Neutralization of Interleukin-18 Inhibits Neointimal Formation in a Rat Model of Vascular Injury

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Background—Studies in humans and animal models suggest that interleukin-18 (IL-18) plays a crucial role in vascular pathologies. IL-18 is a predictor of cardiovascular death in angina and is involved in atherotic plaque destabilization. Higher IL-18 plasma levels also are associated with restenosis after coronary artery angioplasty performed in patients with acute myocardial infarction. We investigated the effective role of IL-18 in neointimal formation in a balloon-induced rat model of vascular injury.

Methods and Results—Endothelial denudation of the left carotid artery was performed by use of a balloon embolectomy catheter. Increased expression of IL-18 and IL-18Rα/β mRNA was detectable in carotid arteries from days 2 to 14 after angioplasty. The active form of IL-18 was highly expressed in injured arteries. Strong immunoreactivity for IL-18 was detected in the medial smooth muscle cells at days 2 and 7 after balloon injury and in proliferating/migrating smooth muscle cells in neointima at day 14. Moreover, serum concentrations of IL-18 were significantly higher among rats subjected to vascular injury. Treatment with neutralizing rabbit anti-rat IL-18 immunoglobulin G significantly reduced neointimal formation (by 27%; \( P < 0.01 \)), reduced the number of proliferating cells, and inhibited interferon-γ, IL-6, and IL-8 mRNA expression and nuclear factor-κB activation in injured arteries. In addition, in vitro data show that IL-18 affects smooth muscle cell proliferation.

Conclusions—These results identify a critical role for IL-18 in neointimal formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in the reduction of neointimal development. (Circulation. 2006;114: 430-437.)

Key Words: angioplasty ■ balloon ■ carotid arteries ■ interleukins
congestive heart failure. Furthermore, an epidemiological study has suggested that IL-18 can predict cardiovascular death in patients with stable and unstable angina. Interestingly, Yamagami et al demonstrated an association between higher serum IL-18 levels and greater carotid intima-media thickness in patients without histories of cardiovascular accidents. Moreover, Kawasaki et al showed that higher IL-18 plasma levels were associated with restenosis after emergency coronary angioplasty performed in patients with acute myocardial infarction.

To date, however, the expression and function of IL-18 in balloon-induced neointimal formation have not been investigated. This is of particular relevance because it is well established that long-term failure of arterial stenting is due to neointimal formation, whereas a combination of arterial remodeling and SMC proliferation is responsible for restenosis after balloon angioplasty in humans. In addition, recent studies have provided strong evidence for an important role of IL-18 in SMC proliferation and migration in vitro.

The aims of the present study were to evaluate the expression of IL-18 and its related receptor, to evaluate the distribution of the IL-18 active form in rat carotid arteries subjected to vascular injury, and to assess the relationship between IL-18 neutralizing and neointimal formation in damaged arteries.

The results provided in this study strongly support a prominent role for IL-18 in the local immune/inflammatory processes leading to neointimal formation.

**Methods**

**Animals**

Male Wistar rats (Harlan Italy, Udine, Italy) weighing 250 g were used for the present study. Rats were housed at the Department of Experimental Pharmacology, University of Naples. We performed all procedures according to Italian and European regulations on the protection of animals used for experimental and other scientific purposes.

**Balloon Angioplasty**

Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) (Gellini International, Latina, Italy) and xylazine (5 mg/kg) (Sigma, Milan, Italy). Endothelial denudation of the left carotid artery was performed with a balloon embolectomy catheter (2F, Fogarty, Edwards Lifesciences, Milan, Italy) according to the procedure well validated in our laboratory. Naive animals were used as control. Some animals were subjected to anesthesia and surgical procedure without balloon injury (sham-operated rats). Rats were euthanized 0, 4, and 24 hours and 2, 7, and 14 days after vascular injury, and carotid arteries were removed and processed as described below.

**Morphology**

Carotid arteries were fixed by perfusion with 100 mL phosphate-buffered saline (PBS; pH 7.2), followed by 80 mL PBS containing 4% paraformaldehyde through a large cannula placed in the left ventricle. Paraffin-embedded sections were cut (6 μm thick) from the approximate middle portion of the artery and stained with hematoxylin and eosin to demarcate cell types and picrosirius red viewed with polarized light to detect collagen. Ten sections from each carotid artery were reviewed and scored under blinded conditions. The cross-sectional areas of media and neointimal and collagen content were determined by a computerized analysis system (Leica, Milan, Italy).

**IL-18 and Anti–α-Smooth Muscle Actin Immunohistochemistry**

Carotid arteries were snap-frozen in liquid nitrogen in OCT embedding medium (Tissue Tek, Sakura FineTek Europe, Zoeterwoude, the Netherlands) and stored at −80°C. Ten cross sections were cut (6 μm) from the approximate middle portion of the artery and used for IL-18 and anti–α-smooth muscle actin (anti–α-SMA) detection by immunofluorescence. For staining, sections were fixed in acetone for 10 minutes, air dried, and rehydrated with PBS before incubation in serum-free Protein Block (DakoCytomation, Milan, Italy) for 30 minutes. To detect IL-18, sections were stained with 15 μg/mL anti-rat IL-18 antibody (goat IgG, R&D Systems, Minneapolis, Minn) diluted in 1% blocking reagent (Perkin Elmer, Milan, Italy)/0.3% Triton X-100 (MP Biomedicals, Verona, Italy) in PBS overnight before being washed in TNT wash buffer (Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.05% Tween 20, Sigma). Sections incubated with an isotype-matched control antibody were used as negative control. Subsequently, sections were incubated with 1/75 Texas Red-donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, Soham, United Kingdom) for 30 minutes before washing. Monoclonal anti–α-SMA fluorescein isothiocyanate conjugate (1/250, clone 1A4, Sigma) was added in blocking buffer for 1 hour before washing as described above. Dapi was used to identify nuclei. Images were taken by an AxioCam HRC video-camera (Zeiss, Milan, Italy) connected to an Axioskop fluorescent microscope (Zeiss) using the AxioVision 3.1 software.

Neointimal α-SMA–positive cell numbers were determined by counting all nucleated cells with fluorescein isothiocyanate fluorescence present on a carotid section. For each group studied, carotid arteries obtained from 5 different rats per group were analyzed. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blinded conditions.

**Proliferating Cell Nuclear Antigen Analysis**

Proliferating cell nuclear antigen (PCNA) analysis was used to quantify the proliferative activity of cells at the balloon injury sites, and it was performed using monoclonal mouse anti-PCNA antibody (1/1000, PC10, Sigma) and biotinylated anti-mouse secondary antibody (1/1000, DakoCytomation). Sections incubated with an isotype-matched control antibody were used as negative control. Slides were treated with avidin-biotin block and exposed to diamobenzidine chromagen with hematoxylin counterstain. For each group studied, carotid arteries obtained from 5 different rats were analyzed. Six sections from each carotid artery and 10 fields per section were reviewed and scored under blinded conditions. Data are represented as percentage of total medial and neointimal cells positive for PCNA 7 days after angioplasty.

**Enzyme-Linked Immunosorbent Assay**

Serum IL-18 levels (2, 7, and 14 days after angioplasty) were measured by ELISA. Briefly, 96-well plates (Maxisorb, Nunc Immunomax, Roskilde, Denmark) were coated with anti-rat IL-18 antibody (1 μg/mL in 0.1 mol/L NaH2CO3, R&D Systems) overnight at 4°C and blocked, and serial dilution of sera was added. Bound IL-18 was detected with 1/2000 polyclonal rabbit anti-goat immunoglobulins/biotinylated (DakoCytomation), followed by 1/200 streptavidin-peroxidase polymer (Sigma), and developed with tetramethylbenzidine substrate (Sigma). Plates were read at 630 nm. The results are expressed as picogram per milliliter.

**Total RNA Isolation and Complementary DNA Reverse Transcription**

Total RNA was isolated from the carotid arteries with TRIzol (Invitrogen, Milan, Italy). Briefly, the carotid arteries (n=3 per group) were frozen in liquid nitrogen, pooled, crushed into powder in a mortar with a pestle, transferred to a microcentrifuge tube, and immediately suspended in TRIzol. Subsequently, the samples were processed to obtain total RNA that was reverse transcribed into complementary DNA as previously described.
Reversal-Transcription Polymerase Chain Reaction of IL-18, IL-18Ra/β, IFN-γ, IL-6, and IL-8

The levels of IL-18, IL-18Ra/β, IFN-γ, IL-6, and IL-8 mRNA were evaluated with polymerase chain reaction (PCR) amplification of reverse-transcribed mRNA. Parallel amplification of rat housekeeping gene β-actin was performed as internal control. The PCR primer set and conditions are available on request. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Real-Time PCR of IFN-γ, IL-6, and IL-8

Expression of IFN-γ, IL-6, and IL-8 was examined by real-time PCR performed with SYBR green dye (QuantiTect SYBR Green PCR kit, Qiagen, Calif.) and a Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif.). The PCR primers set and conditions are available on request. The relative quantification of target genes mRNA expression was calculated with the standard curve method (Applied Biosystems) and normalized to an endogenous control (β-actin). The relative values were then standardized to a calibrator and expressed as percentage of mRNA expression. PCR-amplified products also were electrophoresed on 2% agarose gels to confirm that single bands were amplified.

Preparation of Cytosolic and Nuclear Extracts

All the extraction procedures were performed on ice with ice-cold reagents as previously described. Briefly, carotid arteries crushed into powder as described above were resuspended in adequate volume of hypotonic lysis buffer and chilled on ice for 5 minutes. Then, the homogenates were vigorously shaken for 15 seconds in the presence of 100 μL of 5% Nonidet P-40 and incubated on ice for 15 minutes. The nuclear fraction was precipitated by centrifugation at 1500g for 10 minutes, and the supernatant, which contained the cytosolic fraction, was removed and stored at −80°C. The nuclear pellet was resuspended in an adequate volume of high-salt extraction buffer and incubated with shaking at 4°C for 30 minutes. The nuclear extract was then centrifuged for 15 minutes at 13 000g, and the supernatant was placed in aliquots and stored at −80°C. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Segrèt, Milan, Italy).

Western Blot Analysis

The level of IL-18 was evaluated in cytosolic extracts by immunoblot analysis. β-Actin immunoblot analysis was performed to ensure equal sample loading. Equivalent amounts of protein (60 μg) from each sample were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes according to the manufacturer’s instructions (Bio-Rad). The membranes were saturated by incubation with 10% nonfat dry milk in PBS/0.1% Triton X-100 for 3 hours at room temperature and then incubated with anti–IL-18 goat antibody (0.2 μg/mL) (R&D Systems) or anti-β-actin (1/5000) (Sigma) mouse antibody overnight at 4°C. The membranes were washed 3 times with 0.1% Tween 20 in PBS and then incubated with anti-goat or anti-mouse (1/1000) immunoglobulins coupled to peroxidase (DakoCytomation) for 1 hour at room temperature. The immune complexes were visualized by the enhanced chemiluminescence method (Amersham, Milan, Italy). Subsequently, the relative intensities of the bands were quantified by densitometric scanning of the x-ray films with the GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM). IL-18 protein levels were expressed as arbitrary densitometric units.

Electrophoretic Mobility Shift Assay

NF-κB/DNA binding activity was evaluated in nuclear extracts by electrophoretic mobility shift assay as described previously. Briefly, 10 μg protein from each sample was incubated for 20 minutes with 32P-labeled, double-stranded NF-κB consensus binding-site oligonucleotides in 20 μL binding reaction buffer. Nuclear protein–oligonucleotide complexes were analyzed by gel electrophoresis. Gels were dried and autoradiographed with intensifying screen at −80°C for 24 hours.

Anti–IL-18 Treatment

To neutralize endogenous IL-18, rats were subjected to balloon angioplasty, followed by intraperitoneal injection of 3 mg of purified rabbit anti-IL-18 IgG prepared by Primm (Milan, Italy). Subsequent injections were at days 4, 8, and 12. Control rats received normal rabbit IgG. The biological activity of the antibody was tested in vitro. A dose of 200 μg anti–IL-18 antibody was shown to completely block IFN-γ–inducing activity of 50 ng IL-18 in rat spleen cells stimulated with Concanavalin A (data not shown).

In vitro Assay for Cell Proliferation

Primary rat aortic SMCs (ASMCs; Dominion Pharmakine, Spain) were cultured as previously described. All experiments were performed using early cell passages. ASMCs were treated with or without 25 ng/mL recombinant rat IL-18 (R&D Systems) for 72 hours. Cell proliferation was determined by [3H]-thymidine incorporation.22

Figure 1. A, Time course analysis of IL-18, IL-18Ra/β, IFN-γ, IL-6, and IL-8 mRNA expression in carotid arteries after balloon angioplasty. PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from pooled (n=3 per group) normal carotid (N), contralateral (CL), and injured carotid (L) arteries at different time points after angioplasty. Parallel amplification of the rat housekeeping gene β-actin was performed as internal control. Data shown are representative of 3 different experiments performed. B, Top, Time course analysis of IL-18 protein expression in carotid arteries after balloon angioplasty. Western blot analysis was performed on cytosolic extracts of pooled (n=3 per group) carotid arteries at different time points after angioplasty. Equal loading was confirmed by β-actin staining. Data shown are representative of 3 different experiments. Bottom, Densitometric analysis of IL-18 protein expression levels, normalized to expression levels of the housekeeping gene β-actin, was determined by computer program. Results are expressed as mean±SEM of 3 experiments. **P<0.01 vs normal carotid arteries.
Statistical Analysis
Results are expressed as mean±SEM of n rats for in vivo experiments and mean±SEM of multiple experiments for molecular biology. Student t tests were used to compare 2 groups, or ANOVA was used with the Dunnett’s post tests for multiple groups using Prism software (Graph Pad, San Diego, Calif). The level of statistical significance was 0.05 per test.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
Time Course of Neointimal Formation
Balloon angioplasty led to a time-dependent increase in neointimal formation. In normal rats (n=5), there was no neointimal formation. In rats subjected to angioplasty, the neointimal area was not detectable at day 2 (n=10), whereas it was 0.013±0.001 mm² (n=10) at day 7 and 0.213±0.010 mm² (n=10) at day 14. In sham-operated rats (n=5) not subjected to vascular injury, there was no neointimal formation at all time points analyzed. Medial area (0.145±0.008 mm² in the sham group) was not affected by vascular injury at all time points analyzed.

Expression of IL-18, IL-18Ra/β, IFN-γ, IL-6, and IL-8 mRNA in Rat Carotid Arteries After Balloon Angioplasty
To determine whether IL-18 mRNA and IL-18Ra/β mRNA were expressed in rat carotid arteries after balloon angioplasty, reverse transcription (RT)-PCR was performed (Figure 1A). Both IL-18 mRNA and IL-18Ra/β mRNA were detected in carotid arteries subjected to vascular injury, whereas their expression was very low in contralateral carotid arteries at all time points analyzed (days 2, 7, and 14 after angioplasty) (Figure 1A). Little expression was detected in normal arteries. The expression of both IL-18 and IL-18Ra/β mRNA was found to peak at 7 days and to diminish 14 days after angioplasty (Figure 1A). A similar pattern of expression was evident for all cytokines/chemokines analyzed. IFN-γ mRNA and IL-8 mRNA were evident mostly at day 7. IL-6 mRNA expression peaked at day 2 and diminished thereafter (Figure 1A).

Time Course of IL-18 Protein Expression in Rat Carotid Arteries After Balloon Angioplasty
Western blot assays were performed on cytosolic protein extracts from carotid arteries. The active form of IL-18 was highly expressed in carotid arteries subjected to angioplasty. No expression was detected in normal arteries (Figure 1B). Densitometric analysis of IL-18 levels showed peak IL-18 expression intensity at day 7 (Figure 1B), concomitant with the beginning of neointimal formation.

Localization of IL-18 and α-SMA in Rat Carotid Arteries
Localization of IL-18– and α-SMA–positive cells in rat carotid arteries was performed by immunofluorescence to determine the temporal expression, spatial distribution, and cellular localization of IL-18. Noninjured arterial tissue lacked immunoreactive IL-18. In contrast, injured carotid arteries stained strongly for IL-18 (Figure 2). Control IgG showed no signal (data not shown). In preliminary data, no
IL-18–positive staining was detectable in injured vessel up to day 2 (0 to 24 hours; data not shown), whereas a clear immunoreactivity for IL-18 was detected in the medial α-actin–positive SMC at day 2 after balloon injury (Figure 2). Intriguingly, at day 7, medial SMCs started to lose α-actin staining and to increase IL-18 expression (maybe a consequence of changes in phenotype); in addition, IL-18 was detected in neointimal cells (Figure 2). Immunoreactivity for IL-18 was observed mostly in neointima at day 14 (Figure 2); at this stage, colocalization of IL-18 and α-actin also was evident in some cells (Figure 2). SMCs in the neointima, although stained with the anti–α-actin antibody, typically showed weaker signal than contractile-state cells. No IL-18 expression was seen in the adventitia at all time points analyzed. These results suggest that IL-18 is expressed mainly in proliferating/migrating SMCs, contributing greatly to neointimal formation.

IL-18 Serum Levels
Serum IL-18 levels were measured by ELISA to determine whether balloon angioplasty could affect circulating levels of IL-18 (Figure 3). Serum concentration of IL-18 was higher in rats subjected to vascular injury than among naive rats. IL-18 was increased slightly in serum at day 2 after angioplasty, reached a maximal level at day 7 (113±18 pg/mL; \( P<0.01; n=10 \)), and diminished at day 14 (83±6.5 pg/mL; \( P<0.05; n=10 \)) compared with naive rats (40±7 pg/mL; \( n=10 \); Figure 3).

**Figure 3.** Serum IL-18 concentration in rats 2, 7, and 14 days after angioplasty. Data are expressed as mean (pg/mL)±SEM of 10 rats per group. N indicates naive rats. \( ^* P<0.05, \) \( ^{**} P<0.01 \) vs naive group.

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**Figure 4.** A, Photomicrographs of PCNA-stained rat carotid arteries at day 7 after injury. Data are represented as percentage of total medial and neointimal cells positive for PCNA as described in Methods. Magnification ×400. B, Photomicrographs showing the effect of IL-18 neutralization on neointimal formation in rat carotid arteries at day 14 after injury. Magnification ×50. C, Effect of IL-18 neutralization (anti–IL-18 IgG; \( n=5 \)) on neointimal areas of rat injured carotid arteries. Control animals (\( n=5 \)) were treated with normal rabbit IgG after angioplasty as described in Methods. Results are expressed as mean±SEM. D, Neointimal α-SMA–positive cell number was determined 14 days after angioplasty as described in Methods. \( ^{**} P<0.01 \) vs control group (C).
Neutralization of IL-18 Inhibits Neointimal Formation

We tested the hypothesis that endogenous IL-18 affects neointimal formation by using neutralizing rabbit anti-rat IL-18 IgG. Several group of rats (n=5) were treated with neutralizing anti-rat IL-18 IgG or normal rabbit IgG (control) beginning at the time of angioplasty; the antibody injections were repeated at days 4, 8, and 12. A remarkable increase in the number of PCNA-positive cells was demonstrated in the media and neointima 7 days after injury in control rats, which was much higher than the number of PCNA-positive cells in the anti–IL-18 IgG–treated group at the same time (Figure 4A).

Intriguingly, IL-18 neutralization caused a significant inhibition of neointimal formation by 27% (P<0.01) at day 14 compared with the control group (Figure 4C); concomitantly, anti–IL-18 IgG–treated rats exhibited significantly (P<0.01) diminished neointimal content of α-SMA–positive cells (Figure 4D), clearly suggesting a potential role of IL-18 in balloon-induced SMC proliferation. We examined vessels histologically in an effort to determine the effect of IL-18 blockade on collagenous matrix. IL-18 neutralization slightly increased, although not reaching significance, carotid collagen content (anti–IL-18 IgG, 123±20%; control, 100±16%).

Neutralization of IL-18 Inhibits Cytokine Production and NF-κB Activation

To provide mechanistic insights, the effect of IL-18 neutralization on several factors involved in neointimal formation was investigated. Interestingly, IL-18 neutralization inhibits IFN-γ, IL-6, and chemokine IL-8 mRNA expression 7 days after angioplasty (Figure 5A). In addition, anti–IL-18 IgG treatment reduced balloon-induced NF-κB activation in injured arteries at day 14. A low level of NF-κB/DNA binding activity was detected in nuclear protein extracts from contralateral carotid arteries and from carotid arteries of sham-operated rats (n=5; Figure 5B). Conversely, a retarded band was clearly shown in injured carotid arteries from control rats treated with normal rabbit IgG (n=5). Treatment of rats with neutralizing anti-rat IL-18 IgG (n=5), as described above, caused a marked inhibition of NF-κB activation in injured carotids (Figure 5B).

IL-18 Induces SMC Proliferation In Vitro

To provide data to suggest the mechanism for IL-18 role in neointimal formation, we investigated whether IL-18 induces rat ASMC proliferation in vitro. Our data demonstrate that IL-18 indeed increased rat ASMC proliferation (recombinant IL-18, 148±14%; control, 100±12%; P<0.01; data representative of 3 experiments run in triplicate), which is in agreement with the observations of Chandrasekar et al.4

Discussion

The present results identify a critical role for IL-18 in neointimal formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in the reduction of neointimal development. In this study, we examined the temporal expression of IL-18 and IL-18Rα/β mRNA and the IL-18 active form in rat carotid artery after balloon angioplasty. We report increased expression of the proinflammatory cytokine IL-18 and its signaling receptor, IL-18R (α/β-chain), in rat carotid arteries after vascular injury. Moreover, the levels of circulating IL-18 were found to be increased. IL-18 mRNA and IL-18Rα/β mRNA were equally expressed during the time course of neointimal formation. A clear induction of IL-18 and IL-18Rα/β mRNA and active peptide was observed 2 days after balloon angioplasty, and the elevated levels were sustained up to 14 days, with maximal expression evident at day 7, concomitant with the beginning of neointimal formation.

Our in situ findings indicate that carotid arteries strongly express IL-18 after angioplasty. In contrast, noninjured arte-

Figure 5. A, Effect of IL-18 neutralization (anti-IL-18 IgG) on IFN-γ, IL-6, and IL-8 mRNA expression in rat carotid arteries 7 days after balloon angioplasty. Control animals were treated with normal rabbit IgG after angioplasty as described in Methods. Real-time quantitative PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from pooled (n=3 per group) injured carotid arteries from 2 separate experiments. Data, expressed as mean±SEM, are from 3 different real-time PCR assays. B, Effects of IL-18 neutralization on NF-κB/DNA binding activity in rat carotid arteries after balloon angioplasty. Electrophoretic mobility shift assay was performed on nuclear extracts of sham carotid (S), contralateral (CL), and injured (L, lesion) carotid arteries from control rats treated with normal rabbit IgG or rats treated with neutralizing anti-rat IL-18 (anti–IL-18 IgG) collected 14 days after angioplasty. Data shown are representative of 3 different experiments. **P<0.01, ***P<0.001 vs control group (C).
rial tissue did not contain IL-18. It is interesting to note that expression of IL-18 is located primarily in SMCs that are actively involved in proliferation and migration, suggesting its potential role related to neointimal formation.\textsuperscript{17} SMC IL-18-positive staining agrees with previous reports, demonstrating IL-18 expression by intimal SMCs in human atherosclerotic plaques.\textsuperscript{5}

Several cells in injured arteries may express the IL-18 receptor, namely endothelial cells, SMCs, macrophages, and T lymphocytes, as demonstrated elsewhere.\textsuperscript{2} Unfortunately, the unavailability of appropriate antibodies hampered immunohistochemical analysis of IL-18\(\alpha/\beta\) expression in situ. Low levels of IL-18R\(\alpha/\beta\) mRNA in noninjured tissue, supported by RT-PCR data, suggested modest basal expression of the receptor on vascular cells, a finding consistent with reports of constitutive expression of the IL-18 receptor on ASMCs.\textsuperscript{4} Interestingly, the combination of several cytokines found in neointima, namely IL-1\(\beta\) and TNF-\(\alpha\), could promote the expression of both IL-18 receptor chains.\textsuperscript{2}

Increased serum levels of IL-18 in animals subjected to vascular injury are in keeping with an active role for this cytokine in the tissue pathogenesis and correlate well with epidemiological evidence showing higher IL-18 plasma levels associated with human restenosis.\textsuperscript{16,23}

To elucidate clearly the precise contribution of IL-18 involvement in the development of vascular damage after balloon angioplasty, we examined the effect of IL-18 neutralization on neointimal formation. Animals treated with neutralizing IL-18 IgG exhibited a significant reduction in neointimal size, showing diminished neointimal content of \(\alpha\)-SMA-positive cells. Collagen content was not significantly affected by IL-18 blockade. Interestingly, IL-18 neutralization diminished the number of PCNA-positive proliferating cells in the media and intima 7 days after injury, concomitant with the beginning of neointimal formation, clearly suggesting a potential role of IL-18 in balloon-induced SMC proliferation.

The precise molecular pathways responsible for the inhibitory effect of IL-18 neutralization on neointimal formation in vivo remain to be elucidated. Several hypotheses can be put forward to explain these results. Recent studies have provided strong evidence for an important role of IL-18 on SMC proliferation and migration in vitro.\textsuperscript{1,4,18} Importantly, we have shown that IL-18 induces rat ASMC proliferation in vitro; these results are in agreement with the Chandrasekar et al\textsuperscript{4} data. Effects of IL-18 on cell survival and proliferation are cell type dependent. Unlike endothelial cells,\textsuperscript{24} IL-18 in vitro failed to induce SMC death.\textsuperscript{4} Sahar et al\textsuperscript{4} showed that IL-18 activates several key signaling pathways, including mitogen-activated protein kinases, transcription factor NF-\(\kappa\)B, and activator protein 1, and induces the expression of proinflammatory cytokines and chemokines such as IL-6, IL-8, and monocyte chemoattractant protein-1 in vascular SMCs. Activation of these signaling kinases also is related to vascular SMC migration.\textsuperscript{18}

In the present study, we demonstrate in vivo in a rat model of vascular injury that IL-18 neutralization inhibits balloon-induced cytokines (IFN-\(\gamma\), IL-6) and chemokine (IL-8) mRNA expression in carotid arteries 7 days after injury. Furthermore, IL-18 neutralization reduced NF-\(\kappa\)B activation, a transcription factor involved in neointimal development and progression.\textsuperscript{25} A recent study suggests that angioplastic injury elicits an early, transient vascular NF-\(\kappa\)B activation in media and a late, persistent activation in intima, critical in controlling intimal hyperplasia and the associated vascular inflammation.\textsuperscript{25}

The induced expression of IL-18 in neointimal formation may involve additional functions. For example, IL-18 production may induce the expression of adhesion molecules,\textsuperscript{26,27} matrix metalloproteinases,\textsuperscript{18,28} and growth factors (eg, granulocyte-macrophage colony-stimulating factor), inducible nitric oxide synthase, or inducible cyclooxygenase,\textsuperscript{29} all factors regulated at the transcriptional level by NF-\(\kappa\)B.\textsuperscript{30} This could explain why at day 14, cytokine is decreasing while pathology is maximal.

The present study has some limitations. The rat balloon angioplasty model could be considered ideal to study the proliferation of SMCs in vivo, but it is not a reliable experimental model of human angioplasty because the injury is performed on a normal nonatheromatous arterial bed. This method does not take into account the contribution of vascular remodeling in human restenotic process. In addition, the carotid artery is not similar to other arterial beds (eg, coronary arteries) with respect to its reaction to stimuli. It is not feasible to regulate IL-18 artificially in humans to determine its association with restenotic diseases; therefore, dissection of the role of this cytokine in lesion development is dependent on animal models.

Conclusions

These results identify a critical role for IL-18 in neointimal formation in a rat model of vascular injury and suggest a potential role for IL-18 neutralization in reduction of neointimal development. The disease-modifying activity of IL-18 neutralization in this model can offer a clue to the role of IL-18 in the human restenotic process.

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Disclosures

None.

References

Vascular disease has become recognized as an active, inflammatory vascular process. Evidence of inflammation is found locally in the vascular wall and systemically in the circulation. Circulating markers suggesting an augmented state of vascular inflammation have been found in acute myocardial infarction, chronic coronary artery disease, and restenosis and in asymptomatic patients at high risk for vascular disease. Among these markers, cytokines and cytokine networks involved in the inflammatory cascade are receiving growing emphasis. Interleukin-18 (IL-18) is among the more recently recognized cytokines that play a role in cardiovascular pathologies. However, it is still unclear whether IL-18 plays a causative role or whether elevated levels simply reflect an ongoing inflammatory process. Here, we show a prominent role for IL-18 in collagen-induced arthritis in the BB rat. Clin Exp Immunol. 2004;136:440–447.


**CLINICAL PERSPECTIVE**

Vascular disease has become recognized as an active, inflammatory vascular process. Evidence of inflammation is found locally in the vascular wall and systemically in the circulation. Circulating markers suggesting an augmented state of vascular inflammation have been found in acute myocardial infarction, chronic coronary artery disease, and restenosis and in asymptomatic patients at high risk for vascular disease. Among these markers, cytokines and cytokine networks involved in the inflammatory cascade are receiving growing emphasis. Interleukin-18 (IL-18) is among the more recently recognized cytokines that play a role in cardiovascular pathologies. However, it is still unclear whether IL-18 plays a causative role or whether elevated levels simply reflect an ongoing inflammatory process. Here, we show a prominent role for IL-18 in neointimal formation in a rat balloon injury model. We found increased expression of IL-18 in injured carotid arteries, mainly in proliferating/migrating smooth muscle cells. Neutralizing IL-18 in vivo with a rabbit anti-rat IL-18 immunoglobulin G significantly reduced neointimal formation and the number of proliferating smooth muscle cells and inhibited the expression of other cytokines and inflammatory markers (interferon-γ, IL-6, IL-8) in injured arteries. Although rat balloon injury is not a reliable experimental model of human angioplasty, the disease-modifying activity of IL-18 neutralization in this model could offer a clue to the role of IL-18 in human restenotic process. Clearly, the clinical feasibility of this approach is far from being suggested; however, our results support the hypothesis that targeting cytokines and inflammatory markers may provide new therapeutic approaches for the treatment of occlusive vascular diseases.
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