# Expression of Interleukin-1β and Interleukin-1 Receptor Antagonist in oxLDL-Treated Human Aortic Smooth Muscle Cells and in the Neointima of Cholesterol-Fed Endothelia-Denuded Rabbits

**Shing-Jong Lin,[1,2,4] Hui-Tzu Yen,[3] Yung-Hsiang Chen,[1] Hung-Hai Ku,[3] Feng-Yen Lin,[3] and Yuh-Lien Chen[3]***

[1]Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan
[2]Cardiovascular Research Center, National Yang-Ming University, Taipei, Taiwan
[3]Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei, Taiwan
[4]The Division of Cardiology, Taipei Veterans General Hospital, Taipei, Taiwan

**Abstract** The migration of vascular smooth muscle cells (VSMCs) from the media to the intima and the proliferation of intimal VSMCs are key events in restenotic lesion development. These events, which are preceded and accompanied by inflammation, are modulated by the proinflammatory cytokine, interleukin-1β (IL-1β), which induces vascular smooth muscle cells to express adhesion molecules and to proliferate. IL-1β action is complex and regulated, in part, by its naturally occurring inhibitor, the IL-1 receptor antagonist (IL-1ra). Whether there was a temporal and spatial correlation between IL-1β and IL-1ra expression in, and release by, oxidized low density lipoproteins (oxLDL)-stimulated human aortic smooth muscle cells (HASMCs) was determined by using ELISA and Western blot. In addition, IL-1β and IL-1ra expression was detected in the neointima of endothelia-denuded cholesterol-fed New Zealand white rabbits by immunohistochemistry and Western blot. In HASMCs, oxLDL induced IL-β and IL-1ra expression and release in a dose- and time-dependent manner. Treatment with 20 μg/ml oxLDL resulted in increased IL-1β release after 6 h, which peaked at 24 h, and in increased IL-1ra release, first seen after 12 h, but continuing to increase for at least 48 h. In the cells, IL-β expression showed a similar pattern to release, whereas IL-1ra expression was seen in unstimulated cells and was not increased by oxLDL treatment. Confocal microscopy showed colocalization of IL-β and IL-1ra expression in oxLDL-stimulated HASMCs. oxLDL caused significant induction of nuclear factor kappa B and activator protein-1 DNA binding activity in HASMCs (6.6- and 3.3-fold, respectively). In cholesterol-fed endothelia-denuded rabbits, the notably thickened intima showed significant IL-1β and IL-1ra expression. These results provide further support for the role of IL-1 system in the pathogenesis of restenosis. This is the first demonstration of IL-1β and IL-1ra expression and secretion of oxLDL-treated HASMCs and their expression in the rabbit neointima, suggesting that the smooth muscle cells of the intima are an important source of these factors. J. Cell. Biochem. 88: 836–847, 2003. © 2003 Wiley-Liss, Inc.

**Key words:** IL-1β; IL-1ra; inflammation; smooth muscle cells; restenosis

*Correspondence to: Dr. Yuh-Lien Chen, Institute of Anatomy and Cell Biology, National Yang-Ming University, No. 155, Section 2, Li-Nong Street, Shih-Pai, Taipei 112, Taiwan. E-mail: chenyl@ym.edu.tw

The migration of smooth muscle cells into the intima, followed by their proliferation and matrix deposition (neointimal hyperplasia), is a central theme of atherosclerosis and restenosis. Many lines of research suggest that these events are preceded and accompanied by inflammation [Ross, 1999; Simon et al., 2000]. Cytokines of the interleukin-1 (IL-1) family play a pivotal role in regulating immunoinflammatory responses, and extensive studies have been performed to determine whether IL-1 modifies the inflammatory response [Dinarello, 1996; Oemar, 1999; Wang et al., 2000]. IL-1β induces a substantial increase in the expression of

adhesion molecules by vascular smooth muscle cells (VSMCs) in vitro, and these factors promote monocyte recruitment and infiltration into the arterial wall [Wang et al., 1994, 1995; Wu et al., 1999] and stimulate VSMC proliferation [Nilsson, 1993; Dinarello, 1996]. Recent in vivo studies have shown increased levels of IL-1β mRNA in human atherosclerotic lesions [Wang et al., 1989] and of IL-1β mRNA and protein in VSMCs, endothelium, and macrophages in atherosclerotic arteries from nonhuman primates [Ross et al., 1990; Moyer et al., 1991] and in coronary arteries of patients with ischemic heart disease [Galea et al., 1996]. Treatment of porcine arteries with IL-1β induces intimal lesions [Ito et al., 1996]. Although the relationship between IL-1β and cardiovascular disease has been extensively studied, IL-1β expression in oxidized low density lipoprotein (oxLDL)-induced human aortic smooth muscle cells (HASMCs) and in restenotic lesions has not been studied in detail.

IL-1β production may be an important initiating factor in the cascade of events resulting in inflammation and vascular disease. It is specifically inhibited by natural antagonists, including the IL-1 receptor antagonist (IL-1ra) [Granowitz et al., 1991], the secreted protein product of a gene adjacent to the IL-1B gene which binds to IL-1 type I and II receptors without producing a signal [Dewberry et al., 2000]. Recent studies support the hypothesis that an imbalance between IL-1β and IL-1ra production at the tissue level is pathogenically important in chronic inflammatory bowel disease [Casini-Raggi et al., 1995], in rheumatoid arthritis [Chomarat et al., 1995], and in chronic hepatitis [Gramantieri et al., 1999]. Some patients with acute or chronic liver disease have elevated serum levels of both IL-1β and IL-1ra [Tilg et al., 1993]. IL-1β and IL-1ra expression is closely involved in neointimal formation in the rat carotid artery subjected to balloon angioplasty [Wang et al., 2000]. However, the temporal expression and cellular source of IL-1β and IL-1ra in oxLDL-stimulated HASMCs and during neointimal hyperplasia have not been studied. The aims of this study were therefore to measure IL-1β and IL-1ra expression and release in oxLDL-stimulated HASMCs and their expression in the neointima of cholesterol-fed endothelia-denuded rabbits. Our results show that vascular IL-1β and IL-1ra expression is upregulated in both cases, consistent with

their playing a role in modulating neointimal development.

## METHODS

### Preparation and Oxidation of LDL

Human LDL (d = 1.019−1.063 g/ml) was isolated by sequential ultracentrifugation of fasting plasma samples from healthy adult males [Winocour et al., 1992] and extensively dialyzed under nitrogen for 24 h at 4°C against phosphate-buffered saline (PBS, 5 mM phosphate buffer and 125 mM NaCl, pH 7.4). The LDL represents the native LDL (nLDL) used in the current study. LDL was oxidized by dialysis for 24 h at 37°C against 10 μM $CuSO_4$ in PBS, as described by Steinbrecher et al. [Steinbrecher et al., 1984], then the oxidized LDL (oxLDL) was dialyzed for 24 h at 4°C against PBS containing 0.3 mM EDTA. The extent of oxidation was monitored by measuring thiobarbituric acid-reactive substance (TBARS) and agarose gel electrophoresis. The cholesterol content of nLDL and oxLDL was determined using a cholesterol enzymatic kit (Merck).

### Human Aortic Smooth Muscle Cell (HASMCs) Cultures

HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR, USA), were grown in culture flasks in smooth muscle cell growth medium (Cascade Biologics, Inc., OR, USA) supplemented with fetal bovine serum (FBS, 5%), human epidermal growth factor (10 ng/ml), human basic fibroblast growth factor (3 ng/ml), insulin (10 μg/ml), penicillin (100 units/ml), streptomycin (100 pg/ml), and Fungizone (1.25 μg/ml) at 37°C in a humidified 5% $CO_2$ atmosphere. The growth medium was changed every other day until confluence. Cells were used between passages 3 and 8. The purity of HASMC cultures was verified by immunostaining with a monoclonal antibody against smooth muscle-specific α-actin. Before oxLDL or nLDL treatment, the cells were first serum-starved for 24 h.

### MTT Assay of Cell Viability

As an index of cell viability, mitochondrial dehydrogenase activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Chen et al. [2001]. The absorbance after nLDL or oxLDL treatment, normalized to that for cells

incubated in control medium, was used as a measurement of cell viability, the control cells being considered 100% viable.

### Foam Cell Formation

Foam cell formation was identified by oil red O staining of oxLDL-incubated HASMCs. Briefly, cells ($5 \times 10^4$) were suspended in 1.5 ml of medium containing 5% FBS and placed on a coverslip in a 35-mm dish for overnight, then were switched to serum-starved medium for 24 h before being treated with various doses of oxLDL. After incubation for 8 h at 37°C, the cells were fixed for 15 min at room temperature (RT) with 4% paraformaldehyde in PBS, treated for 2 min at RT with propylene glycol, and stained for 15 min at RT with oil red O (5 mg/ml in propylene glycol), followed by hematoxylin counterstaining. The coverslips were then washed in tap water. The number of lipid droplets per HASMC, with or without oxLDL treatment, was measured quantitatively using the WIPLab™ Analysis Program (Foreseen Science & Technology); four randomly-selected regions on each coverslip were systematically scanned under light microscope and the number of lipid droplets in HASMCs in each scanned area determined.

### Quantification of IL-1β and IL-1ra in Culture Supernatants

An aliquot (200 μl) from three independent samples of supernatant was added to IL-1β (IL-1β ELISA kit, R&D systems, Inc., Minneapolis, MN) and IL-1ra (IL-1ra ELISA kit, Endogen, Inc., Woburn, MA) immunoassay plates and the plates, processed according to the manufacturer's protocol, read at 450 nm after 30 min. The sensitivity of each assay was 1 pg/ml.

### Western Blots of HASMCs

The cells were washed with PBS, then lysed for 10 min at 4°C in 150 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.5 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at $4,000\,g$ for 30 min at 4°C. All subsequent manipulations were at RT. Samples of the supernatants (15 μg of protein) were subjected to 12% SDS–PAGE, then electrotransferred to polyvinylidene difluoride (PVDF) membranes (NEN), which were then incubated for 2 h with PBS, 0.1% Tween-20, 5% skim milk before incubation for 1 h with goat antibodies against human IL-1β (1:1,000 dilution, R&D) or

human IL-1ra (1:1,000 dilution, R&D). Bound antibody was detected by incubation for 1 h with horseradish peroxidase-conjugated rabbit anti-goat secondary antibodies (1:3,000 dilution, Sigma, St. Louis, MO), and the signal visualized using Chemiluminescence Reagent Plus (NEN), followed by exposure to Biomax MR film (Kodak, Rochester, NY). The intensity of the band was quantified using a densitometer. Anti α-tubulin antibodies (1: 1,000 dilution, Oncogen) were used to quantify α-tubulin as an internal control.

### Immunocytochemical Localization of IL-1β and IL-1ra

The topographical relationship between IL-1β and IL-1ra was studied by double immunofluorescence labeling in conjunction with confocal microscopy. Cells, cultured on coverslips, were treated with 20 μg/ml of oxLDL for 12 h, then fixed for 15 min at RT with 4% paraformaldehyde in PBS. After washing with PBS, the cells were treated for 5 min with 80% methanol at −20°C, then for 1 h at RT with PBS containing 3% skim milk. The coverslips were then incubated for 1 h at 37°C with either mouse anti-human IL-β antibody (1:100 dilution, R&D) or goat anti-human IL-1ra antibody (1:100 dilution, R&D). After rinsing with PBS, the cells were incubated for 1 h at 37°C with FITC-conjugated horse anti-mouse IgG antibody (1:300 dilution, Vector, USA) and TRITC-conjugated rabbit anti-goat IgG antibody (1: 300 dilution, Sigma). After washing with PBS, the cells were mounted using Vectashield mounting medium (Vector) and viewed with a Leica confocal laser-scanning microscope. In controls in which the primary antibodies were omitted, negligible immunofluorescence was seen.

### Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were prepared as previously described [Chen et al., 2002]. Protein concentrations were determined by the Bio-Rad method. The 22-mer synthetic double-stranded oligonucleotides used as NF-κB and AP-1 probes in the gel shift assay were (5′-AGTTG-AGGGGACTTTCCCAGGC-3′; 3′-TCAACTCC-CCTGAAAGGGTCCG-5′) and (5′-ATTCGATC-GGGGCGGGGCGAGC-3′; 3′-TAAGCTAGCC-CCGCCCCGCTCG-5′), respectively. Doubled-stranded DNA was end-labeled with γ-$^{32}$P-adenosine-5′-triphosphate (ICN) using T4

polynucleotide kinase (Boehringer-Mannheim), unincorporated nucleotides being removed by gel filtration on a Sephadex G-25 column (BM-Quick Spin columns DNA G25, Boehringer-Mannheim). The DNA-binding reaction was performed for 20 min at room temperature in a volume of 20 μl containing 2 μg of nuclear extract, ~1 ng of $^{32}$P-labeled NF-κB (2–5 × $10^5$ cpm/ng), 10 μg of salmon sperm DNA (Sigma-Aldrich), and 15 μl of binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 5 μg/ml of leupeptin). Nuclear protein-bound oligonucleotide was separated from unbound probe by electrophoresis through a native 5% polyacrylamide gel (acrylamide/bisacrylamide 29:1) in 0.25 × TBE (1 × TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA buffer, pH 8.0). The gels were vacuum-dried and subjected to autoradiography and the films scanned using a UMAX scanner.

### Immunohistochemistry of Aortic Specimens From Control and Cholesterol-Fed Endothelia-Denuded Rabbits

This investigation conformed to the "Guide for the care and use of laboratory animals" published by the US National Institute of Health. Ten male New Zealand white rabbits, 3 months of age and weighing 2.5–3.0 kg, were fed for 6 weeks with a 2% high cholesterol diet (Purina Mills, Inc., St. Louis, MO) to induce hypercholesterolemia. The animals were bled periodically to measure plasma cholesterol level and liver and renal function. At the end of the 3rd week of the high cholesterol diet, the animals were fasted for 12 h and anesthetized by intramuscular injection of xylazine (5 mg/kg) and ketamine hydrochloride (35 mg/kg), then surgery was performed as previously described [Chen et al., 2001]. Ten aged-matched male rabbits on regular diet chow without balloon treatment were used as control. At the end of the 6th week of the experiment, the animals were anesthetized by intravenous injection of 35–40 mg/kg sodium pentobarbital and sacrificed. One segment of the abdominal aorta was rinsed with ice-cold PBS, immersion-fixed with 4% buffered paraformaldehyde and paraffin-embedded, then cross-sectioned for immunohistochemistry, while the remaining portion was immediately frozen in liquid nitrogen for protein extraction. The tissue sections (5–6 μm

thick) were mounted on poly-L-lysine coated slides, deparaffinized, rehydrated, and washed with PBS. To study cellular expression and localization of IL-1β and IL-1ra, serial sections were incubated with 1% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity and to permeabilize the cells, then nonspecific binding was blocked by incubation for 1 h at RT with PBS containing 5 mg/ml of bovine serum albumin. In the primary antibody step at 37°C for 1 h, the first serial section was incubated with goat anti-human IL-1β antibody (1:100, R&D), the second with mouse anti-α-smooth muscle actin (1:400, 1A4, Sigma), and the third with goat anti-human IL-1ra antibody (1:100, R&D). The second section was then incubated for 1.5 h at RT with FITC-conjugated goat anti-mouse secondary antibody (1:400, Sigma Chemical Co., St. Louis, MO) and observed by fluorescent microscopy, while bound antibodies on the first and third slides were localized by an indirect immunoperoxidase technique (avidin-biotin-horseradish peroxidase complex) employing diaminobenzidine (Vector) as chromogen. Each incubation was followed by three times 5 min washes in PBS. Negative controls were performed by omitting the primary antibody.

### Western Blots of Aortal Specimens

The frozen abdominal aortas of control and cholesterol-fed endothelia-denuded rabbits were pulverized in liquid nitrogen, then lysed for 1 h at 4°C in 0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 1% SDS, 10 μg/ml leupeptin, 1 μM PMSF, and centrifuged at $13,500\,g$ for 10 min at 4°C. All subsequent stages were at RT. Samples of the supernatants (15 μg protein) were applied to 8% SDS–PAGE and electrotransferred to PVDF membranes (NEN), which were then treated for 1 h with PBS containing 0.05% Tween-20 and 2% skim milk, and incubated for 1 h with goat anti-human-IL-1β (1:1,000; R&D) or anti-human IL-1ra antibodies (1:1,000; R&D). After washing, the membranes were incubated for 1 h with horseradish peroxidase-conjugated donkey anti-goat monoclonal antibodies (1:3,000; Bethyl), then bound antibody was detected using Chemiluminescence Reagent Plus (NEN) and exposure to Biomax MR film (Kodak). Antibodies against β-actin (1:1000;Sig) were used as an internal control.

## Statistical Analysis

Values are expressed as the mean ± SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a $P$ value < 0.05 being considered significant.

## RESULTS

### Effect of nLDL and oxLDL on HASMC Viability

Figure 1 shows the effect of different periods of incubation with nLDL or oxLDL on HASMC viability. Treatment with 20 µg/ml oxLDL did not result in cell cytotoxicity (115.1 ± 7.6% at 3 h, 106.6 ± 5.1% at 6 h, 98.0 ± 2.2% at 12 h, and 97.0 ± 3.6% at 24 h), whereas exposure to 40 or 60 µg/ml oxLDL significantly reduced cell viability with time (108.0 ± 9.3% and 100.3 ± 9.3% at 3 h, 82.2 ± 7.2% and 39.8 ± 8.3%* at 6 h, 17.4 ± 4.3%* and 2.8 ± 1.1%* at 12 h, 23.4 ± 7.4%* and 1.1 ± 0.4%* at 24 h, respectively, *$P$ < 0.05 vs. the control at the indicated time). In contrast, treatment with 60 µg/ml nLDL resulted in a slight increase in viability with time (99.1 ± 6.0% at 3 h, 102.5 ± 2.2% at 6 h, 112.2 ± 5.3% at 12 h, and 123.8 ± 4.0%* at 24 h, *$P$ < 0.05 vs. the control at the indicated time).

### Foam Cell Formation by HASMCs Induced by oxLDL

Treatment of HASMCs for 8 h with different concentration of oxLDLs induced the formation of foam cells. Clear differences were seen in lipid loading between untreated HASMCs (Fig. 2A) and those treated with 20 µg/ml of oxLDL
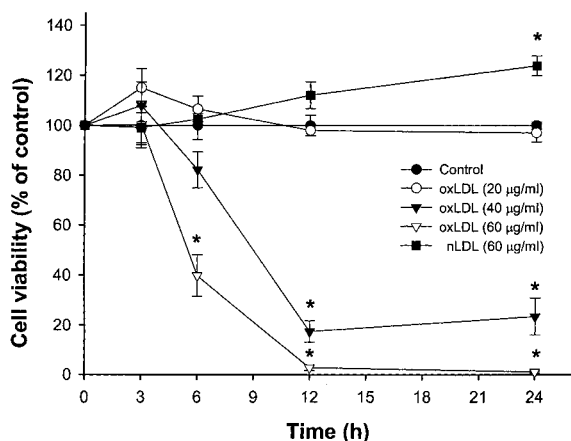
**Fig. 1.** Viability of HASMCs after incubation for 3, 6, 12, or 24 h in the absence or presence of either 60 µg/ml of nLDL or the indicated concentrations of oxLDL. The values are the mean ± SEM for three experiments, each in triplicate.
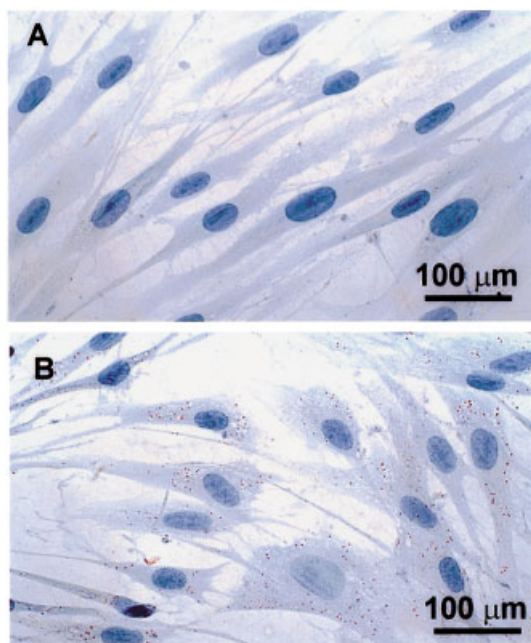
**Fig. 2.** Identification of foam cell formation by HASMCs using oil red O staining. HASMCs incubated without oxLDL showed no foam cells (**A**), whereas those incubated with 20 µg/ml of oxLDL for 12 h showed many foam cells (**B**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 2B). When measured quantitatively, the number of lipid droplets per HASMC treated with oxLDL (7.71 ± 0.44) was significantly higher than in control cells (0.23 ± 0.11).

### IL-1β and IL-1ra Release by oxLDL-Stimulated HASMCs

HASMCs were incubated for 3–48 h with various concentrations of oxLDL, then IL-1β and IL-1ra levels in the culture medium were measured by ELISA.

Figure 3A shows the time-dependent and dose-dependent effects of oxLDL on IL-1β release. Using 20 µg/ml oxLDL, a significant effect on IL-1β release was seen after 6 h, which increased up to 24 h (269.5 ± 56.1 pg/ml compared to 14.8 ± 8.1 pg/ml in untreated cell), then decreased at 48 h (220.1 ± 28.2 pg/ml; data not shown). Treatment with 40 µg/ml of oxLDL resulted in a significant increase in IL-1β release after 6 h, which plateaued at 12–24 h (454.3 ± 11.1 pg/ml vs. 14.8 ± 8.1 pg/ml), while treatment with 60 µg/ml of oxLDL resulted in a significant increase after 3 h, which peaked at 12 h (480.3 ± 17.6 pg/ml vs. 17.4 ± 5.2 pg/ml), then declined. Native LDL had no effect on IL-1β release at any time-point.
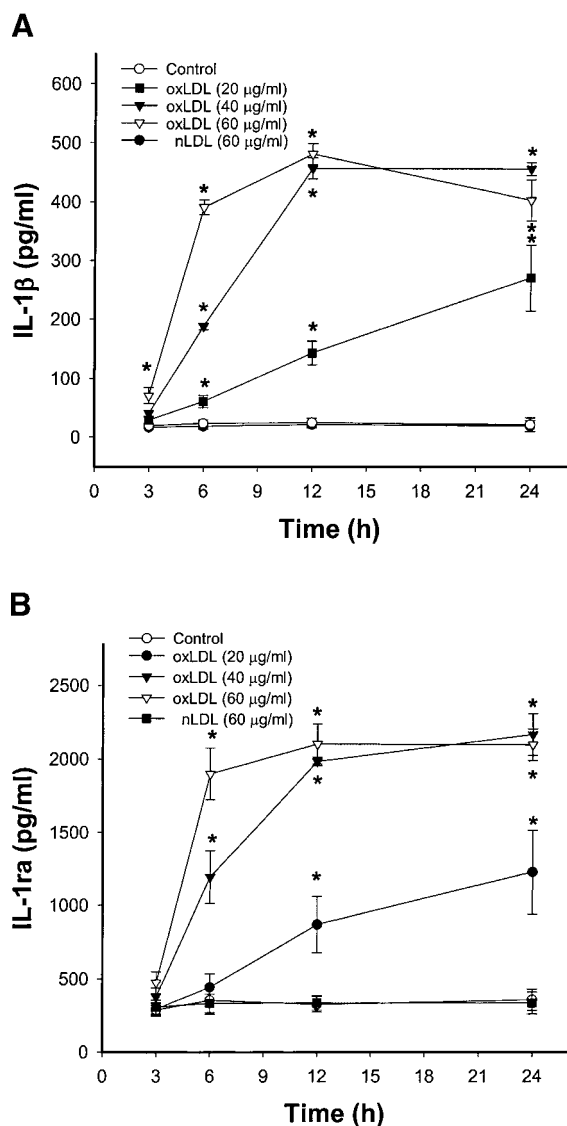
## A



## B



**Fig. 3.** Time-course of IL-1β and IL-1ra release from HASMCs induced by oxLDL or nLDL. (**A**) IL-1β; (**B**) IL-1ra. HASMCs were incubated in the absence (control) or presence of oxLDL or nLDL at the indicated concentration. The data are representative of three experiments; the values are expressed as the mean ± SEM of triplicate determinations. *P < 0.05 vs. controls at the indicated time.

Figure 3B shows the corresponding results for IL-1ra release. Using 20 μg/ml of oxLDL, the amount of secreted IL-1ra first showed a significantly increase after 12 h, and continued to increase at 24 h (1,226.3 ± 288.2 pg/ml vs. 380.6 ± 72.2 pg/ml in the untreated control) and 48 h (1,381.9 ± 246.2 pg/ml; data not shown). Treatment with 40 or 60 μg/ml of oxLDL resulted in significantly increased release at 6 h, then a further increase, plateauing at 12–24 h (2,164.4 ± 142.6 and 2,094.3 ± 107.2 pg/ml,

respectively, for 40 and 60 μg/ml of oxLDL vs. 340.6 ± 72.2 pg/ml for the control). nLDL treatment had no effect on IL-1ra release.

Using 20 μg/ml of oxLDL, the fold increase in IL-β and IL-1ra release seen with treated cells compared to untreated cells was, respectively, 1.5 ± 0.3 and 1.0 ± 0.1 at 3 h, 3.6 ± 0.5* and 1.1 ± 0.3 at 6 h, 8.9 ± 1.1* and 2.0 ± 0.4* at 12 h, 18.2 ± 3.1* and 3.0 ± 0.7* at 24 h, and 14.6 ± 3.9* and 3.6 ± 0.9* at 48 h (*P < 0.05 vs. the control at the indicated time, n = 3).

### Western Blot Analysis of IL-1β and IL-1ra Expression in oxLDL-Stimulated HASMCs

oxLDL (20 μg/ml) induced IL-1β expression in HASMCs at 6 h and expression peaked at 24 h, at which time it was approximately 17-fold higher than in unstimulated cells (Fig. 4A). In contrast, unstimulated HASMCs showed strong IL-1ra expression which was not increased by oxLDL treatment (Fig. 4B).

### Indirect Immunofluorescence of IL-1β and IL-1ra Expression in oxLDL-Stimulated HASMCs

The effects of oxLDL were studied by immunofluorescence confocal microscopy. In untreated cells, IL-1β expression was weak (Fig. 5A), whereas IL-1ra was present in the cytoplasm (Fig. 5B); these two images are superimposed in Figure 5C. In contrast, cells treated for 12 h with 20 μg/ml oxLDL showed strong IL-1β expression (Fig. 5D) and IL-1ra was present diffusely throughout the cytoplasm (Fig. 5E); these two
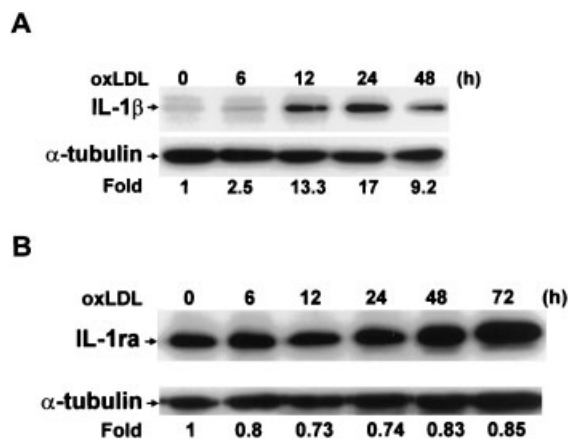
## A



## B



**Fig. 4.** Western blot analysis of the time-course of IL-1β and IL-1ra expression in oxLDL-treated HASMCs. (**A**) IL-1β; (**B**) IL-1ra. HASMCs were treated with 20 μg/ml oxLDL. The figure is a representative example of three separate experiments. α-tubulin was used as an internal control.
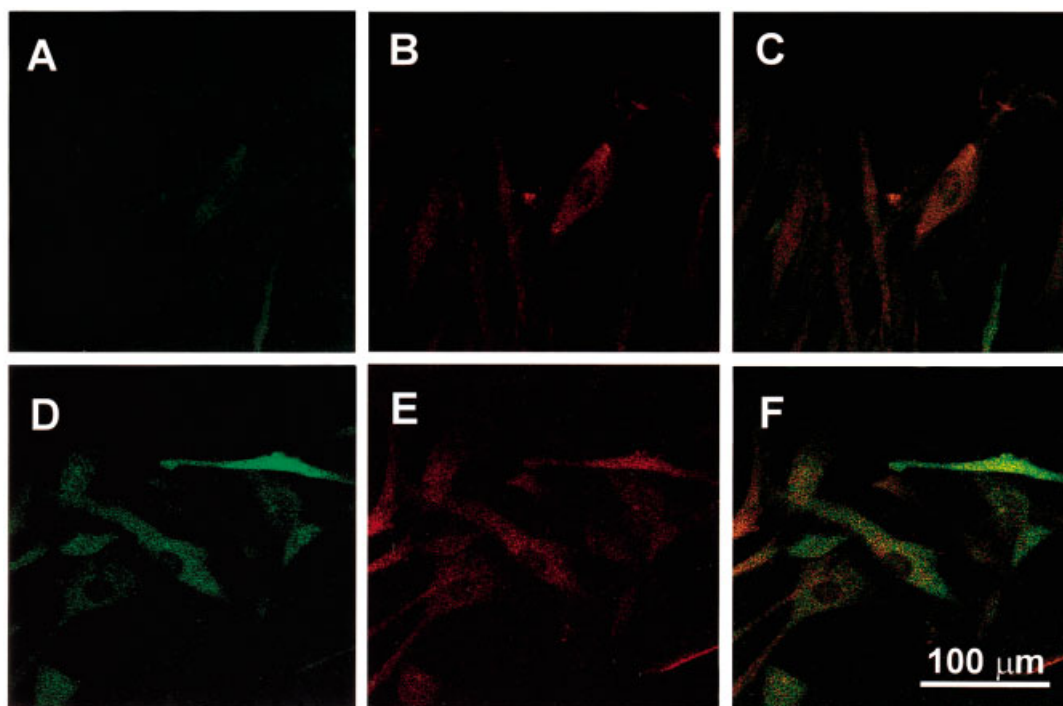
**Fig. 5.** Immunocytochemical detection by laser confocal microscopy of IL-1β and IL-1ra in HASMCs with or without oxLDL treatment. (**A–C**) Untreated cells; (**D–F**) cells treated with 20 μg/ml oxLDL for 12 h. In untreated cells, IL-1β expression was weak (A, green fluorescence), whereas IL-1ra was found in the cytoplasm (B, red fluorescence); the superimposed images only show IL-1ra expression (C). oxLDL-treated HASMCs showed a dramatic increase in IL-1β expression (D), whereas IL-1ra expression was unchanged (E). The superimposed images show that IL-1β and IL-1ra were colocalized in most cells (F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

images are superimposed in Figure 5F. IL-1β and IL-1ra immunofluorescence was colocalized in most cells after oxLDL stimulation.

### Effects of oxLDL on NF-κB and AP-1 Activity in HASMCs

Since transcriptional regulation involving NF-κB or AP-1 activation has been implicated in the oxLDL-induced expression of inflammatory cytokines, gel-shift assays were performed to determine whether oxLDL induced NF-κB or AP-1 activation. As shown in Figure 6A, low levels of basal NF-κB binding activity were detected in unstimulated control serum-starved cells, and treatment with oxLDL (20 μg/ml for 30 min) resulted in a 6.6-fold increase in NF-κB binding activity; this was specific for NF-κB, as it was undetectable in the presence of a 100-fold excess of unlabeled NF-κB oligonucleotide (data not shown). Serum-starved HASMCs also contained small or undetectable amounts of active AP-1 (Fig. 6B), which was increased 3.3-fold by oxLDL stimulation.

### Expression of IL-1β and IL-1ra in Smooth Muscle Cells From Cholesterol-Fed Endothelia-Denuded Rabbits

Immunohistochemistry was used to examine the cellular expression and localization of IL-1β and IL-1ra during neointimal hyperplasia. In the control group, no IL-1β was detected (Fig. 7A), and smooth muscle cells were only detected in the tunica media (Fig. 7B), which showed faint staining for IL-1ra (Fig. 7C). The treated group showed a markedly thickened intima which stained strongly for IL-1β (Fig. 7D), whereas no IL-1β was detected in the underlying media of the lesioned area. The cells in the thickened intima were mainly smooth muscle cells (Fig. 7E), and the markedly thickened intima and a few smooth muscle cells in the media stained strongly for IL-1ra (Fig. 7F).

### Western Blotting of Abdominal Aorta Tissue

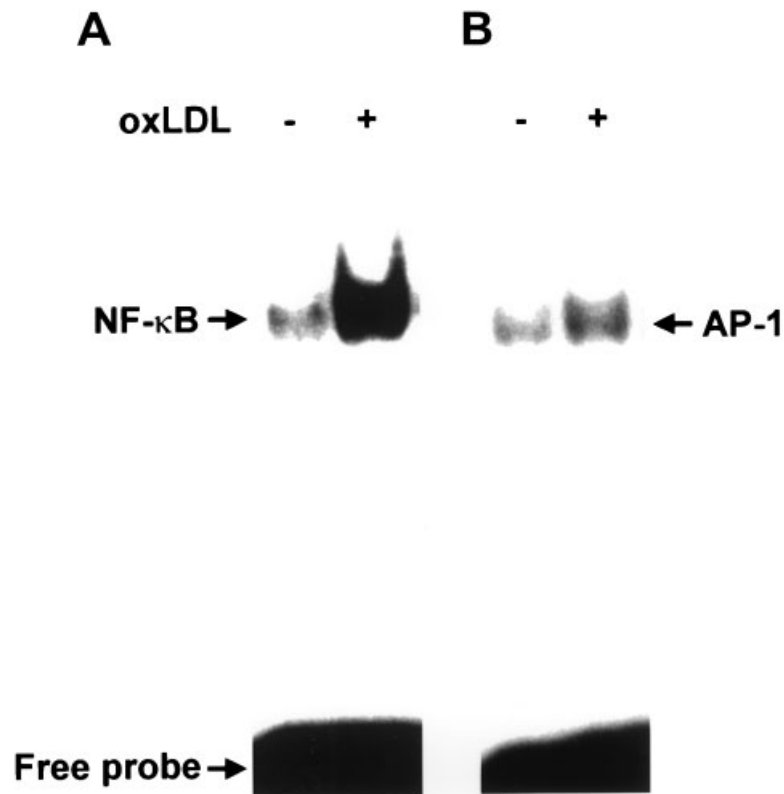As shown in Figure 8 and in accordance with the immunohistochemical results, IL-1β was

**Fig. 6.** Autoradiographs showing activation of NF-κB and AP-1 by oxLDL. HASMCs were incubated for 30 min with or without 20μg/ml of oxLDL, then NF-κB and AP-1 binding activity in nuclear extracts was analyzed by EMSA using a NF-κB or AP-1 consensus oligonucleotide as probe. The arrows indicate the positions of the NF-κB- or AP-1-specific complexes.

scarcely detectable in the control group, but IL-1ra was present, whereas, in the cholesterol-fed endothelia-denuded group, expression of both IL-1β and IL-1ra was relatively high.

## DISCUSSION

This study demonstrates that IL-1β and IL-1ra protein expression is upregulated in smooth muscle cells in the neointima of cholesterol-fed endothelia-denuded rabbits. A concomitant increase in IL-1β and IL-1ra release was also detected in culture media from HASMCs after oxLDL stimulation in vitro. These results suggest a role for IL-1β and IL-1ra in the development of restenotic lesions.

Immunohistochemical results from serial sections indicated that IL-1β and IL-1ra expression occurred together in both smooth muscle cells and in the neointima. IL-1β has previously been detected in diet-induced atherosclerotic lesions of the iliac artery in monkeys [Moyer et al., 1991] and in coronary arteries of humans

with ischemic heart disease [Galea et al., 1996] or with restenosis [Clausell et al., 1995]. Transgenic mice lacking functional IL-1 do not show the neointimal hyperplasia which is induced in wild-type mice by low shear stress [Rectenwald et al., 2000]. In the present study, the fact that IL-1β synthesis was restricted to sites of lesion development suggests that IL-1β plays an important role in the pathogenesis of restenosis.

IL-1β has previously been found in luminal and adventitial vessel endothelial cells and in macrophages in coronary arteries from patients with ischemic heart disease [Galea et al., 1996], in foam cells, smooth muscle cells, and the endothelium of diet-induced iliac artery atherosclerotic plaques in monkeys [Moyer et al., 1991], in the endothelium and neointima in a rat vein graft model [Faries et al., 1996], and in neointimal cells in balloon injured porcine coronary arteries [Chamberlain et al., 1999], and has also been found in atheromatous macrophages cultured from human carotid arteries
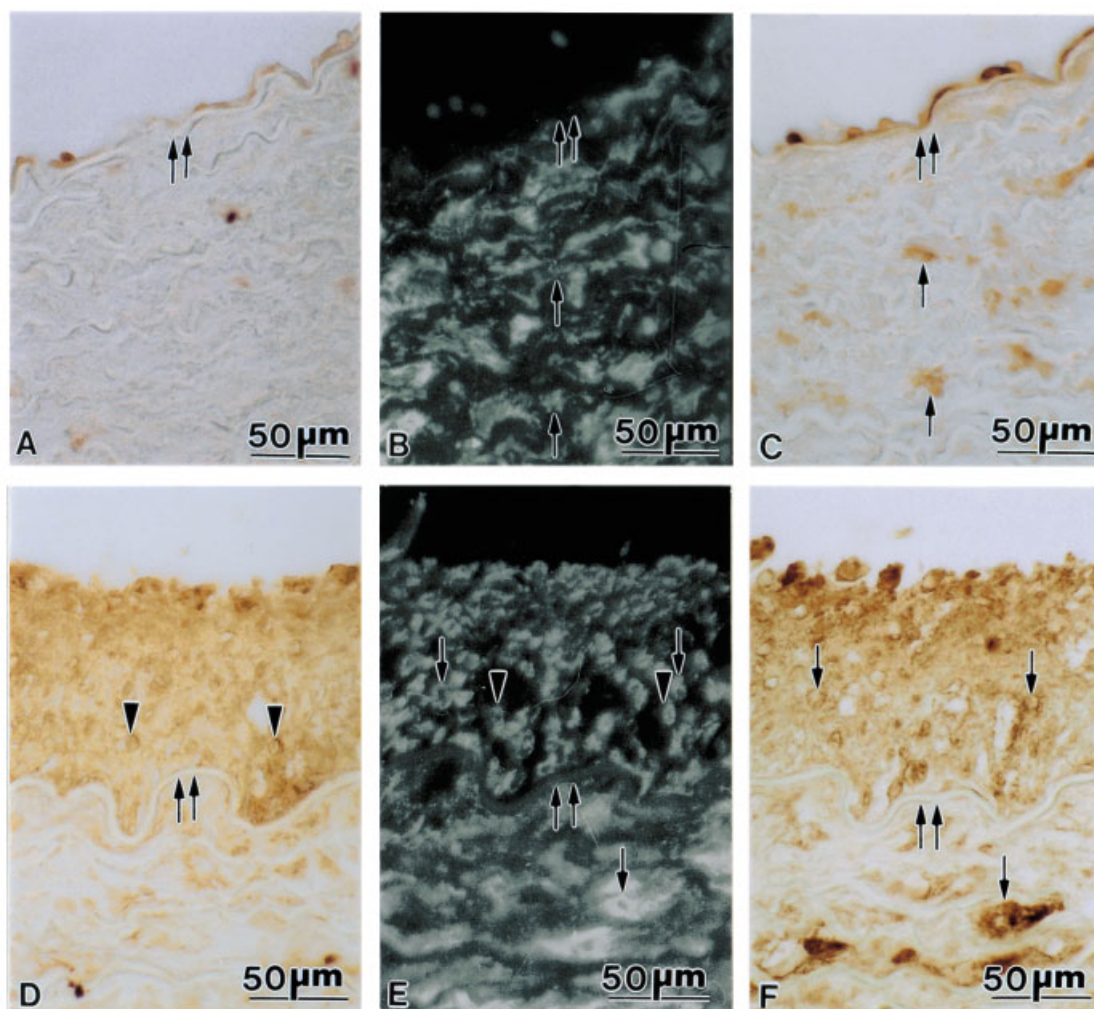
**Fig. 7.** Immunohistochemical staining for IL-1β, smooth muscle cells, and IL-1ra in serial sections of abdominal aortas from control and cholesterol-fed endothelia-denuded rabbits. (**A–C**) control rabbits; (**D–F**): endothelia-denuded rabbits. (A, D) IL-1β staining; (B, E) staining for smooth muscle cells; (C, F) IL-1ra staining. The internal elastic membrane is indicated by double arrows. The arrowheads and single arrows indicate smooth muscle cells overlapping with IL-1β or IL-1ra expression, respectively. In the control group, IL-1β was not detected (A), whereas low amounts of IL-1ra were present in the media (C); smooth muscle cells were only detected in the tunica media (B). In cholesterol-fed endothelia-denuded rabbits, strong IL-1β expression was only detected in the thickened intima (D), whereas strong IL-1ra expression was seen in the thickened intima and in a few smooth muscle cells in the media (F); the thickened intima and media were mainly composed of smooth muscle cells (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

[Tipping and Hancock, 1993]. The present study provided direct evidence that IL-1β expression was localized in smooth muscle cells of the neointima, a result consistent with a role in promoting smooth muscle cell migration into the intima and their subsequent proliferation and activation in the thickened intima.

Several studies have demonstrated that IL-1ra is produced by mononuclear cells [Poutsiaka et al., 1991], epithelial cells [Haskill et al., 1991], endothelial cells [Dewberry et al., 2000], and VSMCs [Di febbo et al., 1998]. In this study, we found, for the first time, that oxLDL is an effective stimulus for IL-1ra secretion from HASMCs. This is an interesting result considering that IL-1β and IL-1ra are coexpressed in both oxLDL-stimulated HASMCs and in the neointima. In addition, we demonstrated that, following oxLDL treatment, the increase in IL-1ra secretion occurred later than the increase in IL-1β secretion. Expression of IL-1β and IL-1ra in both HASMCs and cholesterol-fed endothelia-denuded rabbits appeared to differ, with IL-1ra being detectable before oxLDL treatment or balloon injury, whereas basal IL-1β expression was low, and only markedly increased after
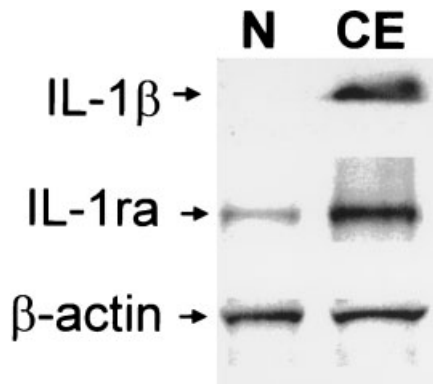
**Fig. 8.** Western blot analysis of IL-1β and IL-1ra in the abdominal aorta of normal and cholesterol-fed endothelia-denuded rabbits. N, normal rabbit; CE, cholesterol-fed endothelia-denuded rabbit. The result is representative of three separate experiments. In the normal rabbits, although IL-1β was scarcely detectable, IL-1ra was present, whereas, in the CE rabbits, the expression of both IL-1β and IL-1ra was relatively high. β-actin was used as an internal control.

stimulation. The fact that IL-1ra was detectable in untreated cells is probably due to a cellular storage phenomenon, as recently shown for IL-1ra in human umbilical vein endothelial cells [Dewberry et al., 2000]. Since up to a 100-fold molar excess of IL-1ra may be required to counteract the action of IL-1 in a disease situation [Dewberry et al., 2000], we suggest that de novo synthesis would not be very efficient in meeting this requirement, and that storage of IL-1ra within the cell would be preferable so as to avoid a long delay in the action of IL-1ra.

IL-1ra is a naturally occurring inhibitor that counterbalances the biological activity of IL-1β, acting by binding competitively to IL-1 receptors without inducing signal transduction [Gramantieri et al., 1999]. Increased serum levels of IL-1ra have been detected in the acute phase of meningococcal disease and in rheumatoid arthritis, and are supposed to reflect an antiinflammatory reaction [van Deuren et al., 1994; Barrera et al., 1995]. Induction of IL-1ra, coupled with IL-1β induction, has been demonstrated in healthy subjects in whom endotoxemia was produced by injection of *Escherichia coli* endotoxin [Granowitz et al., 1991], and IL-ra induction is seen in cancer patients receiving IL-1β as a therapeutic agent [Kopp et al., 1996]. In addition, increased levels of IL-1ra and IL-1β are seen in lipopolysaccharide (LPS)-treated whole blood from both patients with diabetes mellitus and cigarette smokers, both of which

are risk factors for cardiovascular disease [Mol et al., 1997]. Allele 2 of the *IL-1ra* gene is associated with a lower incidence of restenosis after coronary stenting [Kastrati et al., 2000]. The biological relevance of IL-1ra to the normal physiology of IL-1 or to the possible role of IL-1 in pathophysiology remains to be established. However, it is worth noting that macrophages, which are involved in the atherogenic process [Ross, 1993], simultaneously produce IL-1 and IL-1ra [Mikuniya et al., 2000]. IL-1ra can control IL-1β-induced VSMC growth [Porreca et al., 1993], again demonstrating its anti-inflammatory properties. In this context, the increased IL-1ra levels seen in cholesterol-fed endothelia-denuded rabbits were closely associated with inflammation. The increased IL-ra expression may be due to increased oxidative stress and hyperlipidemia, but may also reflect inflammation related to cardiovascular disease. The overall balance between IL-1β and IL-1ra may determine the severity of restenotic lesions in response to the level of smooth muscle cell proliferation. Thus, the IL-1ra level results in our study could indicate increased inflammation.

Much attention has been paid to the role of LDL oxidation in the development of cardiovascular disease and, in particular, to the possibility that LDL oxidation in the arterial wall activates the inflammatory process that characterizes lesion progression. Activation of the redox-regulated transcriptional factors, NF-κB and AP-1, has been suggested to play a key role in this process, as they bind to the promoters of many genes involved in inflammation. In the present study, we demonstrated that treatment of HASMCs for 30 min with 20 μg/ml of oxLDL activated NF-κB and AP-1, suggesting that IL-1β and IL-1ra upregulation in response to oxLDL is mediated by these transcriptional factors. However, NF-κB is a ubiquitously expressed multiunit transcription factor which is activated by diverse signals, possibly via phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimer, p50 and p65, to the nucleus [Ghosh and Baltimore, 1990]. In a recent study, treatment of human arterial smooth muscle cells for 4 h with 50 μg/ml of oxLDL was shown to activate AP-1, but not NF-κB [Ares et al., 1995]. In contrast, treatment of HUVECs for 48 h with 220 mg/dl of oxLDL activates AP-1 [Lin et al., 1996] and treatment of bovine aortic endothelial

cells for 5 min with 100 μg/ml of oxLDL activates NF-κB [Cominacini et al., 2000]. Depending on the cell type, extent of oxLDL oxidation, and incubation time used in the experiments, in vitro studies have shown either stimulatory or inhibitory effects of oxLDL on NF-κB and AP-1 activation. In the present study, we did not prove conclusively that the oxLDL-induced NF-κB and AP-1 activation in HASMCs is directly linked to the role of HASMCs in neointimal formation. Although the role of IL-1β as a modulator in neointimal hyperplasia is fairly complex, there is increasing evidence that IL-1β is a potent stimulator of the synthesis of adhesion molecules, including VCAM-1 and ICAM-1, in endothelial cells [Wu et al., 1999] and vascular smooth muscle cells [Wang et al., 1994; Rolfe et al., 2000]. Other in vitro studies support the idea that IL-1β may contribute to the migration of leukocytes into the inflammatory tissue and to the cellular interaction with other inflammatory cells by upregulating adhesion molecules [Wang et al., 1995]. It is conceivable that increased IL-1β expression in the thickened intima could promote the migration and proliferation of smooth muscle cells into the lesions and facilitate the chronic inflammatory response during intimal hyperplasia.

In conclusion, this study has demonstrated that IL-1β and IL-1ra expression was increased in smooth muscle cells in the neointima of cholesterol-fed endothelia-denuded rabbits. A concomitant increased release of IL-1β and IL-1ra was also detected in oxLDL-stimulated HASMCs. These results suggest a role for IL-1β and IL-1ra in the transendothelial migration of circulating monocytes, the activation of intimal macrophages and smooth muscle cells, and the stimulation of the expression of other cytokines during the development of restenotic lesions.

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