL-10

The pharmacokinetics of rhuIL-10 (kindly provided by Schering-Plow) were studied in 6 balloon-injured rabbits (see below). Two-milliliter blood samples were collected before, 20 minutes, 2 hours, 6 hours, and 8 hours after the first intravenous injection of 50 mg rhuIL-10 (n=3) or saline (n=3). Blood samples were immediately centrifuged and serum was kept at −80°C until analysis. Serum IL-10 levels were measured in duplicate by the use of standard ELISA methods (Immunotech).

Effect of rhuIL-10 on Lipopolysaccharide-Induced IL-1β Release by Whole Blood Ex Vivo

To assess the inhibitory effect of rhuIL-10 on circulating leukocytes, lipopolysaccharide (LPS)-induced interleukin-1β (IL-1β) release was measured in whole blood, as described.12 One-milliliter blood samples were collected from the 6 above-mentioned animals at the same time points. Whole blood was diluted 1:5 in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from

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Gilco BRL). Two milliliters of diluted blood was incubated in culture wells for 24 hours at 37°C in a 5% CO₂ atmosphere, with or without 100 ng/mL LPS (Sigma), then centrifuged at 1800 rpm for 10 minutes. The supernatant was kept at −80°C until analysis. IL-1β levels were measured in duplicate with the use of an ELISA kit (Genzyme). Results were expressed as percent IL-1β increase in LPS-stimulated versus unstimulated blood, a parameter that depends on the activation status of leukocytes but not on leukocyte count.

Cross-Reactivity of Human IL-10 for Rabbit Leukocytes

To compare the reactivity of rabbit versus human leukocytes to rhuIL-10, additional 1-mL blood samples were collected from 3 rabbits before the first rhuIL-10 injection as well as from 3 healthy volunteers. IL-1β release over 24 hours was measured as above, after stimulation of whole blood by LPS (100 ng/mL), with or without 30-minute preincubation with increasing concentrations of rhuIL-10. Results are expressed as percent IL-1β increase over unstimulated blood.

Models of Intimal Hyperplasia

Animal protocols were approved by Faculté Bichat Institutional Animal Care and Use Committee. Male New Zealand White rabbits, weighing 3.7 to 4.1 kg, were fed a 1% cholesterol diet, started 14 days before angioplasty.

Thirty-four rabbits were studied. Animals were anesthetized with intravenous pentobarbital, and a 5F sheath was inserted in the right carotid artery. Intimal hyperplasia was induced by balloon angioplasty alone (n=18) or followed by stent implantation (n=16). Arterial blood was drawn before angioplasty and at euthanasia to study the effect of IL-10 on lipid metabolism, blood cell count, and standard biology parameters. Bilateral iliac artery angioplasty was performed with a 2.5-mm-diameter, 20-mm-long angioplasty balloon catheter (3×1-minute inflation at 8 atm). Balloon diameter was chosen to approximate a balloon-to-artery ratio at 1 to 1.1. In 16 animals, a 15-mm-long Palmaz-Schatz metallic stent (Johnson-Johnson Interventional Systems Co), mounted over the balloon, was implanted in both iliac arteries immediately after balloon angioplasty (30-second inflation at 10 atm), resulting in frank arterial overstretch (1.2 to 1.3 stent-to-artery ratio). All animals received intravenous heparin (1000 IU IV) before angioplasty. Aspirin was given only to stented animals (50 mg intravenous injection before angioplasty, then 50 mg daily in the drinking water).

IL-10 Regimen

Fifty micrograms of rhuIL-10 was injected intravenously 30 minutes before angioplasty, then 3 times daily for 72 hours. In stented animals, additional rhuIL-10 (50 μg) was given subcutaneously every other day until the animals were killed because of sustained inflammatory stimulus evoked by metallic stents.2,6 Seventeen animals, injured with balloon angioplasty (n=9) or stenting (n=8), were treated with rhuIL-10. Control animals (balloon angioplasty: n=9, stent: n=8) received saline after the same injection protocol as above.

Figure 1. Pharmacokinetics of rhuIL-10. IL-10 serum levels were measured serially after intravenous injection of 50 μg rhuIL-10 (b) or saline (f). *P<0.005 vs saline.

Figure 2. Inhibitory effect of rhuIL-10 on LPS-induced IL-1β release by whole blood ex vivo. LPS-induced IL-1β release is profoundly and rapidly inhibited after intravenous injection of 50 μg rhuIL-10 (b) vs saline (f). *P<0.005 vs saline.

Tissue Harvest and Histology Processing

Twenty-eight days after arterial injury, rabbits were euthanized by pentobarbital overdose. Both iliac arteries were perfusion-fixed with 4% paraformaldehyde at physiological pressure. In balloon-injured arteries, 4 serial 5-mm-long rings, corresponding to the angioplasty site, were cut and embedded in paraffin. Eight 5-μm sections were cut from each ring, stained with hematoxylin/eosin or orcein, and examined through a light microscope (Laborlux S, Leitz).

Stented arteries were impregnated for 24 hours in 80% methyl methacrylate and 20% dibutyl phthalate (all from Merck), as described.13 One percent benzoyl peroxide (Merck) was added, and the arteries were placed in glass tubes. Polymerization of methyl methacrylate was induced by overnight incubation in a 32°C oven. Five-micron arterial sections were cut with tungsten carbide knives, mounted on a HMM55 motor-driven microtome (Microm), and stained with hematoxylin/eosin, Masson trichrome, or orcein. In each artery, 12 sections were taken from 3 levels (4 sections from each end and 4 sections from the middle segment).

Morphometric Analyses

Digital planimetry of orcein-stained arterial sections was performed with the use of a video camera (IEC 800 CC, 125 Inc) mounted on the microscope and an interfaced computer-assisted image quantification program (Ereslib, ERESI). Measurements of the luminal area as well as the 2 areas bounded by the internal and external elastic laminae served to compute intimal and medial areas. Three indexes of intimal growth were used: (1) intimal area; (2) ratio of intimal to medial areas; and (3) ratio of the intimal area to the area bounded by the internal elastic lamina (luminal cross-sectional area narrowing).

In stented arteries, an injury score was calculated as described.14

Immunohistochemistry

Immunohistochemical staining was performed on adjacent sections taken from the 2 central arterial rings of balloon-injured arteries (4 sections for each monoclonal antibody). Arterial sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase, then with monoclonal mouse antibodies directed against
Inhibitory Effect of rhuIL-10 on LPS-Induced IL-1β Release by Whole Blood

Percent increase of IL-1β release in LPS-stimulated versus unstimulated whole blood (Figure 2) was similar at baseline in animals treated with rhuIL-10 (237%) or saline (241%). However, as early as 2 hours after rhuIL-10 injection, LPS-induced IL-1β release was significantly reduced to 64% and continued to decline thereafter, down to 9% at 8 hours.

Cross-Reactivity of Human IL-10 for Rabbit Leukocytes

LPS-induced IL-1β release was similarly and dose-dependently abrogated by rhuIL-10 in rabbit and human whole blood (Figure 3), although there was a trend toward more profound inhibition in humans.

Effect of rhuIL-10 on Balloon Angioplasty–Induced Intimal Hyperplasia

Concentric intimal hyperplasia developed after balloon angioplasty in control animals (Figure 4). Lipid-laden foam cells accumulated both in the intima and the media, whereas a fibroproliferative reaction was visible in the superficial layers of the intima, as previously reported. Analysis of immunostainings for RAM-11 and α-actin on adjacent arterial sections revealed that the vast majority of foam cells in the intima were of macrophage origin, whereas in the media some of the foam cells stained for α-actin and thus were of smooth muscle origin (data not shown). Morphometric analyses (in each group: n=9 animals, 18 arteries, 144 sections) indicated that intimal growth—expressed either as intimal area, intima/media ratio, or luminal cross-sectional area narrowing—was significantly reduced by rhuIL-10 (by 50%, 71%, and 51%, respectively), resulting in nonsignificant improvement of luminal area.

Effect of rhuIL-10 on Stent-Induced Intimal Hyperplasia

Stent implantation resulted in severe medial compression and stretching (Figure 5). Frequent internal elastic lamina disruption and medial laceration by stent struts were observed. Arterial wall injury was similar in the rhuIL-10 and control
groups, based on measurement of arterial cross-sectional area (4.3±0.6 and 4.6±0.3 mm², respectively, \( P=\text{NS} \)), a marker of arterial stretching, and arterial injury score (1.1±0.6 and 1.2±0.6, respectively, \( P=\text{NS} \)).

In the control group, intimal hyperplasia developed predominantly around stent struts and consisted of a loose connective tissue massively infiltrated with foam cells and covered with a fibromuscular cap. The intimal area was significantly larger in control stented arteries than in control balloon-injured arteries (1.6±0.3 vs 0.6±0.3 mm², \( P=0.0001 \)). The intima/media ratio positively correlated to the injury score (\( r=0.66, P=0.01 \)).

Neointimal structure was dramatically modified by rhuIL-10, both quantitatively and qualitatively. Morphometric analyses (in each group: \( n=8 \) animals, 16 arteries, 96 sections) indicated a significant reduction of intimal growth in rhuIL-10-treated animals (by 50%, 48%, and 43%, for intimal area, intima/media ratio, and luminal cross-sectional area narrowing, respectively). The limitation of intimal growth by
rhuIL-10 in stented arteries resulted in significantly larger luminal area. Moreover, rhuIL-10 treatment was associated with a thin fibrocellular neointima and markedly reduced foam cell infiltration.

Effect of rhuIL-10 on Inflammatory Cell Infiltration and Proliferative Activity

Inflammatory cells infiltrating balloon-injured arteries were almost exclusively of macrophage origin. Treatment with rhuIL-10 resulted in dramatic reduction of total (≈67% reduction) as well as percentage of (≈78% reduction) macrophage infiltration 28 days after balloon injury (Figure 6). In contrast, CD43-positive cells were only occasionally observed in the intima and the adventitia, both in rhuIL-10–treated (intima: 3.3 ± 2 cell/mm²; adventitia: 2.7 ± 1.5 cell/section) and control (intima: 2.7 ± 2.3 cell/mm²; adventitia: 3.7 ± 2.5 cell/section; both P = NS vs rhuIL-10 group) rabbits (Figure 7). Proliferative activity, indicated by an MIB1/Ki-67

Figure 5. Intimal hyperplasia 28 days after stent implantation. A and B, Photomicrographs (Masson’s trichrome, ×10 magnification) of arterial sections from rabbits treated with saline (A) or rhuIL-10 (B). Note in the saline-treated rabbit (1) massive intimal infiltration with foam cells (arrowheads), predominantly around stent struts (filled rectangle); and (2) superficial fibromuscular cap (red/green). In contrast, the rhuIL-10–treated rabbit has a thin, fibromuscular intima (red/green) with minimal foam cell infiltration around stent struts (empty rectangle). Arrow indicates internal elastic lamina. M indicates media; I, intima; and L, lumen. C, Bar graph showing reduced intimal area, intima/media ratio, and luminal cross-sectional area narrowing (intima/iel) in rhuIL-10–treated (hatched bars) vs control (black bars) rabbits, resulting in significant improvement of luminal area. iel indicates internal elastic lamina; eel, external elastic lamina. *P = 0.01; †P = 0.0001.
nuclear stain, was scarce in the intima and virtually absent in the media of control arteries (Figure 8). When present, foci of proliferative cells were located predominantly in the outermost layers of the intima. Analysis of adjacent sections stained for RAM-11 suggested that most proliferative cells were macrophages. Total as well as percentage of MIB1/Ki-67–positive area were smaller in rhuIL-10–treated animals (81% and 71% reduction, respectively).

Cholesterol Levels and Toxicity
There was no apparent toxicity of rhuIL-10 on renal and liver functions (Table). Minor although significant hematologic changes were observed, including mild leukocytosis, monocytosis, and neutrophilia as well as relative lymphopenia, consistent with previous reports. No overt infection was observed in rhuIL-10–treated rabbits. Total and HDL cholesterol and triglyceride levels were not modified by rhuIL-10.

Discussion
The main findings of the present study are that (1) systemic administration of the anti-inflammatory cytokine rhuIL-10 successfully inhibits intimal hyperplasia after balloon injury or stent implantation in hypercholesterolemic rabbits; (2) this protective effect is associated with a major inhibition of IL-1β release by circulating leukocytes and reduced infiltration of the arterial wall by activated macrophages; and (3) rhuIL-10 has no apparent effect on lipid metabolism and no systemic toxicity in this animal model.

Inflammation plays a key role in the development of primary atherosclerotic lesions. After balloon angioplasty, additional inflammatory cells are recruited as part of the tissue repair phenomenon. Experimental and clinical studies suggest that postangioplasty inflammation and intimal hyperplasia are even greater after stent implantation. Therefore inflammation is instrumental in the development of
in-stent neointimal hyperplasia, and anti-inflammatory agents may be useful to prevent in-stent restenosis.

IL-10 is an anti-inflammatory cytokine endogenously expressed in the human atherosclerotic plaque, with potent inhibitory effects on proinflammatory cytokine synthesis by activated monocytic cells. In addition to being a monocyte deactivator, IL-10 may be protective against restenosis by several other functions, including inhibition of cell adhesion molecules, monocyte chemoattractant MCP-1, tissue factor, fibrinogen, metalloproteinase, T-lymphocyte granulocyte-macrophage colony-stimulation factor, inducible nitric oxide synthase, and smooth muscle cell proliferation.

In the present study, rhuIL-10 reduced intimal hyperplasia by almost 50% after balloon angioplasty or stent implantation, resulting in more preserved arterial lumen in stented arteries. Several lines of evidence suggest that the protective effect of rhuIL-10 is mediated, at least in part, by mononuclear cell infiltration.

Figure 7. T-lymphocyte infiltration 28 days after balloon injury. CD43-immunostained arterial sections (×100 magnification) from rabbits treated with saline (A) or rhuIL-10 (B, C). In both groups, only rare T-lymphocytes (brown membrane stain, large arrow) are found in the intima (A, B) and the adventitia (C). Arrow indicates internal elastic lamina. Arrowhead indicates external elastic lamina. A indicates adventitia; M, media; I, intima; and L, lumen.
deactivation. First, rhuIL-10 inhibited IL-1β release by blood cells, consistent with previous reports. Because mononuclear cells represent the main source of IL-1β in whole blood and ∼80% of circulating leukocytes in rabbits, it can be inferred that rhuIL-10 is a potent deactivator of mononuclear cells in the rabbit model. Second, rhuIL-10 reduced macrophage infiltration at the angioplasty site by 67%. Finally, rhuIL-10 reduced residual macrophage proliferative activity by 81%.

These results, however, should be interpreted with caution. The single injury model lacks the atherosclerotic substrate on which intima develops. Also, the 1% cholesterol diet results in extremely high total cholesterol levels, primarily because of an increase in β-VLDL, not LDL, rich in proinflammatory oxidized phospholipids. Thus extrapolation of our data to human restenosis deserves further studies. Finally, the efficacy of a 72-hour course of rhuIL-10 was demonstrated only in balloon-injured arteries, whereas stented animals received rhuIL-10 for 28 days. Whether a shorter treatment would successfully mitigate in-stent intimal growth remains to be investigated.

In conclusion, on the basis of recent evidence that the inflammatory process in the atherosclerotic plaque may be regulated by a balance between proinflammatory and anti-inflammatory cytokines, our data indicate that systemic administration of the anti-inflammatory cytokine rhuIL-10 reduces arterial inflammation and intimal growth after arterial injury. The potent attenuation of in-stent lumen loss by rhuIL-10 and its favorable toxicity profile suggest that rhuIL-10 may be useful therapeutically to prevent in-stent restenosis.

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