Expression of Interleukin-10 in Advanced Human Atherosclerotic Plaques
Relation to Inducible Nitric Oxide Synthase Expression and Cell Death

Ziad Mallat, Christophe Heymes, Jeanny Ohan, Elisabetta Faggin, Guy Lesèche, Alain Tedgui

Abstract—Inflammation is a major feature of human atherosclerosis and is central to development and progression of the disease. A variety of proinflammatory cytokines are expressed in the atherosclerotic plaque and may modulate extracellular matrix remodeling, cell proliferation, and cell death. Little is known, however, about the expression and potential role of anti-inflammatory cytokines in human atherosclerosis. Interleukin-10 (IL-10) is a major anti-inflammatory cytokine whose expression and potential effects in advanced human atherosclerotic plaques have not been evaluated. We studied 21 advanced human atherosclerotic plaques. IL-10 expression was analyzed by use of reverse transcription–polymerase chain reaction and immunohistochemical techniques. Inducible nitric oxide synthase expression was assessed by using immunohistochemistry, and cell death was determined by use of the TUNEL method. Reverse transcription–polymerase chain reaction identified IL-10 mRNA in 12 of 17 atherosclerotic plaques. Immunohistochemical staining of serial sections and double staining identified immunoreactive IL-10 mainly in macrophages, as well as in smooth muscle cells. Consistent with its anti-inflammatory properties, high levels of IL-10 expression were associated with significant decrease in inducible nitric oxide synthase expression ($P<0.0001$) and cell death ($P<0.0001$). Hence, IL-10, a potent anti-inflammatory cytokine, is expressed in a substantial number of advanced human atherosclerotic plaques and might contribute to the modulation of the local inflammatory response and protect from excessive cell death in the plaque. (Arterioscler Thromb Vasc Biol. 1999;19:611-616.)

Key Words: interleukin-10 ☐ atherosclerotic plaque ☐ cytokines ☐ inflammation

A

variety of proinflammatory cytokines, including tumor necrosis factor-$\alpha$, interleukin (IL)-$\beta$, IL-6, and interferon-$\gamma$, are expressed in the human atherosclerotic plaque. These cytokines alone or in conjunction contribute to the local inflammatory response and may have great impact on plaque formation and progression. Indeed, proinflammatory cytokines have the potential to induce excessive extracellular matrix degradation and cell death promoting plaque instability. However, the inflammatory response is known to be balanced by anti-inflammatory cytokines, including IL-10.

We therefore hypothesized that this may occur within the plaque. Hitherto, little is known about the expression and potential role of anti-inflammatory cytokines in human atherosclerosis. Among the anti-inflammatory cytokines, IL-10 is produced by Th2 cells as well as by macrophages and has potent deactivating properties on these cells. Because macrophages and T-lymphocytes are involved in atherogenesis, we hypothesized that IL-10 may be produced locally in the plaque and may protect from an excessive proinflammatory response and cell damage in the plaque. To test this hypothesis, we analyzed the expression and localization of IL-10 in advanced human atherosclerotic plaques and examined its relation to inducible nitric oxide synthase (iNOS) expression, a reliable marker of the proinflammatory response, and to cell death.

Methods

Materials

Twenty-one human atherosclerotic plaques removed from 18 patients undergoing carotid endarterectomy and 3 patients undergoing resection of an abdominal aortic aneurysm were collected. For controls, 4 carotid arteries free of atherosclerosis (1 with minimal fibromuscular thickening) were obtained at autopsy and 3 internal mammary arteries were obtained during coronary bypass surgery. A segment from the most stenotic area of each arterial specimen was immediately placed for 2 hours in fresh 4% paraformaldehyde, then transferred to a 30% sucrose–PBS solution before being snap-frozen in optimal cutting temperature tissue processing medium (O.C.T. Compound, Miles Inc, Diagnostics Division) with liquid nitrogen and stored at $-80^\circ$C for cryostat sectioning. Several 5- to 6-$\mu$m sections were obtained from each specimen for histological analysis, immunohistochemical studies, and in situ detection of apoptosis. Fresh, unfixed material was used for protein, RNA, and DNA extraction.

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Total RNA Extraction and Reverse Transcription–Polymerase Chain Reaction to Detect IL-10 mRNA
Total RNA was extracted from 17 atherosclerotic plaques by use of Trizol reagent according to the manufacturer’s instructions (Life Technologies, Inc). The purified RNA was dissolved in water and the concentration measured by absorbance at 260 nm. The antisense primer for reverse transcription–polymerase chain reaction (RT-PCR) was 5′-AAGCTGAGAACCAAGACCCAGACATCAAGGC-3′ (nucleotides 615 to 647 of the coding sequence) and the sense primer was 5′-AGCTATCCAGAGCCCCAGATCGGATTGGG3′ (nucleotides 320 to 351 of the coding sequence), resulting in a 328-bp amplification product. The oligonucleotides were obtained from Clontech Laboratories. For RT reaction, 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc) was used to synthesize (39°C, 90 minutes) single-strand cDNA from 1 μg of total RNA. The reaction was performed in 20 μL of a mixture containing 1 μmol/L of the reverse primer, 50 μmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 0.5 mmol/L (Np)-CD68, KP1), or a primary mouse monoclonal antibody against once in PBS, then incubated with either a primary mouse monoclonal antibody against once in PBS, the slides were incubated with the following secondary antibodies: a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc) at a dilution of 1:200 for detection of stains with antibodies against CD68 and smooth muscle α-actin, a biotinylated horse anti-goat IgG (Vector) at a dilution of 1:200 for detection of anti-IL-10 antibody, and a biotinylated goat anti-rabbit IgG (Vector) at a dilution of 1:200 for detection of anti-iNOS antibody. Immunostains were visualized with the use of avidin–biotin HRP (brown staining) or alkaline phosphatase (red color) visualization systems (Vectorstain ABC kit PK-6100 and AK-5000 Vector). For negative controls, adjacent sections were stained with isotype-matched irrelevant antibodies instead of the primary antibodies.

In Situ Detection of Apoptotic Cell Death
In situ detection of apoptotic cells, using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method of fragmented DNA was performed on cryostat sections as previously described.16 It is noteworthy that fixation time was abrogated and treatment of sections with protease K was omitted and that has recently been shown to enhance the specificity of the staining.11 Negative controls for TUNEL labeling were obtained after omission of the enzyme TdT.

Relation Between IL-10 Expression, iNOS Expression, and TUNEL Labeling
Twenty-one plaques were analyzed. From each plaque, at least 10 adjacent serial sections were examined after staining for IL-10, iNOS, or TUNEL. To evaluate the relation between IL-10 expression, iNOS expression, and TUNEL labeling, we performed semi-quantitative and quantitative analyses.

For semiquantitative analysis, areas with positivity for IL-10, iNOS, or TUNEL were first identified at low magnification (×100 and ×200) over all the sections. We then analyzed 500 microscopic fields (×400) that showed positivity for at least 1 of the 3 stainings (IL-10, iNOS, or TUNEL) (almost 90% of the positive fields) and discarded those fields that were acellular or negative for all 3 stainings (748 fields). Cell counting was performed by 2 investigators (Z.M. and A.T.) who obtained similar results. The level of IL-10 expression in these fields was graded as follows: 0 (no staining), + (<10% staining), ++ (10% to 50% staining), or +++ (>50% staining). We then determined the distribution of iNOS expression and TUNEL labeling in the corresponding serial fields. Fields were considered positive for iNOS or TUNEL when at least 5 cells per field stained positive.

For quantitative analysis, double staining was performed on 52 sections from 9 of the 21 plaques. Percentages of IL-10-positive, iNOS-positive, or TUNEL-positive cells were first determined (the total number of cells counted in each section varied between 800 and 5000 cells). Then, the percentages of iNOS-positive or TUNEL-positive cells among IL-10-positive cells were calculated.

**Figure 1.** Expression of IL-10 mRNA in human atherosclerosis. One microgram of total RNA was used in each case. After RT, the cDNA was amplified by 30-cycle PCR with IL-10 primers (see Methods). The 328-bp product was visualized under UV light after electrophoresis of the PCR products in agarose gels containing ethidium bromide. IL-10 mRNA was expressed in most atherosclerotic plaques examined (results from 10 plaques are shown) but not in control carotid arteries. MW indicates DNA size markers. Positive control was purchased from Clontech.

**Western Blot Analysis**
Proteins were extracted from 6 atherosclerotic plaques. Frozen plaques were pulverized under liquid nitrogen. The powders were resuspended in ice-cold lysis buffer [20 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% Tween 20, 1 μg/mL aprotinin, 1 μL/mL PMSF, 0.5 mmol/L N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 0.5 mmol/L N(a)-p-tosyl-l-lysine chloromethyl ketone (TLCK)] at a ratio of 0.3 mg/mL 10 mg of wet weight. 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 using a Bio-Rad protein assay; 70 μL of Laemmli sample buffer was added to 100-μL aliquots, samples were boiled for 3 minutes and loaded on a 12% SDS-polyacrylamide gel. Proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose. Nitrocellulose membranes were saturated for 2 hours at room temperature in TBST [50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, and 0.1% Tween saline] containing 5% of fat-free dry milk. Goat polyclonal antibodies to human IL-10 (R & D Systems Europe Ltd) were used at a concentration of 1 μg/mL. Following incubation with anti-goat HRP conjugated antibodies (Sigma), 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 were incubated with 1:10 normal horse serum or 1:10 normal goat serum for 30 minutes at room temperature, washed once in PBS, then incubated with either a primary mouse monoclonal antibody against CD68 for macrophage identification (DAKO-CD68, KP1), or a primary mouse monoclonal antibody against human smooth muscle α-actin (HHF35, DAKO) for identification of smooth muscle cells. These antibodies were used at a dilution of 1:200. To identify IL-10 within atherosclerotic plaques, a specific goat polyclonal antibody (R & D Systems Europe Ltd) was used at a dilution of 10 μg/mL. iNOS was detected by using a specific rabbit polyclonal antibody (Biomol) at a dilution of 1:500. After washing in PBS, the slides were incubated with the following secondary antibodies: a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc) at a dilution of 1:200 for detection of stains with antibodies against CD68 and smooth muscle α-actin, a biotinylated horse anti-goat IgG (Vector) at a dilution of 1:200 for detection of anti-IL-10 antibody, and a biotinylated goat anti-rabbit IgG (Vector) at a dilution of 1:200 for detection of anti-iNOS antibody. Immunostains were visualized with the use of avidin–biotin HRP (brown staining) or alkaline phosphatase (red color) visualization systems (Vectorstain ABC kit PK-6100 and AK-5000 Vector). For negative controls, adjacent sections were stained with isotype-matched irrelevant antibodies instead of the primary antibodies.

**Expression of IL-10 mRNA in Human Atherosclerosis**
Western blot analysis was performed to detect IL-10 mRNA by using RT-PCR. The 328-bp amplification product was performed in a total volume of 100 μL containing 0.2 μmol/L of the sense primer, 1× PCR buffer (10 mmol/L Tris-HCl, 1.5 mmol/L MgCl2, and 50 mmol/L KCl), 0.3 mmol/L dNTP, 2 mmol/L DTT, and 2.5 U Taq DNA polymerase (Boehringer Mannheim). The mixture was over-laid with 1 drop of mineral oil and the amplification was performed as follows: denaturation at 95°C for 45 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes, 400 times. Twenty microliters of the RT-PCR mixtures was electrophoresed in 2% agarose gel.
Statistical Analysis
Results are expressed as mean±SEM values. Semiquantitative analysis of the effect of IL-10 on iNOS expression and TUNEL labeling was performed by using a χ² test. Quantitative analysis of the effect of IL-10 on iNOS expression and TUNEL labeling was performed by comparing the conditional probability of iNOS expression or TUNEL labeling given that cells were positive for IL-10, with the probability of iNOS expression or TUNEL labeling in all cells by use of a t test. P<0.05 was considered statistically significant.

Results
Detection of IL-10 in Human Atherosclerotic Plaques
We detected IL-10 mRNA in 12 of 17 atherosclerotic plaques analyzed by using RT-PCR, whereas IL-10 mRNA was not found in control carotid arteries (Figure 1). IL-10 protein expression was detected in 16 of 21 plaques examined by using immunohistochemical techniques (Figure 2). There was no staining for IL-10 in the media of control carotid and internal mammary arteries. Analysis of adjacent serial sections and double-stained sections revealed that most IL-10–positive cells were macrophages (72±3% of IL-10–positive cells) (Figure 2A), identified by staining with anti-CD68 antibody. It is noteworthy that smooth muscle cells, identified by their morphological features and by positive staining for α-actin, also showed cytosolic positive staining for IL-10 (18±3% of IL-10–positive cells) (Figure 2B). IL-10 staining was also detected extracellularly. No or very rare T lymphocytes stained for IL-10. The specificity of the immunostaining was confirmed by Western blot analysis of plaques showing a single band at 36 kDa (Figure 3) corresponding to the noncovalently linked IL-10 homodimer.12

Relation Between IL-10 Expression, iNOS Expression, and TUNEL Labeling
As previously reported,10,13–18 iNOS expression and cell death were detected in the plaques by using immunohistochemistry and TUNEL labeling, respectively (see below). There was no staining for iNOS or TUNEL in the control carotid arteries.

As shown in Table 1, most microscopic fields with positive iNOS expression (≈75%) showed low or no IL-10 expression (Figure 4A and 4C). Conversely, most fields with no iNOS expression (72%) showed moderate to high levels of IL-10 expression. The effect of the level of IL-10 expression in the plaques on iNOS expression was highly significant (χ²=130.5, P<0.0001).

As shown in Table 1, cell death revealed by TUNEL labeling was detected in only 2.5% of fields with moderate to high levels of IL-10 expression. Conversely, cell death was much more frequently observed in fields with low or no IL-10 expression (28% of these fields) (Figure 4A and 4B). These findings indicate that there is a strong association between high levels of IL-10 expression and reduced cell death in the plaque (χ²=70.7, P<0.0001). These results were confirmed by analysis of double-stained sections as shown in Table 2. Quantitative analysis of these sections revealed that the

<table>
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<tr>
<th>Level of IL-10 Expression</th>
<th>0 (no staining)</th>
<th>+ (10% to 50%), n=63</th>
<th>++ (&gt;50%), n=175</th>
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<tr>
<td>Microscopic fields</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>positive for iNOS</td>
<td>181 (82%)</td>
<td>16 (40%)</td>
<td>17 (27%)</td>
</tr>
<tr>
<td>negative for iNOS</td>
<td>41 (18%)</td>
<td>34 (60%)</td>
<td>46 (73%)</td>
</tr>
<tr>
<td>Microscopic fields</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive for TUNEL</td>
<td>68 (31%)</td>
<td>6 (15%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>negative for TUNEL</td>
<td>154 (69%)</td>
<td>34 (85%)</td>
<td>56 (90%)</td>
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Figure 3. IL-10 expression in human atherosclerotic plaques. Western blot analysis with antibodies against human IL-10 revealed a single band of the appropriate size of 36 kDa corresponding to IL-10 homodimer. Plaques used for Western blotting were different from those used for RT-PCR analysis in Figure 1.

Figure 2. Intense staining for IL-10 (red) in macrophages (A) and smooth muscle cells (B, arrowheads) of human atherosclerotic plaques. Original magnifications ×630 (A) and ×600 (B).
probability of iNOS protein expression or TUNEL positivity was ≈3-fold lower in IL-10–positive cells than in total cells (Table 2). This is illustrated in part in Figure 5. The results were similar regardless of the region of the plaque analyzed (fibrous cap, shoulder, or lipid core).

**Discussion**

Recently, Uyemura et al. found IL-10 mRNA expression in 2 of 7 noncomplicated atherosclerotic plaques, but no information was reported about the cellular expression and localization of the corresponding protein, and it is still unknown whether IL-10 is expressed in advanced atherosclerosis. In the present study, we analyzed advanced atherosclerotic plaques and found IL-10 mRNA expression in 12 of 17 plaques examined. We also performed immunohistochemical studies and detected IL-10 protein in 16 of 21 plaques. As expected, immunoreactive IL-10 was detected in the extracellular matrix, and it was expressed mainly by macrophages. It is noteworthy that staining of serial adjacent sections as well as double staining showed that IL-10 also localized to the cytoplasm of several smooth muscle cells. Although not definitive proof, this finding strongly suggests that these smooth muscle cells were producing IL-10. However, we were not able to detect either IL-10 mRNA or IL-10 protein in human aortic smooth muscle cells in culture, whether unstimulated or stimulated by minimally modified LDLs, oxidized LDLs, or a mixture of tumor necrosis factor-α, IL-1β, and interferon-γ (unpublished data). That very specific environmental conditions or cell phenotype are required for smooth muscle cells to produce IL-10 cannot be ruled out.

Expression of IL-10 in human atherosclerotic plaques may have several potential effects. Because IL-10 is a potent antiinflammatory cytokine with deactivating properties in macrophages,8,9,20 it is likely that its expression by plaque macrophages would limit the inflammatory response and promote plaque healing. It has recently been shown that endogenous production of IL-10 by human monocytes in response to LDL stimulation inhibits IL-12 production,19 indicating a cross-regulatory action of IL-10 that may counterbalance the proinflammatory response. In our study, IL-10 expression in advanced human atheroma was associated with a 3-fold decrease in the probability of iNOS expression. Because iNOS is a major mediator of the inflammatory role for IL-10 in human atherosclerosis.

Apoptosis is known to occur in the atherosclerotic plaque.10,14–18 The TUNEL method may, in some instances, reveal other processes than apoptosis,11 essentially because of delayed prefixation times and misuse of proteinase K. In the present study, tissues were immediately fixed in 4% paraformaldehyde after carotid endarterectomy and digestion with proteinase K before TUNEL labeling was omitted. Such material processing greatly enhances the specificity of TUNEL for apoptosis.11 Moreover, we have previously shown that TUNEL staining in the plaque is highly associated with caspase-3 expression10 and it is well accepted that caspases are specific features of apoptosis.21 Several potential inducers of apoptosis in the plaque have been identified including oxidized LDLs and proinflammatory cytokines.22,23 However, little is known about the expression of antiapoptotic factors in human atherosclerosis. Our study supports that the counterregulatory effect induced by anti-inflammatory

<table>
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<th>TABLE 2. Quantitative Analysis of the Percentages of Cells Positive for iNOS or TUNEL in IL-10–Positive Cells or in Total Cells</th>
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<tr>
<td><strong>IL-10–Positive Cells</strong></td>
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<tr>
<td>Percentages of iNOS cells</td>
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<td>Percentages of TUNEL cells</td>
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Figure 4. Three serial adjacent sections stained with anti-iNOS antibody (A), TUNEL (B), or anti-IL-10 antibody (C). Counterstaining was performed by using hematoxylin. Intense staining for iNOS (brown staining) in this region of plaques was associated with positive staining for TUNEL, as shown in a magnified zone from the center of this area (brown staining, arrowheads) (B) but no staining for IL-10 (C). Original magnifications ×250 (A) and ×800 (B and C).
c cytokines, especially IL-10, on the inflammatory response may protect from excessive cell damage and death in the plaque. Our results are in agreement with previous studies showing antiapoptotic properties for IL-10 in cultured macrophages

In conclusion, we show that IL-10 is expressed in advanced human atherosclerosis and is associated with low iNOS expression and low levels of cell death. Therefore, IL-10 may play a critical role in atherosclerotic plaque formation and progression.

Acknowledgments

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References


Figure 5. Double staining for TUNEL (brown staining) and IL-10 protein (red staining) by use of immunohistochemical techniques (see Methods). Counterstaining was performed, using hematoxylin yielding blue nuclei. A, Representative photomicrograph from a region of a human atherosclerotic plaque showing intense staining for IL-10 (red staining) but no staining for TUNEL. B, Representative photomicrograph from a region of a human atherosclerotic plaque showing staining for TUNEL (brown nuclei, arrowheads) but no or very little staining for IL-10. C, Immunoreactive IL-10 (red staining, arrows) preferentially localized to nonapoptotic cells in TUNEL–/IL-10+ areas. Arrowhead shows an apoptotic cell with no IL-10 staining. Original magnifications ×200 (A), ×350 (B), and ×900 (C).


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