Interleukin-1 Receptor Antagonist Expression in Human Endothelial Cells and Atherosclerosis

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Abstract—The proinflammatory cytokine interleukin (IL)-1 is expressed mainly within the endothelium of atherosclerotic plaques and may be linked with inflammatory mechanisms of atherogenesis. IL-1 action is complex and regulated in part by its naturally occurring inhibitor, the IL-1 receptor antagonist (IL-1ra). Therefore, we studied differential and specific isoform expression of IL-1ra in the endothelium of diseased coronary arteries and in endothelial cells (ECs) stimulated under defined conditions. In view of an association with IL-1ra gene (IL-1RN) polymorphism, the influence of endothelial cell genotype at IL-1RN on IL-1ra protein production was also examined. Secreting IL-1ra and intracellular IL-1ra mRNAs were detected by semiquantitative reverse transcription–polymerase chain reaction in human atherosclerotic and dilated cardiomyopathic coronary arteries; protein expression appeared increased in atherosclerotic compared with dilated cardiomyopathic arteries, where IL-1ra appeared to be confined to the endothelium. Only intracellular IL-1ra type 1 mRNA was detected in human umbilical vein ECs (HUVECs) and human coronary artery ECs (HCAECs) when they were stimulated with bacterial lipopolysaccharide/phorbol myristate acetate and transforming growth factor-β. IL-1β and IL-1α were without effect. IL-1ra protein was detected in HUVECs (intracellular IL-1-ra), HCAECs (intracellular IL-1-ra), and human coronary artery smooth muscle cells (intracellular IL-1-ra) by immunoprecipitation and Western blot. IL-1ra was detected in HUVEC cell lysates by ELISA and appeared to be influenced by the genotype of the IL-1RN variable number tandem repeat, an 86-bp repeat polymorphism in intron 2 of the IL-1ra gene, with lower levels of IL-1-ra produced by IL-1RN allele 2–containing cells (ratio of IL-1-ra to total protein: for 1,1 homozygotes, 1.38; for 1,2 heterozygotes, 0.81; and for 2,2 homozygotes, 0.63; P<0.05 compared with 1,1 homozygotes). This is the first demonstration of IL-1ra in human diseased arteries, stimulated HUVECs, and HCAECs and indicates the endothelial cell as an important source. Endothelial IL-1ra production may be controlled by the endothelial IL-1RN genotype. These data further support the role of the IL-1 system of cytokines in the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2000;20:2394-2400.)

Key Words: interleukin-1 receptor antagonist endometheium inflammation human coronary arteries

Atherogenesis is a complex process, but endothelial cell (EC) activation appears to be a central theme. Stimulation and activation of ECs by the proinflammatory cytokine interleukin (IL)-1 causes multiple responses within the endothelium but most notably induces the expression of adhesion molecules, which promote monocyte recruitment and infiltration into the arterial wall. Local plaque synthesis of IL-1 is well established in atherosclerosis, and in particular, ECs are an abundant source of IL-1 at the luminal surface and in the adventitial capillaries.

IL-1 action is complex and is regulated at multiple stages. Processed mature IL-1 signals via the type I IL-1 receptor but may also bind to a nonsignaling receptor (IL-1 receptor type II). The IL-1 receptor antagonist (IL-1ra) is a secreted protein product of a gene adjacent to the IL-1B gene and binds to IL-1 type I and II receptors without signaling. IL-1ra is an acute-phase reactant, and levels in a number of biological systems vary in parallel with IL-1. The IL-1ra gene (IL-1RN) produces a number of mRNA splice variants. The secreted form (sIL-1ra) inhibits IL-1 action by binding to the type I IL-1 receptor, but the other variants remain intracellular (types I, II, and III). The mode of action of the intracellular forms remains to be elucidated.

Because IL-1β is upregulated in the endothelium of atherosclerotic vessels, we studied the distribution and synthesis of IL-1ra in atherosclerosis and in cultured ECs. It has previously been reported that human umbilical vein ECs (HUVECs) do not express IL-1-ra. In addition, we studied the control of IL-1ra protein production by ECs after our report of the association between IL-1RN polymorphism and single-vessel coronary disease.

Methods

Cell Culture
HUVECs, isolated from umbilical cords routinely collected from our hospital maternity unit, were cultured at 37°C on gelatin-coated
flasks in medium 199 supplemented with 20 μg/mL EC growth supplement (Sigma Chemical Co), 90 μg/mL heparin (Sigma), 10% FCS, and 10% newborn calf serum. At confluence, HUVECs were passaged by trypsin/EDTA dispersion, seeded at a density of 8×10^4 cells/cm^2, and used at passage 2 throughout. Human coronary artery ECs (HCAECs) and human coronary artery smooth muscle cells (HCASMCs), both from single donors, were purchased from Clonetech and cultured according to the suppliers’ instructions. HCAECs were characterized by the manufacturer by positive immunostaining for acetylated LDL, uptake factor VIII–related antigen, and negative immunostaining for α-smooth muscle cell actin. HCASMCs were characterized in a similar way but were negative for EC markers. ECs were stimulated with lipopolysaccharide (LPS, 100 ng/mL), phorbol myristate acetate (PMA, 10 ng/mL; both from Sigma), and transforming growth factor (TGF)-β (10 ng/mL, R&D Systems). HCAECs and HCASMCs were used only up to passage 3 in our experiments.

Human peripheral blood mononuclear cells were prepared with the use of Lynphoprep (Nycomed) and stimulated with 100 ng/mL LPS for 5 hours.

Cell-associated IL-1ra was measured by use of a commercially available ELISA (R&D Systems). Genotyped HUVECs were lysed in 9 mmol/L CHAPS buffer for 1 hour at 37°C and stored at −80°C. Particulate material was removed by centrifugation before analysis. IL-1ra ELISA sensitivity was 17 pg/mL, and all measurements were made in duplicate. Total protein was determined by using a micro-BCA assay (Pierce). IL-1ra levels were normalized to the amount of total protein and compared with the IL-1RN genotype. HUVECs were genotyped for IL-1RN (variable number tandem repeat [VNTR]) by use of a previously described method.6 The common 4 repeat polymorphism was designated allele 1 (*1); the less common 2 repeat, allele 2 (*2).

Human Coronary Arteries

Human diseased arteries from patients with ischemic heart disease (IHD, n = 4) and dilated cardiomyopathy (DCM, n = 4) were obtained from the explanted hearts of cardiac transplant recipients in accordance with our institutional ethics policy.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared and used for reverse transcription (RT)–polymerase chain reaction (PCR) as described previously.4 After an initial denaturation step, amplifications were at 55°C for 2 minutes for 35 cycles, with a final extension at 72°C for 2 minutes. Product accumulations had not reached a plateau phase, as determined by inclusion of a hot nucleotide and electrophoretic separation of products obtained at different cycles. For amplification of IL-1ra, sense primers were 5'-AGAAGACCTCCTGTCCTATG 3' (types I, II, and III) and 5'-ATGGAAATCTGCAGAGGCCTC 3' (secreted), and the antisense primer (5'-TACTGCTTCTCTGGAGAATA 3') was used for all isoforms.5 Controls without reverse transcriptase were performed, and the primers crossed an intron. β-Microglobulin primers were 5'-CCTTGGAGCTATCCAGCCGTCT 3' and 5'-CCATGATGCTGCTTACCT 3'.

Products were run on 8% polyacrylamide gels and visualized by ethidium bromide staining. For DNA sequencing, RT-PCR products were ligated into pCRII (Invitrogen) and manually sequenced by use of Sequenase (Amersham).

For in situ RT-PCR, HUVECs were stimulated as described above and fixed in 10% neutral buffered formaldehyde for 20 minutes. RT was performed using 5 mmol/L MgCl2, 1× RT buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, pH 9.0), 0.1% Triton, 1 mmol/L dNTPs, 1 U RNAsin, 2.5 U avian myeloblastosis virus RT, and 1 mmol/L IL-1ra antisense primer and was performed by use of a Techne PHC-3 Dri Block Thermocycler (Techne Ltd). cDNA was amplified using an intracellular IL-1ra (iIL-1ra) type I sense primer and the universal antisense IL-1ra primer (detailed elsewhere) incorporating 10 mmol/L digoxigenin-labeled dUTP (Boehringer-Mannheim) for 35 cycles, with an annealing temperature of 55°C. A positive signal was detected by using anti-digoxigenin alkaline phosphatase Fab fragments (1:100, 30 minutes, Boehringer-Mannheim) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyolphosphate (Biogenex). Cells were counterstained with 1% Nuclear Fast Red (BDH) and mounted in Aquamount (BDH). Negative controls included no avian myeloblastosis virus RT to eliminate the possibility that the amplified product was from genomic DNA and cells treated with RNase.

Immunoprecipitation and Western Blot Analysis of Cell Lysates

Immunoprecipitation or Western blot analysis was performed with HUVECs, HCASMCs, and human keratinocytes (kindly donated by S. MacNeil, University of Sheffield, Sheffield, UK). Approximately 1×10^6 HUVECs and HCASMCs were lysed in NP-40 buffer (150 mmol/L NaCl, 50 mmol/L Tris [pH 8.0], and 1% Nonidet P-40) and incubated with 3 μg of anti-human IL-1ra goat polyclonal antibody (R&D Systems) for 2 hours at room temperature, followed by a 1-hour incubation with protein G–Sepharose beads (Sigma). The beads were pelleted and washed, and the bound proteins were eluted by boiling the sample in reducing buffer for 5 minutes before loading on a polyacrylamide gel for Western analysis. Approximately 1×10^6 HCASMCs were lysed in NP-40 buffer and digoxigenin by use of Western blot. Samples were electrophoresed on 18% polyacrylamide gels, followed by semidry transfer to 0.2 μm nitrocellulose. The membrane was blocked overnight in blocking buffer (10% nonfat milk/0.1% Tween 20 in PBS) and hybridized with 14 μg primary antibody (R&D Systems) for 1 hour at room temperature. Bound antibody was detected with a horseradish peroxidase–labeled rabbit anti-goat secondary antibody (1:1000, Dako), and the signal was visualized with enhanced chemiluminescence with the use of ECL detection reagents (Amersham), followed by exposure to XAR-Omat film for 15 minutes.

Immunohistochemistry

Frozen acetone-fixed sections were double-stained for IL-1ra and IL-1β. Endogenous peroxidases were inhibited with 0.3% hydrogen peroxide in methanol. A primary antibody to human IL-1β (1:100, Genzyme) was applied for 60 minutes, and then a biotinylated anti-mouse secondary antibody and peroxidase-conjugated tertiary antibody were applied for 40 minutes each; visualization was accomplished with diaminobenzidine. The secondary antibody to IL-1ra (1:70, gift of W. Arend, Denver, Colo; monoclonal antibody batch 642), which recognizes all isoforms of IL-1ra,10 was applied for 60 minutes, and then an alkaline phosphate–conjugated rabbit anti-mouse secondary antibody was applied; the complex was visualized with New Fuschin (Dako). Other combinations of ABC peroxidase and secondary antibodies gave unacceptable background. In addition, control experiments were performed to ensure that the IL-1ra antibody did not bind to the initial anti–IL-1 monoclonal antibody or that the second layer in the first sequence did not bind to the second IL-1ra monoclonal antibody. Sections were counterstained with hematoxylin and mounted in DPX. Negative controls were without primary and secondary antibodies; for preabsorption experiments, the antibodies were incubated with specific antigen for 30 to 60 minutes at room temperature before the addition to tissue sections. In addition, each antibody was used separately to confirm that the dual staining seen was real and represented the true expression of both antigens.

Results

Human Arteries

In arteries from patients with DCM and IHD, IL-1ra protein was located almost exclusively in the endothelium (Figure 1a and b, DCM section; Figure 1c, IHD section), with suggestion that the increase in the amount of IL-1ra observed in IHD arteries in this small study. In the sections from IHD arteries, positive staining was also seen in macrophage-rich areas (Figure 1c). Numerous negative controls (no antibody, preabsorption) showed background staining only (Figure 1d).

A representative example of RT-PCR of IL-1ra mRNA from atherosclerotic and DCM arteries is shown in Figure 2e. Bands migrating at 510 bp (iIL-1ra type I) and 533 bp...
(sIL-1ra) were confirmed by DNA sequencing. Figure 2f shows semiquantification of these data, with IHD arteries exhibiting more sIL-1ra and icIL-1ra mRNA than DCM arteries ($P=\text{NS}, n=4$). Internal mammary arteries were also examined and showed weak expression of sIL-1ra only (data not shown).

**Human ECs**

Isolated HUVECs stimulated with a combination of LPS (100 ng/mL) and PMA (10 ng/mL) over an 8-hour time course express only icIL-1ra type I at 2 hours after stimulation, with decreased expression observed at 8 hours (Figure 2a). Identity of the single band migrating at 510 bp was confirmed by sequencing. Expression of icIL-1ra at 0 hours was variable and observed in 2 of 6 batches of HUVECs. Semiquantitative RT-PCR was attempted but was not possible with the use of simultaneous amplification because of the low signal obtained for icIL-1ra and technical problems with competition from primer sets arising from this. Stimulation of HUVECs with recombinant human IL-1$\alpha$ or IL-1$\beta$ did not induce the expression of any form of IL-1ra (data not shown). TGF-$\beta$ (10 ng/mL) induced the expression of icIL-1ra type I in HUVECs (Figure 2b) at 4 hours; this expression was still evident at 24 hours, and this response was dose dependent (Figure 2c), with no detectable expression at 50 ng/mL. Expression of icIL-1ra type I mRNA in HUVECs was cytoplasmic, as determined by in situ RT-PCR (Figure 2d).

HCASMCs express icIL-1ra type I mRNA constitutively, which can be upregulated by stimulation with LPS and PMA (as described above) over an 8-hour time course (Figure 3a).
and with TGF-β (10 ng/mL) over a 24-hour time course (data not shown). Recombinant human IL-1α (1 ng/mL) and IL-1β (40 pg/mL) also induced the expression of icIL-1ra type I in HCASMCs (data not shown).

Isolated HCAECs stimulated with a combination of LPS (100 ng/mL) and PMA (10 ng/mL) express only icIL-1ra type I mRNA (Figure 3b). HCAECs stimulated with TGF-β (as described above) also induce the expression of icIL-1ra type I mRNA (data not shown).

sIL-1ra mRNA was never detected under basal or stimulated conditions in HUVECs or HCAECs. Positive controls (polymorphonuclear cells) were documented to synthesize sIL-1ra under the same conditions used above for ECs (Figure 2a).

Immunoprecipitation and/or Western blot was used to confirm the intracellular expression of particular isoforms of IL-1ra in HUVECs and HCASMCs. icIL-1ra was detected in HUVECs (higher molecular weight bands in immunoprecipitation lanes are nonspecific because of immunoglobulin binding, as determined by the negative control). In HCASMCs, by standard Western analysis, icIL-1ra was detected in LPS/PMA-stimulated cells (Figure 4). To further reinforce the intracellular nature of IL-1ra in HUVECs, IL-1ra was never detected in EC culture media with the use of a specific IL-1ra ELISA and was measurable only in cell lysates (see below).

**IL-1RN Genotype and IL-1ra Protein Production**

IL-1ra protein levels were measured in IL-1RN–genotyped unstimulated HUVECs. The rare homozygous genotype (IL-1RN *2/*2) was associated with 2- to 3-fold less IL-1ra protein than the common genotype (Figure 5, *P* <0.05, paired t test). The heterozygote *1/*2 had an intermediate level of IL-1ra production.

**Discussion**

These data demonstrate icIL-1ra type I mRNA, sIL-1ra mRNA, and IL-1ra protein in human coronary arteries. IL-1ra protein colocalizes with IL-1β predominantly in the endothelium of these arteries, with less IL-1ra mRNA expression occurring in less-diseased DCM arteries. To our knowledge, this is the first documentation of IL-1ra expression by HCAECs. We also show only icIL-1ra type I mRNA expression in HUVECs and HCAECs stimulated in vitro with LPS/PMA or TGF-β. Previous reports have failed to detect IL-1ra mRNA in these cell types with the use of other stimuli and less sensitive detection methods, such as Northern blots, and there are no previous reports of immunohistochemistry for IL-1ra in human vessels. With the use of immunoprecipitation/Western blot techniques, our data also confirm that the expression of IL-1ra in HUVECs and HCAECs is intracellular. We have also demonstrated that the rare allele (IL-1RN *2/*2) of a VNTR polymorphism in the IL-1RN gene is associated with significantly reduced levels of IL-1ra in HUVECs under basal culture conditions. These data have important implications for the genetic control of IL-1 cytokines in the arterial wall.

The IL-1 cytokine system has been implicated in vascular disease in a number of studies. IL-1 has been detected in plaque cells, in luminal ECs and macrophages, in the sera
of patients with IHD and unstable angina, and in myocytes and infiltrating leukocytes in human DCM. Less is known about the distribution and role of IL-1ra in vascular material, although IL-1ra mRNA has previously been detected in rat brain vasculature. In addition, high levels of IL-1ra have been detected in rat vascular smooth muscle cells in culture, which constitutively produce icIL-1ra upregulated by TGF-β in a manner analogous to that described in the present study. Human saphenous vein smooth muscle cells also constitutively express icIL-1ra, which can be upregulated after stimulation.

The IL-1ra system is part of a complex regulatory system that controls IL-1 signaling; secreted IL-1ra blocks transmission of a signal by the IL-1 receptor. sIL-1ra is measurable in plasma and behaves as an acute-phase reactant. Levels during unstable angina parallel IL-6 levels and appear to have prognostic implications.

The role of the intracellular splice variant of the IL-1ra gene is much less clear. Although exogenously added icIL-1ra can inhibit IL-1–induced production of IL-6, IL-8, and monocyte chemotactic protein in ECs, it does not have agonist activity. One suggested role for icIL-1ra has been that of providing a counterbalance for constitutive intracellular IL-1α (C.A. Dinarello, personal communication, December 1998).

Figure 3. IL-1ra in HCASMCs and HCAECs. a, HCASMCs stimulated with 100 ng/mL LPS and 10 ng/mL PMA (n=1) for 0, 2, 4, and 8 hours (lanes 2 to 5, respectively). Note that icIL-1ra type I mRNA expression is detectable at 0 hours and is upregulated with stimulation. No sIL-1ra was detected. Negative and positive controls are shown in lanes 6 and 7, respectively. Lane 1 shows DNA markers. b, Representative data of HCAECs stimulated with LPS/PMA (n=3) for 0, 2, 4, and 8 hours (lanes 2 to 5, respectively). icIL-1ra type I is evident as indicated by the band at 510 bp. Lanes 6 and 7 are negative and positive controls, respectively. The loading control (β2MG) indicates equal loading.

Figure 4. Immunoprecipitation and Western analysis of IL-1ra isoforms in HUVECs and HCASMCs. Standard Western analysis of HCASMCs stimulated with 100 ng/mL LPS and 10 ng/mL PMA for 0 and 24 hours (lanes 2 and 3, respectively) is shown. Immunoprecipitated protein from HUVECs and a negative control (no cell lysate) are shown in lanes 4 and 5, respectively. icIL-1ra (18 kDa) is detected in HUVECs and HCASMCs. Keratinocytes are a positive control (lane 1).

Figure 5. IL-1RN genotype correlated with IL-1ra protein production in unstimulated HUVEC lysates. Graphic representation of the mean IL-1ra protein concentration is normalized to total protein production in HUVECs genotyped as 1,1 homozygotes, 1,2 heterozygotes, and 2,2 homozygotes. The IL-1ra protein/total protein ratio in 2,2 homozygotes is significantly less than in 1,1 homozygotes (*P<0.05).
of signals. Interestingly, although some experimental systems indicate a concordance of IL-1 and IL-1ra synthesis, IL-1α and IL-1β were unable to stimulate IL-1ra production in HUVECs in our laboratory.

The RNA profiles in ECs and HCASMCs appear different; in HCASMCs, IL-1 mRNA is always detectable at time 0 (as detected by others), whereas in HUVECs/HCAECs, this is not the case. IL-1 mRNA was detectable in only 2 of 6 batches of HUVECs at time 0.

In contrast to this, our experiments in HUVECs show IL-1ra protein at time 0, and although this seems at variance with the mRNA data presented, we suggest that this is due to a cellular storage phenomenon similar to that observed recently for the chemokine IL-8, which is stored in Weibel-Palade bodies. Because it is well known that up to a 100-fold molar excess of IL-1ra is required to counteract the action of IL-1 in a disease situation, we suggest that de novo synthesis would not be very efficient in meeting this requirement; hence, storage of IL-1ra within the cell would be preferable to prevent a delay in the action of IL-1ra.

The impact of the IL-1ra intron 2 VNTR polymorphism on the basal production of IL-1ra by ECs is of considerable interest because it implies a further level of regulation of the basal production of IL-1ra by ECs. In monocytes, previous reports have indicated that IL-1RN*2 is associated with increased production of IL-1ra protein. In columnar epithelial cells, IL-1RN*2 is associated with reduced protein production. Our present data in ECs concurs with that in epithelial cells. It is interesting to speculate that the impact of this polymorphism is different in cells synthesizing different mRNA splice variants; those synthesizing the intracellular form produce less IL-1ra when carrying IL-1RN*2, in contrast to monocytes that synthesize predominantly sIL-1ra and produce more protein when of genotype IL-1RN*2. This could be one explanation for the observed differences, but complex relationships may exist between the IL-1RN*2 variant and other polymorphisms and mutations in the IL-1ra gene or within neighboring genes within the IL-1 cluster that could also determine the absolute levels of protein.

The studies of human coronary arteries indicate that IL-1ra is synthesized by atherosclerotic and DCM arteries. The number of arteries available was small, and immunohistochemistry and RT-PCR are at best semiquantitative, but small differences were suggested. sIL-1ra is well known to be expressed in macrophages, and inasmuch as ischemic arteries have increased numbers, this could account for the increase in IL-1 mRNA documented therein. These data concur with those on IL-1β mRNA and protein, which have also been shown to be upregulated in atherosclerosis, although IL-1β has been documented in DCM arteries previously. These data may suggest that the endothelial IL-1ra system is more constitutively active than is the IL-1 system, although our cell culture experiments with inflammatory agents do indicate the ability to induce further synthesis.

The data in the present study can only speculate on the role of IL-1ra in atherogenesis. The current popular model suggests that the atherosclerotic program is driven by a proinflammatory mechanism. In the context of the IL-1 system, this might indicate that IL-1ra would have a generally beneficial effect or retarding effect on atherogenesis. This is supported by evidence that IL-1ra inhibits fatty streak formation in apoE knockout mice. Further support for these data is also provided by the recent observation that IL-1ra gene knockout mice develop a chronic inflammation of the arterial wall. Our own group has also published an association between IL-1ra VNTR polymorphism and the presence of single-vessel coronary disease, and others have shown an association with a single nucleotide IL-1RN polymorphism (in complete linkage with the IL-1RN VNTR) and carotid atherosclerosis in African-Americans. Expansion of these data are reported in the present study, with evidence that the IL-1ra genotype may predict a relatively proinflammatory phenotype with lower IL-1ra levels, thus accelerating the atherosclerotic program in a manner consistent with the apoE mouse experimental data.

We interpret these data with caution, however, because the biology of atherosclerosis is complex and multifactorial. IL-1ra could interact with the IL-1 system in a number of ways, and the net effect will be an integrated response determined by the level of IL-1 activation and the abundance of the type II IL-1 receptor (decoy) as well as levels of agonists and antagonists. It is also unclear whether the nature of the biological response elicited by the IL-1 system is the most relevant to the development of atherosclerotic disease.

Therefore, we favor continued investigation of the mechanism of control of IL-1 in vascular endothelium. The data in the present study indicate that the IL-1ra system may be a biological control mechanism with the potential to modulate the development of coronary arterial disease.

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