Clinical Investigation and Reports

Expression of Interleukin-18 in Human Atherosclerotic Plaques and Relation to Plaque Instability

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Background—Interleukin (IL)-18 is a potent proinflammatory cytokine with potential atherogenic properties. Its expression and role in atherosclerosis, however, are unknown.

Methods and Results—In the present study, we examined stable and unstable human carotid atherosclerotic plaques retrieved by endarterectomy for the presence of IL-18 using reverse transcription–polymerase chain reaction (PCR), Western blot, and immunohistochemical techniques. IL-18 was highly expressed in the atherosclerotic plaques compared with control normal arteries and was localized mainly in plaque macrophages. IL-18 receptor was also upregulated in plaque macrophages and endothelial cells, suggesting potential biological effects. To examine the role of IL-18 in atherosclerosis, we determined the relation between IL-18 mRNA expression and signs of plaque instability using real-time quantitative PCR. Interestingly, significantly higher levels of IL-18 mRNA were found in symptomatic (unstable) plaques than asymptomatic (stable) plaques (P<0.01).

Conclusions—These results suggest, for the first time, a major role for IL-18 in atherosclerotic plaque destabilization leading to acute ischemic syndromes. (Circulation. 2001;104:1598-1603.)

Key Words: atherosclerosis ■ interleukin ■ stroke

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by progressive accumulation of lipids, cells (macrophages, T lymphocytes, and smooth muscle cells [SMCs]), and extracellular matrix.1 A large body of evidence suggests that the inflammatory process plays a major role throughout the development of the atherosclerotic lesion.1 Inflammation is also involved in atherosclerotic plaque disruption and thrombosis2,3 and may greatly influence the occurrence of acute ischemic syndromes and their related mortality.4 Therefore, identification of the critical inflammatory pathways involved in plaque destabilization may open the way for the development of novel therapeutic strategies aiming to reduce atherosclerosis-related mortality.

Unstable or vulnerable atherosclerotic plaques are characterized by increased accumulation of inflammatory cells, particularly macrophages and T lymphocytes, and by a large lipid core and a thin fibrous cap. Cross talk between the inflammatory cells of unstable plaques results in the production of high levels of inflammatory cytokines that are responsible for detrimental effects on plaque composition, ie, a decrease in SMC and collagen content.1,3 Among the proinflammatory cytokines, interferon (IFN)-γ and one of its potent inducers, interleukin (IL)-12, appear to play a central role in both plaque development and stability.5,6 IFN-γ is produced by lymphocytes of the Th1 phenotype; it stimulates the expression of adhesion molecules on endothelial cells and major histocompatibility complex II on macrophages and vascular cells and inhibits collagen synthesis by SMCs,7 all features that may promote plaque development and instability. Indeed, apolipoprotein E–knockout mice that are deficient for the IFN-γ receptor show a significant decrease in atherosclerotic lesion size and increased collagen accumulation, consistent with a stable plaque phenotype.5

In this context, IL-18, initially described as an endotoxin-induced serum factor that stimulates IFN-γ production, might be involved in atherosclerosis. IL-18 is a member of the IL-1 family of cytokines and is processed, like IL-1β, by caspase-1.8,9 IL-18 is a pleiotropic cytokine acting in both acquired and innate immunity.10 IL-18 promotes the action of IL-12, which favors T-lymphocyte differentiation along the Th1 lineage.10 IL-18 and IL-12 act synergistically to induce the production of IFN-γ in T cells, natural killer cells, and subsets of macrophages.10-12 In addition, IL-18 acts directly as a proinflammatory cytokine by inducing IL-1β, IL-8, and the expression of adhesion molecules.13,14 IL-18 is also able to stimulate the production of granulocyte-macrophage colony–stimulating factor, tumor necrosis factor-α, and inducible nitric oxide synthase by mononuclear and mesenchymal cells.10

Received May 17, 2001; revision received July 18, 2001; accepted July 25, 2001.
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In light of the potent inflammatory activities of IL-18, we hypothesized that it might play important roles in atherosclerosis development and stability. Therefore, the aim of this study was to examine the expression and cellular localization of IL-18 in human carotid atherosclerotic plaques. We also examined whether the expression of IL-18 was related to plaque vulnerability.

**Methods**

**Specimens**

Forty human atherosclerotic plaques removed from 35 patients undergoing carotid endarterectomy were collected. For controls, 2 carotid and 3 internal mammary arteries free of atherosclerosis (2 with minimal fibromuscular thickening) were obtained at autopsy or during coronary bypass surgery. They were rapidly immersed in liquid nitrogen and stored at −80°C. Plaques that were used for protein and RNA extraction were rapidly washed and immersed in liquid nitrogen before they were stored at −80°C. For immunohistochemical studies, plaques were placed for 2 hours in fresh 4% paraformaldehyde, then transferred to a 30% sucrose–PBS solution before being snap-frozen (O.C.T. Compound, Miles Inc, Diagnostics Division) with liquid nitrogen and stored at −80°C for cryostat sectioning. Several 8- to 10-μm sections were obtained from each specimen for histological analysis and immunohistochemical studies.

**RNA Preparation**

Total RNA was extracted from 28 atherosclerotic plaques in an acid guanidinium–thiocyanate solution and extracted with phenol and chloroform according to the method of Chomczynski and Sacchi.16 The purified RNA was dissolved in water, and the concentration was measured by absorbance at 260 nm. RNA integrity was assessed by electrophoresis on 1% agarose gels. CDNA was synthesized from 1 μg of total RNA by the Promega RT system according to the manufacturer’s protocol.

**Western Blot Analysis**

Proteins were extracted from 12 atherosclerotic plaques and 5 control normal arteries. Frozen samples were pulverized under liquid nitrogen. The powders were resuspended in ice-cold lysis buffer (mM/L): Tris–HCl 20 [pH 7.5], EGTA 5, NaCl 150, glycerophosphate 20, NaF 10, sodium orthovanadate 1, PMSF 1, TPCK 0.5, and TLCK 0.5, plus 1% Triton X-100, 0.1% Tween 20, and 1 μg/mL aprotinin (at a ratio of 0.3 mL/10 mg wet wt. Extracts were incubated on ice for 15 minutes and then centrifuged (12 000g, 15 minutes, 4°C). The detergent-soluble supernatant fractions were retained, and protein concentrations in samples were equalized by use of a Bio-Rad protein assay.

Protein extracts were boiled for 5 minutes and loaded on a 7.5% or 15% SDS–polyacrylamide gel. Samples were electrophoretically transferred from polyacrylamide gels onto nitrocellulose. Membranes were incubated with goat anti-human IL-18, goat anti-human IL-18 receptor (IL-18R) (α–chain) polyclonal antibodies (1 μg/mL) (R&D Systems), or rabbit anti–human caspase-1 p10 subunit polyclonal antibody (1 μg/mL) (C-20, Santa Cruz). After incubation with horseradish peroxidase (HRP)–conjugated corresponding antibodies, chemiluminescence substrates (ECL, Western blotting; Amersham Corp) were used to reveal positive bands according to the manufacturer’s instructions, and bands were visualized after exposure to Hyperfilm ECL (Amersham Corp).

**Immunohistochemistry**

Frozen sections from 6 atherosclerotic plaques were incubated with either a primary anti-CD68 antibody (Dako, CD68, KPI), a primary anti–smooth muscle α–actin antibody (1A4, Dako), or a primary anti–CD31 antibody (Dako). To identify IL-18 and IL-18R-α within atherosclerotic plaques, specific goat polyclonal antibodies (R&D Systems) were used at a dilution of 5 or 10 μg/mL, respectively. Immunostains were visualized with the use of avidin-biotin HRP visualization system (Nichastain ABC kit PK-6100, Vector). For negative controls, adjacent sections were stained with isotype-matched irrelevant antibodies instead of the primary antibodies.

**Patient Classification**

To study the potential relation between IL-18 expression and signs of plaque instability, we collected, in a prospective and blinded manner, clinical data from 22 consecutive patients (of 35) undergoing the endarterectomy procedure between May and August 2000. The presence or absence of an intraplaque ulcer on macroscopic examination was systematically reported by the surgeon who performed the endarterectomy procedure. This enabled us to classify the plaques as ulcerated or nonulcerated plaques. In addition, the patients were classified according to clinical symptoms in 2 separate groups. Patients who presented with clinical symptoms of cerebral ischemic attack related to the carotid stenosis were classified as symptomatic. Endarterectomy was performed 2 to 32 days (12.4±3.0 days) after the onset of clinical symptoms in these patients. Patients who never experienced symptoms of cerebral ischemia in the carotid artery territory were classified as asymptomatic. Asymptomatic carotid stenosis was defined as the basis of systematic clinical examination of patients with coronary or peripheral disease, and its severity was determined by repeated Doppler echography by an experienced validated echographist. Even though asymptomatic patients never had an ischemic episode in the territory of the carotid stenosis, carotid endarterectomy has been shown to be beneficial in these patients, as shown by Asymptomatic Carotid Atherosclerosis Study (ACAS) investigators.15

**Semiquantitative and Real-Time PCR of IL-18 in Human Atherosclerotic Plaques**

Semiquantitative PCR reactions were performed in a total volume of 50 μL in the presence of 1 U of AmpliTaq DNA polymerase (Perkin Elmer, Roche), 2.5 mM/L dNTPs (Amersham), and 50 pmol of forward and reverse PCR primers. Reactions were incubated in a PTC-200 Peltier Thermal Cycler (MJ Research) under the following conditions: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C. To ensure comparison of the amount of PCR products during the linear phase of the PCR reaction, IL-18 and β–actin were analyzed after 25, 28, and 31 cycles. The optimal number of cycles for IL-18 and β–actin before saturation of the bands was determined (28 and 25, respectively). PCR primers were designed on the basis of the published sequences (AF110799, D49950, X00351) as follows: IL-18, reverse 5′-CCTAGTCCCAGGGCTTTGATT-3′; forward 5′-CCACCCATGGCAAATTCC-3′; β–actin, reverse 5′-GGAGGAGCA-ATGATCTTGATCTTC-3′; forward 5′-GCTCACCATGGATGATCGCC-3′. To exclude the amplification of potential genomic DNA that might contaminate the samples, PCR reactions were performed in the absence of the cDNA template. PCR products (10 μL) were analyzed on 1% agarose gels electrophoresed in 1× TAE buffer (40 mM/L Tris-acetate [pH 8.5], 1 mM/L EDTA). The size of PCR products was verified by comparison with a 1-kb ladder (Gibco) after staining of the gels.
DNA contamination was excluded by PCR reactions performed with specific intron-GAPDH primers. The absence of nonspecific amplification was confirmed by analysis of the PCR products by 3.5% agarose gel electrophoresis. SYBR Green real-time PCR was performed with 5 μL/well of RT products (0.5 ng total RNA), 25 μL/well of SYBR Green PCR master mix (PE Biosystem) with AmpErase uracil N-glycosylase (UNG) (0.5 U/well), and 20 μL of primers (300 nmol/L). PCR was performed at 50°C for 2 minutes (for AmpErase UNG incubation to remove any uracil incorporated into the cDNA) and 95°C for 10 minutes (for AmpliTaq Gold activation) and then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute on the ABI PRISM 7700 Detection System. The reverse-transcribed cDNA samples were thus amplified, and their cycle threshold (Ct) values were determined. All Ct values were normalized to the housekeeping gene GAPDH. A single specific DNA band for IL-18 and GAPDH was observed by gel electrophoresis analysis.

The principle of real-time detection with the SYBR Green PCR master mix is based on the direct detection of PCR product by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA.

Statistical Analysis

Data are expressed as mean±SEM. Levels of IL-18 were compared between groups by the Mann-Whitney test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Expression of IL-18 Protein in Atherosclerotic Plaques

Western blot assays were performed on protein extracts from 12 carotid atherosclerotic arteries and 5 normal controls. Both forms of IL-18, the pro and active forms, were highly expressed in most atherosclerotic plaques (in 12 of 12 plaques for the pro form and in 9 of 12 plaques for the active form). Little or no expression was detected, however, in normal arteries (Figure 1). Interestingly, detection of the active form of IL-18 seemed to correlate with the processing of caspase-1 (detection of p10 subunit), which is involved in IL-18 processing (Figure 1). An important expression of IL-18R protein (the α-chain) was also detected in all atherosclerotic plaques, whereas its expression was low in normal arteries (Figure 1).

Figure 1. Representative Western blot showing expression of IL-18, IL-18R, and caspase-1 p10 subunit in human carotid atherosclerotic plaques and control arteries. Significant expression of IL-18 and IL-18R was found in atherosclerotic plaques. Expression of mature form of IL-18 (18 kDa) was associated with caspase-1 cleavage, as indicated by presence of p10 subunit. Ponceau red staining is included to show total protein load.

Figure 2. Representative photomicrographs showing expression of IL-18 in human atherosclerotic plaques. Sections were double-stained with irrelevant mouse and goat IgGs for negative controls (A); SMCs (B and E) were identified by anti-α-actin antibody, clone 1A4 (Dako). Macrophages were identified by anti-CD68 antibody (C) (Dako). A through D, IL-18 expression (D) is most abundant in macrophage-rich areas (C, lipid core), although some expression is seen in SMC-rich areas (B, fibrous cap). E and F, * indicates media. Sections from SMC–rich area show IL-18 expression (F) in intimal SMCs but not medial SMCs (E, α–actin staining). Sections in E and F were completely negative for CD68 (not shown). Magnification ×200.

Cellular Localization of IL-18 and IL-18R Proteins in Atherosclerotic Plaques

To determine the cellular localization of IL-18, immunohistochemical studies were performed on 6 carotid atherosclerotic plaques. As shown in Figure 2, IL-18 was expressed mainly in macrophages, probably the major source of IL-18 in the plaque (Figure 2). IL-18 was also expressed in some intimal (but not medial) SMCs (Figure 2) and in occasional endothelial cells (not shown). No expression was seen in the underlying normal media. IL-18R-α was highly expressed in both plaque macrophages and endothelial cells. No or barely detectable expression was found in SMCs (Figure 3).

Expression of IL-18 mRNA Transcripts in Atherosclerotic Plaques and Relation to Plaque Instability

To determine whether human IL-18 mRNA was expressed in human carotid atherosclerotic plaques, semiquantitative RT-PCR was performed on 6 atherosclerotic plaques (Figure 4). IL-18 mRNA was detected in all atherosclerotic plaques, although its amount was heterogeneous. Therefore, to accurately quantify the levels of IL-18 mRNA expression, 22 atherosclerotic plaques were further analyzed with the SYBR Green real-time PCR method. The plaques were characterized by clinical and pathological examination as symptomatic (unstable) or asymptomatic (stable) plaques, containing macroscopic ulcer or not. The clinical characteristics of the
patients are summarized in the Table. There were 13 symptomatic and 9 asymptomatic patients. Among the symptomatic group, 10 patients had a transient ischemic attack (6 were recurrent), and 3 patients had a definitive stroke. Risk factors, including age, diabetes, hypercholesterolemia, hypertension, and cigarette smoking, did not differ between the 2 groups.

The amount of IL-18 was found to be 3-fold higher in the symptomatic than in the asymptomatic atherosclerotic plaques (2.18 ± 0.52 versus 0.67 ± 0.17, respectively) (Figure 5A). Statistical analysis demonstrated that this increase in IL-18 expression observed in the symptomatic plaques was highly significant (P < 0.007). IL-18 levels were not related to the type of clinical symptoms (2.2 ± 0.7 in transient ischemic attack versus 2.0 ± 0.5 in stroke, P = NS). In addition, because the presence of ulceration is considered a feature of plaque instability, statistical analysis was further performed on plaques without or with intraplaque ulcers: a significant upregulation of IL-18 was found in the plaques presenting ulcers (P < 0.01) (Figure 5B). An example of such plaques is presented in Figure 6. These data show that the increase in IL-18 expression seen in the atherosclerotic plaques correlates with plaque instability.

### Discussion

This study shows significant expression of the proinflammatory cytokine IL-18 and of its signaling receptor, IL-18R (α-chain) in human atherosclerotic plaques. In addition, using real-time quantitative PCR, we found significantly higher expression of IL-18 mRNA in symptomatic or ulcerated plaques than in asymptomatic or nonulcerated plaques.

Atherosclerosis is an inflammatory disease. Identification of critical regulatory pathways is important to improve our understanding of the disease and may open the way for novel therapeutic strategies to reduce its associated mortality. Our interest in the IL-18 pathway in atherosclerosis is based on several important observations. IL-18 is a proinflammatory cytokine produced mainly by monocytes/macrophages, with potent activities on both macrophages and T cells, two cell types involved in the development and complications of human atherosclerotic plaques. IL-18 shows the most potent synergism with IL-12 for the induction of IFN-γ. These latter cytokines are expressed in atherosclerotic plaques and have been implicated in the immunoinflammatory response that determines both the size and the composition of the atherosclerotic lesion in animal models of atherosclerosis.

IFN-γ greatly affects collagen content of atherosclerotic plaques, in part through inhibition of collagen synthesis.

### Table: Patient Characteristics

<table>
<thead>
<tr>
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<th>Asymptomatic Patients (n=9)</th>
<th>Symptomatic Patients* (n=13)</th>
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<tr>
<td>Coronary artery disease</td>
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<td>0.13</td>
</tr>
<tr>
<td>Macroscopic ulceration</td>
<td>5</td>
<td>10</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Values are numbers of patients, except age, which is mean ± SEM.

*These patients presented with transient or persistent ischemic cerebral attack 2 to 32 days before endarterectomy.

†Number of patients with clinical hypertension being treated with antihypertensive agents.

‡Number of patients with clinical hypercholesterolemia being treated with lipid-lowering drugs.
As a result, IFN-γ is thought to participate in plaque destabilization by preventing the formation of a thick fibrous cap. Therefore, we hypothesized that the IFN-γ–inducing factor, IL-18, may be involved in atherosclerosis progression. In the present study, we detected high levels of IL-18 in atherosclerotic plaques. As expected, IL-18 protein was produced mainly by plaque macrophages and was associated with expression of its α-chain receptor in macrophages and endothelial cells, suggesting biological effects. It is noteworthy that the expression of the mature form of IL-18 was closely associated with the processing of caspase-1, suggesting a significant role for caspase-1 activity in IL-18 release in human atherosclerotic plaques. A role for the Fas/FasL pathway in the production of mature IL-18, however, such as that reported in acute liver injury, cannot be excluded.

Severe clinical manifestations of atherosclerosis (myocardial infarction and stroke) are mainly due to vessel lumen occlusion by a thrombus formed on the contact of a disrupted atherosclerotic plaque. In classic risk factors (including hemodynamic stress) for plaque disruption and thrombosis, a large body of evidence has now been presented relating systemic or local plaque inflammation to the severe clinical ischemic complications of atherosclerosis. The major mechanisms that drive this inflammatory response in humans, however, are not well understood. To gain further insight into the role of IL-18 in atherosclerosis, we examined the relation between IL-18 mRNA expression in human plaques and the presence of clinical and pathological signs of plaque instability by use of real-time quantitative PCR. Importantly, the expression of IL-18 transcripts was significantly associated with the presence of both clinical (unstable symptomatic plaque) and pathological (ulcerated plaque) signs of plaque instability. Our results suggest an important role for IL-18 in plaque destabilization and in the occurrence of acute ischemic syndromes. Our data are consistent with those from other groups suggesting a role for the Th1 response in plaque destabilization. The precise mechanisms involved in the detrimental effects of IL-18 on plaque stability, however, remain to be elucidated.

In conclusion, our study shows, for the first time, significant expression of IL-18 in human carotid atherosclerotic plaques. Moreover, the level of IL-18 mRNA transcripts is significantly associated with the presence of clinical and pathological signs of plaque instability. We propose that the modulation of IL-18 signaling, for example, by use of IL-18 inhibitors, may limit the progression and complications of atherosclerosis.

Acknowledgment

This work was supported by Action Concertée Incitative Jeunes Chercheurs, ACI 2000, Ministère de la Recherche, France.

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2. van der Wal AC, Becker AE, van der Loos CM, et al. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.

Figure 5. SYBR Green real-time PCR analysis of IL-18 mRNA expression in atherosclerotic plaques characterized by histopathological examination as symptomatic and asymptomatic (A), presenting macroscopic ulcer or not (B). Total RNA was isolated from atherosclerotic plaques in an acid guanidinium thiocyanate solution. IL-18 expression was analyzed by SYBR Green real-time PCR. Data were processed with a specially designed software program based on Ct values of each sample and normalized to GAPDH. Quantitative data for IL-18 mRNA expression according to clinical (A) or pathological (B) signs of plaque instability were displayed graphically.


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_Circulation_. 2001;104:1598-1603
doi: 10.1161/hc3901.096721

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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