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A Functional Interleukin-1 Receptor Antagonist Polymorphism Influences Atherosclerosis Development

—— The Interleukin-1β:Interleukin-1 Receptor Antagonist Balance in Atherosclerosis ——

Peder S Olofsson, MD, PhD*,**; Yuri Sheikine, MD, PhD*,†,§; Ken Jatta, PhD††; Mehran Ghaderi, PhD†; Ann Samnegård, MD, PhD‡; Per Eriksson, PhD*; Allan Sirsjö, PhD††

Background: Interleukin (IL)-β plays a central role in inflammation and atherosclerosis, but levels of IL-1β, its natural antagonist, IL-1Ra, and their balance in human atherosclerotic lesions, are unknown. Knowledge of protein levels in atherosclerosis and the influence of a functional *IL-1Ra* polymorphism would increase the understanding of atherosclerosis pathogenesis.

Methods and Results: Fresh and endotoxin-stimulated explanted human atherosclerotic and normal arteries were analyzed for IL-1β, IL-1Ra and IL-1 receptor 1 (IL-1R1) using TaqMan PCR and enzyme-linked immunosorbent assay. Two hundred forty-three survivors of a first myocardial infarction were genotyped for a polymorphism in *IL-1Ra* and their coronary atherosclerosis analyzed by using coronary angiography. Levels of IL-1β, IL-1Ra and IL-1R1 mRNA were significantly increased in atherosclerotic arteries compared with normal arteries. Endotoxin stimulation increased IL-1β levels more than IL-1Ra levels (ie, promoted a pro-inflammatory state). A polymorphism in *IL-1Ra* known to increase levels of IL-1Ra was associated with decreased mean coronary artery plaque area.

Conclusions: Activation of innate immunity changed the balance between IL-1β and IL-1Ra in atherosclerotic arteries towards a more pro-inflammatory state. In line with this, the presence of an *IL-1Ra* intron 2 polymorphism known to increase IL-1Ra levels, and possibly the IL-1Ra:IL-1β ratio, was associated with reduced coronary atherosclerosis. (*Circ J* 2009; **73:** 1531 – 1536)

Key Words: Atherosclerosis; Coronary artery disease; Genetic polymorphism; Immunology

Vascular inflammation is important for the development of atherosclerosis. Macrophages, T cells and other immune cells are recruited to growing atherosclerotic lesions and create a pro-inflammatory environment by producing cytokines and chemokines in the vascular wall. This milieu promotes further cellular influx, aggravation of inflammation and ultimately plaque rupture and atherothrombosis[1]

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The balance between pro- and anti-inflammatory cytokines plays a decisive role in the progression of atherosclerosis[1,2] Interleukin (IL)-1 is a prototypic inflammatory cytokine, predominantly produced by macrophages and dendritic cells, that plays a central role in inflammation[3] IL-1β activity is counterbalanced by the IL-1 receptor antagonist (IL-1Ra), which is a secreted acute-phase reactant capable of competitively binding to IL-1R1[4]

Members of the IL-1 family proteins are present in human atherosclerotic lesions[5,6] IL-1β may contribute to vascular pathogenesis by induction of adhesion molecules, chemokines and promotion of procoagulant activity[7,8] IL-1β and IL-1Ra have been suggested as an important pathogenic pair in this process and their balance has been reported to influence atherosclerosis development[2,3,9] Reduced IL-1Ra levels lead to aggravation of murine atherosclerosis[10] Patients with unstable angina have high levels of IL-1β without a corresponding increase in IL-1Ra, suggesting a shift toward a pro-inflammatory state in this high-risk group[11] The balance between IL-1Ra and IL-1β in atherosclerosis is unknown. Moreover, statins reduce IL-1β expression much more than IL-1Ra expression in peripheral blood mononuclear cells in this patient group[11] which may contribute to the beneficial anti-inflammatory effects of statins.

Activation of the innate immune system may alter the IL-1Ra:IL-1β balance. Activators of innate immunity, including microbial products, induce all three IL-1 proteins[3] Increased levels of bacterial lipopolysaccharide (LPS), an activator of the innate immune system and a strong inducer

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*Center for Molecular Medicine, Department of Medicine, **Department of Anesthesiology and Intensive Care Medicine, Karolinska University Hospital, Solna, Karolinska Institutet, Stockholm, Sweden, †Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA, ††Division of Clinical Medicine, School of Health and Medical Sciences, University of Örebro, Örebro and ‡Department of Clinical Sciences, Karolinska Institutet, Danderyd Hospital, Stockholm, Sweden

§Alternative spelling: Yury Sheykin.

Mailing address: Peder S Olofsson, MD, PhD, Center for Molecular Medicine, L8:03, Karolinska Institutet, Karolinska University Hospital, Solna, 171 76 Stockholm, Sweden. E-mail: Peder.Olofsson@ki.se

Table 1. Baseline Characteristics of the Analyzed Patient Sample Divided on *IL-1Ra* Intron 2, 86-bp Repeat Polymorphism Genotype

Genotype	42&22		44		P value
	Median	n	Median	n	
Age (years)	53 (48–56)	100	54 (50–57)	106	0.14
Sex (M/F)	80/20	100	88/18	106	0.58
BMI (kg/m^2)	26 (24–29)	100	27 (25–28)	106	0.61
Smoker (yes/no)	54/46	100	46/60	106	0.13
CRP (mg/L)	1.4 (0.7–3.3)	96	1.3 (0.7–2.7)	102	0.60
Total cholesterol (mmol/L)	5.1 (4.3–5.7)	99	5.2 (4.5–6.0)	105	0.34
Plasma triglycerides (mmol/L)	1.9 (1.1–2.4)	99	1.7 (1.3–2.1)	105	0.26
LDL-cholesterol (mmol/L)	3.3 (2.6–3.9)	95	3.2 (2.7–4.1)	102	0.31
HDL-cholesterol (mmol/L)	1.1 (0.9–1.3)	95	1.0 (0.9–1.3)	102	0.87
IL-6 (pg/ml)	0.8 (0.6–1.5)	95	0.8 (0.6–1.2)	101	0.19

Numbers are medians (interquartile range). For comparisons between groups, the Mann-Whitney U-test was used for continuous, and the χ^2 test for categorical, variables.
BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

of IL-1β in many cell types, including vascular endothelial cells and smooth muscle cells[3] is accordingly associated with aggravation of atherosclerosis[12–14] and precipitation of clinical disease.[15] Further, the increased atherosclerosis development seen after infection with LPS-containing bacteria in atherosclerosis-prone mice is, interestingly, mediated by the IL-1R1.[16]

A polymorphism in intron 2 of the *IL-1Ra* gene, characterized by the presence of a variable copy number of an 86-bp segment, influences IL-1Ra expression and, in that way, the biological effects of IL-1β.[17] This polymorphism has also been associated with human disease development.[17] In particular, the 2-repeat allele (2 repeats of the 86-bp segment) has been linked with worse clinical outcomes in chronic inflammatory diseases such as systemic lupus erythematosus,[18] diabetic nephropathy[19] and ulcerative colitis.[20] More knowledge the impact of functional *IL-Ra* polymorphisms on atherosclerosis development would increase our understanding of the importance of the IL-1 family in cardiovascular pathogenesis.

The balance between IL-1β and IL-1Ra in human atherosclerotic lesions, and their changes in response to activators of innate immunity, have so far not been investigated. Furthermore, the contribution to human atherosclerotic disease development of the *IL-1Ra* 86-bp polymorphism with its effects on IL-1Ra levels is not fully understood. In this study, we investigated the effects of LPS on the levels of IL-1 family members in human arterial biopsies and the effect of the 86-bp polymorphism of *IL-1Ra* on the severity of coronary artery disease (CAD).

Methods

These studies were approved by the regional ethical committee for human studies.

Human Specimens

Seven patients without a history of cardiovascular disease scheduled for nephrectomy and 31 patients scheduled for carotid endarterectomy were included after informed consent. Biopsies from the renal arteries and atherosclerotic lesions were retrieved peroperatively. Pieces from all renal and 9 carotid arteries were immediately frozen for RNA and protein extraction. Selected renal artery specimens were evaluated by using immunofluorescence and showed no apparent signs of inflammation or atherosclerosis (data not shown); 22 biopsies from atherosclerotic lesions, each 30 mg, were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (Gibco, Rockville, MD, USA) enriched with 30 mg/mL human albumin (Biovitrum AB, Stockholm, Sweden), and incubated for 6 h at 37°C with or without 100 ng/mL LPS from *Escherichia coli*, O55:B5 (Sigma Chemical, St Louis, MO, USA). Medium from 22 and tissue from 12 experiments were analyzed.

Study Group

The Stockholm Coronary Atherosclerosis Risk Factor (SCARF) study, aiming at discovering new biomedical and genetic markers of CAD, included 387 survivors of a first myocardial infarction (MI) under the age of 60 years, who were admitted to 1 of 3 hospitals in Stockholm, Sweden between 1996 and 2000. Basic clinical characteristics have previously been reported.[21,22] Baseline characteristics for the individuals included in this study are presented in **Table 1**. Of 387 post-MI patients, 243 underwent quantitative coronary angiography registering the degree of coronary artery stenosis and mean plaque area, among other parameters.[21] The plaque area was calculated from 2-dimensional longitudinal imaging of the coronary segment using the Medis QCA-CMS system (Leiden, The Netherlands).

RNA and DNA Isolation

Human arteries were frozen in liquid nitrogen and disrupted in a Mikro Dismembrator S (B Braun Biotech International GmbH, Melsungen, Germany). RNA was then isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The integrity and quantity of the isolated RNA was evaluated with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the RNA 6000 Nano Assay Kit (Agilent Technologies). Peripheral venous blood was drawn from the antecubital vein and DNA isolated and stored as previously described.[21]

Semi-Quantitative Real-Time RT-PCR

Using random hexamer primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1 μg of RNA was reverse-transcribed to cDNA. mRNA levels of IL-1Ra, IL-1R1 and IL-1β were assessed by semi-quantitative real-time RT-PCR in a TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA) as previously described.[23]

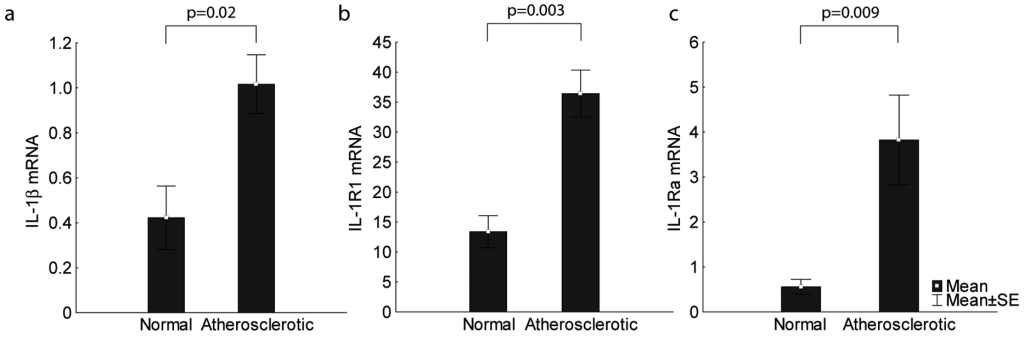

Figure 1. Genes of the interleukin (IL)-1 family were upregulated in atherosclerosis. mRNA levels, expressed as arbitrary units, of (**a**) IL-1β, (**b**) IL-1R1 and (**c**) IL-1Ra were significantly higher in atherosclerotic (n=9) than in normal arteries (n=5) as determined by real-time RT-PCR.

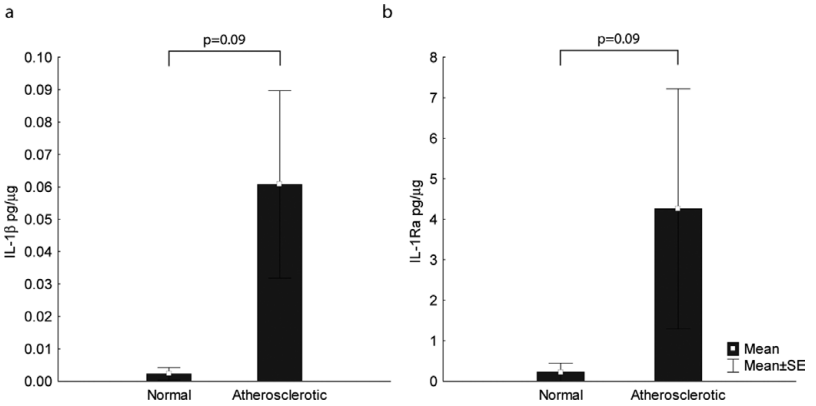

Figure 2. Protein levels of interleukin (IL)-1 family members in normal and atherosclerotic arteries. (**a**) IL-1β and (**b**) IL-1Ra levels were investigated in plaque lysates using enzyme-linked immunosorbent assay. Levels of both proteins were higher in atherosclerotic (n=5) than in normal arteries (n=5), although the difference did not reach statistical significance.

Genotyping

The region in *IL-1Ra* containing the variable number of the 86-bp tandem repeat polymorphism was analyzed as previously described[24] using the flanking oligonucleotide primers 5'-CTCAGCAACAATAATAT-3'and 5'-TCCTG-GTCTGCAGGTAA-3' under the following conditions: denaturation at 96°C for 6 min followed by 35 cycles of 96°C for 1 min, 59°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min. The number of repeats was identified by their electrophoretic migration in 1.5% agarose gels and the genotype stated as xy, where x and y represent the number of 86-bp repeats on each of the 2 alleles in an individual.

Enzyme-Linked Immunosorbent Assay (ELISA)

The IL-1β and IL-1Ra levels in the protein extract from carotid lesions[25] renal arteries and culture media were measured using ELISA Kit (R&D Systems, Minneapolis, MN, USA). Samples were analyzed on a Maxisorb® plate (Nunclon, Roskilde, Denmark) coated with mouse anti-human IL-1β or IL-1Ra antibody according to the manufacturer's recommendations (R&D Systems). The optical density was determined by measuring the absorbance at 450 nm. The absorbance was correlated against a standard curve.

Statistical Analysis

The Mann-Whitney U-test was used for comparisons between 2 groups. Values are expressed as mean±standard error. The χ^2 test was used to compare proportions; r denotes the Pearson product moment correlation coefficient. P<0.05 was considered significant.

Results

IL-1 Family Members Upregulated in Atherosclerosis

Atherosclerotic arteries and normal renal arteries were obtained peroperatively and compared for mRNA levels of IL-1β, IL-1Ra and IL-1R1. The expression levels of all three genes were significantly augmented in the atherosclerotic vessels (**Figure 1**). ELISA showed that levels of IL-1β and IL-1Ra protein also tended to be higher in atherosclerotic than normal arteries, but this difference did not reach statistical significance (**Figure 2**).

IL-1β, IL-1Ra and IL-1R1 Correlated to Leukocyte Markers in Atherosclerotic Lesions

We then wanted to study the relationship between infiltrating leukocytes and the expression of IL-1β, IL-1R1 and IL-1Ra. For this purpose, we analyzed the levels of mRNA for a T-cell marker (CD3), a macrophage marker (CD68), and a natural killer (NK) cell marker (natural cytotoxicity triggering receptor 1, NCR1) in atherosclerotic and normal arteries. There was a significant increase in the gene expression of the leukocyte markers in atherosclerotic compared with normal arteries expressed as median (interquartile range): CD3: 3.4 (1.0–6.0) vs 0.44 (0.21–1.0), P=0.004; CD68: 3.8 (0.68–9.4) vs 0.28 (0.27–0.28), P=0.003; NCR1: 12 (10–20) vs 1.1 (1.0–1.2) arbitrary units, P=0.003. The levels of all leukocyte markers correlated significantly with the expression levels of IL-1Ra, while IL-1R1 expression did not correlate with these leukocyte markers in atherosclerosis (**Figure 3**). IL-1β mRNA levels showed the strongest correlation with CD3 (**Figure 3**).

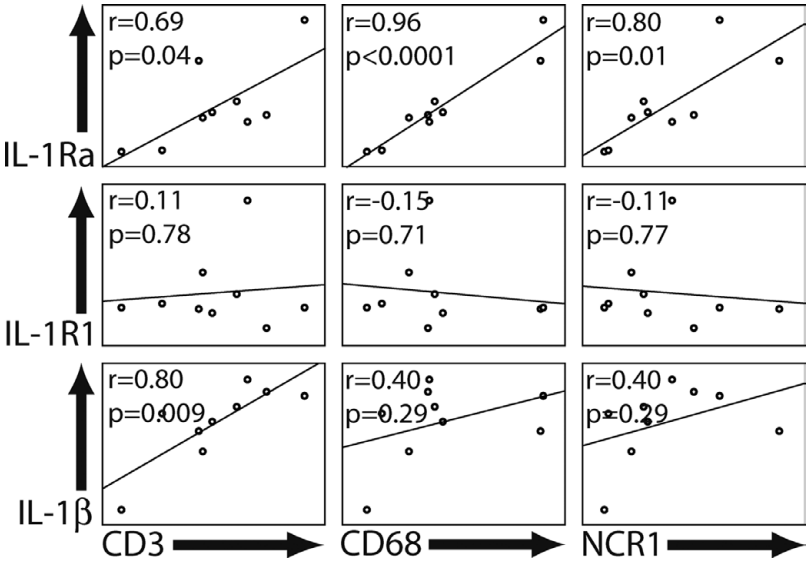

Figure 3. Correlation between interleukin (IL)-1Ra, IL-1R1 and IL-1β mRNA (y axis, arbitrary units) and leukocyte mRNA levels CD3, CD68 and NCR1 (x axis, arbitrary units) in snap-frozen atherosclerotic arteries (n=9). r denotes the correlation coefficient.

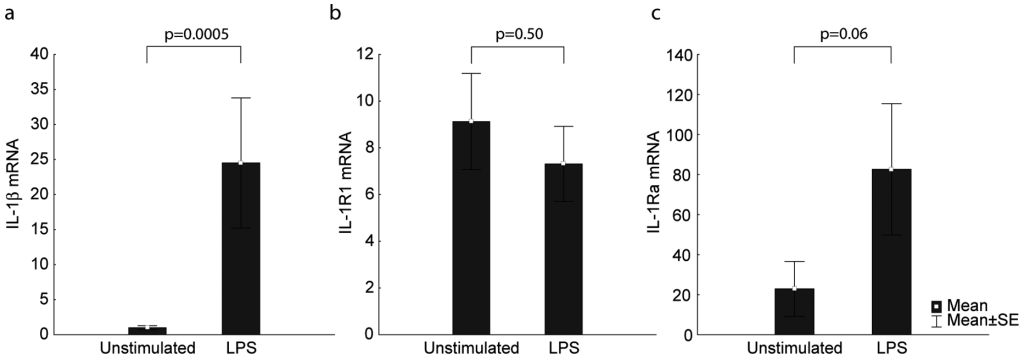

Figure 4. Lipopolysaccharide (LPS) induced interleukin (IL)-1β gene expression in atherosclerotic lesions. mRNA levels of IL-1 family members were compared in cultured atherosclerotic arteries with or without incubation with LPS. Levels were determined using real-time RT-PCR and expressed as arbitrary units. (**a**) LPS significantly induced IL-1β (n=9). (**b**) IL-1R1 expression was not significantly altered by LPS (n=6). (**c**) Although there was a trend toward an increase after treatment, IL-1Ra expression was not significantly induced by LPS (n=12).

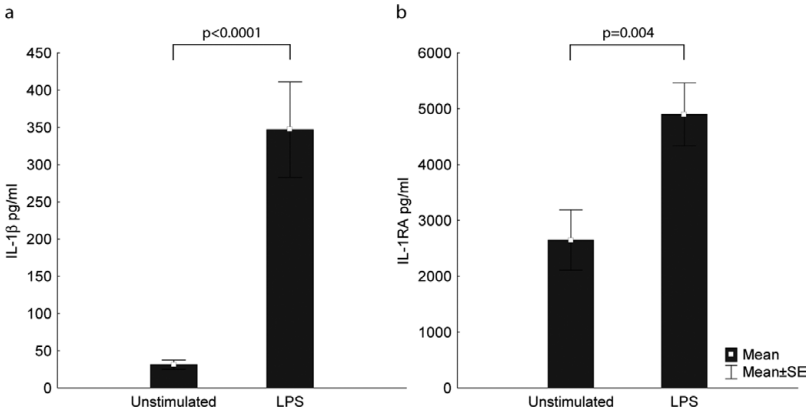

Figure 5. Lipopolysaccharide (LPS) induced interleukin (IL)-1β and IL-1Ra proteins in atherosclerotic arteries. Levels of IL-1β and IL-1Ra were determined in culture media from LPS treated (n=12) and untreated atherosclerotic arteries (n=12) using enzyme-linked immunosorbent assay. The treatment significantly induced (**a**) IL-1β and (**b**) IL-1Ra.

LPS Induced IL-1 Family Members in Atherosclerotic Lesions

Because activation of innate immunity is known to influence atherosclerosis development, we wanted to investigate the effects of a prototypic activator, LPS, on the levels of members of the IL-1 family in atherosclerosis. Fresh atherosclerotic arteries were divided into 2 pieces and incubated for 6 h with or without LPS. This treatment resulted in a significant, 25-fold increase in the mean IL-1β mRNA level, had a non-significant tendency to increase the mean IL-1Ra level and did not significantly change IL-1R1 mRNA levels (**Figure 4**). Mean levels of IL-1β and IL-1Ra proteins in conditioned media after LPS exposure of atherosclerotic arterial biopsies showed significant increases for both the

ligand and the antagonist as measured by using ELISA (**Figure 5**). Finally, LPS shifted the mean IL-1Ra:IL-1β ratio from 260:1 to 28:1 (P=0.03).

An *IL-1Ra* Polymorphism Correlated to Atherosclerotic Lesion Size

Because the 86-bp tandem repeat polymorphism in intron 2 of *IL-1Ra* has been reported to influence the levels of IL-1Ra, we wanted to investigate whether differences in this locus were associated with the development of atherosclerosis. From the SCARF cohort, 243 post-MI patients who had undergone quantitative coronary angiography were genotyped for this polymorphism; 37 patients were excluded from this comparison because of non-22, -42 or -44 genotype (n=11) or unknown genotype (n=26). The mean plaque area was significantly larger in patients with the 44 (homozygous for 4 repeats) gene polymorphism compared with carriers of the 2-allele (42 (heterozygous alleles with 4 repeats and 2 repeats) and 22 (homozygous for 2 repeats)) (**Table 2**). There was no significant difference in baseline characteristics between the analyzed genotypes in this sample (**Table 1**).

Table 2. Mean Plaque Area in Postinfarction Patients as Determined by Angiography Divided on *IL-1Ra* Intron 2, 86-bp Repeat Polymorphism Genotype

Genotype	Mean plaque area (mm^2)	
44	68.2±26.4, n=106	P=0.002
42&22	57.8±24.7, n=100	

Discussion

In this study, we determined the levels of the pro-inflammatory cytokine IL-1β and its natural inhibitor, IL-1Ra, in human atherosclerotic lesions and correlated their expression with that of leukocyte markers in the lesions. Activation of the innate immune system by LPS in atherosclerotic lesions altered the balance between IL-1β and IL-1Ra in a pro-inflammatory direction. In addition, an *IL-1Ra* polymorphism, known to increase systemic levels of IL-1Ra, was linked to decreased atherosclerotic lesion size in patients with CAD.

Pro- and anti-inflammatory cytokines play key roles in the development of atherosclerosis![1,2,26–28] Reduced IL-1Ra levels lead to aggravation of murine atherosclerosis and atherosclerosis-prone mice with a deficiency in *IL-1Ra* have undesirable cholesterol levels and a disease-prone phenotype![10,29] which implies that shifting the IL-1Ra:IL-1β balance toward IL-1Ra may be atheroprotective. These molecules have previously been identified by immunohistochemistry in human atherosclerotic lesions, but have not been investigated with quantitative methods. We found that the mean IL-1Ra:IL-1β ratio was approximately 100:1 in normal and 70:1 in atherosclerotic arteries. A 100-fold excess of IL-1Ra over IL-1β efficiently inhibits IL-1 signaling;[30] which probably translates to weak IL-1 signaling in normal arteries. In atherosclerotic arteries, levels of these 2 proteins were not only 100-fold higher, but the ratio was also shifted towards more IL-1β, presumably resulting in higher IL-1R1 activity. Interestingly, a similar ratio has been observed in the plasma of endotoxemic patients![31]

IL-1β and IL-1Ra mRNA levels correlated to markers of inflammatory cells in atherosclerotic lesions. IL-1β correlated significantly with the T cell marker CD3, but not with CD68, the marker of the main producer of IL-1β (ie, macrophages). Whether this is because of IL-1β-driven T cell chemokine production and augmented T cell recruitment or is a secondary effect on IL-1β production by the resident T cells is at present unknown. IL-1Ra mRNA levels, in contrast, correlated strongly with the T-cell, macrophage and NK cell markers. Hence, IL-1Ra mRNA levels better reflected the infiltration of inflammatory cells in these atherosclerotic arteries. Expression of the IL-1β receptor, IL-1R1, did not correlate with any of these cell markers, suggesting that the transcriptional regulation of IL-1 signaling in these lesions is not primarily at the receptor level.

Because infectious agents and innate immune activation are implied in the development of atherosclerosis![1,32–37] we wanted to investigate how LPS, a known inducer of innate immunity, influences the IL-1Ra:IL-1β balance and levels in human atherosclerosis. Exposure of explanted atherosclerotic arteries to LPS significantly increased the mean levels of IL-1β and IL-1Ra, and significantly reduced the IL-1Ra:IL-1β ratio, implying a pro-inflammatory shift. This observation, together with the fact that the IL-1Ra:IL-1β balance in the lesions is tipped towards more IL-1β already in non-treated lesions compared with normal arteries, further supports the notion that recognition of pathogens, toll-like receptor ligation and innate immune activation in atherosclerosis aggravates plaque inflammation. In view of the many different processes of disease development in which IL-1 signaling may take part, it is possible that IL-1 plays roles in both the chronic and acute development of atherosclerosis![2,8,29]

The in vivo effects on cardiovascular disease of the 2-repeat-allele polymorphism of *IL-1Ra* are not fully clear. The 2-repeat allele has been associated with increased IL-1β production in human macrophages[38] and an increased risk for single vessel, but not multivessel, CAD[39,40] and stroke![41] However, one study did not support an association between the *IL-1Ra* genotype and CAD![42]

In the present study, CAD patients carrying the 2-repeat allele had a smaller mean plaque area, as determined by quantitative coronary angiography, compared with non-carriers of this allele. This finding substantiates previous reports of slower progression of restenosis after coronary angioplasty in individuals carrying the 2-repeat allele![43–45] Others have shown that systemic levels of IL-1Ra are increased in carriers of the 2-repeat allele![46] Hence, the polymorphism of *IL-1Ra* can alter the baseline balance between IL-1Ra and IL-1β and increase the IL-1Ra:IL-1β ratio. Consequently, the efficiency of IL-1 signaling would be decreased. In view of this, we speculate that the higher systemic levels of IL-1Ra in the carriers of the 2-repeat allele reduce the efficiency of IL-1 signaling, which contributes to dampening of inflammation in the affected vessels.

In conclusion, the IL-1Ra:IL-1β ratio was shifted toward a more pro-inflammatory state in atherosclerotic compared with normal arteries. In line with this, the *IL-1Ra* polymorphism known to increase systemic IL-1Ra levels and reduce IL-1β signaling efficiency was associated with decreased coronary atherosclerosis in CAD patients carrying this polymorphism. The precise mechanisms behind our findings and their clinical relevance will require further clarification.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest.

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