Effect of Inflammatory Cytokines on the Metabolism of Low-Density Lipoproteins by Human Vascular Endothelial Cells

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Cytokines have been shown to activate multiple, varied metabolic pathways in endothelial cells. Little information is available concerning the effects of inflammatory cytokines on lipoprotein metabolism by vascular endothelial cells. Human umbilical vein endothelial cells (HuECs) and bovine aortic endothelial cells (BAECs) were incubated with the inflammatory cytokines recombinant human interleukin-1β (IL-1), tumor necrosis factor alpha (TNF), interferon gamma (γ-IF), and interferon beta (β-IF) at increasing concentrations (0.1 to 1,000 U/mL), for increasing periods (6 to 72 hours). After the incubation period, the media were removed and replaced with serum-free media containing radiolabeled native or acetylated low-density lipoprotein (Ac-LDL) and the rates of degradation and accumulation of radiolabeled LDL were determined. The degradation and accumulation of ^{125}I-LDL were significantly increased ($P < .02$) in HuECs preincubated with IL-1 (100 U/mL) compared with control incubations without the cytokine or incubations containing γ-IF, β-IF, or TNF. This resulted from a 38% increase in LDL receptor protein in cells incubated with IL-1. The increased rate of LDL catabolism by HuECs incubated with IL-1 was accompanied by a significant increase ($P < .05$) in the rate of cholesteryl ester synthesis in the cells. Cholesteryl ester synthesis rates in HuECs preincubated with γ-IF, β-IF, or TNF did not differ significantly from the rates in control incubations. The effect of preincubation with cytokine on the activity of the scavenger receptor was also determined. There were no significant differences in the rate of degradation or accumulation of radiolabeled Ac-LDL in control incubations compared with cultures preincubated with IL-1, γ-IF, β-IF, or TNF. There also were no significant differences in the rate of catabolism of native LDL or Ac-LDL in BAECs preincubated with cytokines. Although cytokines have been shown previously to alter the binding of monocytes to endothelial cells, there was no significant increase in the binding of monocytes to cultures incubated with IL-1 plus LDL compared with IL-1 alone. In summary, we now demonstrate that cytokines, specifically IL-1, may alter LDL metabolism by human vascular endothelial cells and alter endothelial cell cholesterol metabolism. These changes in endothelial cell metabolism provide additional evidence supporting the critical role of cytokines in atherogenesis.
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SEVERAL LINES OF EVIDENCE point to the key role of immunologic processes in the pathogenesis of atherogenesis.[1-4] We have shown previously that human monocyte-derived macrophages are transformed into foam cells upon incubation with immune complexes containing low-density lipoprotein (LDL-IC).[5,6] LDL immune complex added to macrophage cultures at a concentration known to induce intracellular accumulation of cholesteryl ester and foam cell transformation stimulates the release of the inflammatory cytokines interleukin-1β (IL-1) and tumor necrosis factor alpha (TNF).[7] It has been shown that the activation of endothelial cells by inflammatory cytokines results in the synthesis of cell adhesion molecules and chemotactic factors, thus increasing their interactions with leukocytes.[8,9] Adhesion of leukocytes to the endothelium results from a cascade of molecular interactions and is mediated by multiple cell adhesion molecules.[10-12]

The endothelium is strategically located at the interface between vascular tissue and circulating blood, and as such, it plays an important role as a regulator of vascular homeostasis. Endothelial cells not only interact with other cells but are also highly active in the intravascular metabolism of lipoproteins, especially LDL, via the classic LDL receptor pathway.[13] Lipoproteins interact with endothelial cells in a manner similar to other cell types studied in culture by binding, internalization, and degradation via receptor-mediated pathways, and the metabolic effects of lipoprotein uptake by endothelial cells are similar to those described for other cell types.[14,15] In addition, endothelial cells, together with macrophages, express receptor activity for modified lipoproteins. However, no information is available concerning the effects of inflammatory cytokines on lipoprotein metabolism by endothelial cells. Therefore, we investigated the effects of the inflammatory cytokines IL-1 and TNF, as well as interferon beta (β-IF) and interferon gamma (γ-IF), on the metabolism of native LDL by human vascular endothelial cells and bovine aortic endothelial cells (BAECs). We also investigated the effects of the cytokines on the metabolism of modified LDL by human and bovine endothelial cells using acetylated LDL (Ac-LDL) as a model of modified LDL.

MATERIALS AND METHODS

Materials

Recombinant human IL-1 was kindly provided by Dr Peter Lomedico of Hoffman-LaRoche (Nutley, NJ) at a specific activity of 1×10^7 U/mg protein. This recombinant protein was found not to contain endotoxin. TNF was obtained from Cetus (Emeryville, CA) with a specific activity of 1×10^8 U/mg protein. γ-IF was a human recombinant protein with an activity of 5.7×10^7 U/mg protein prepared by Biogen (Cambridge, MA) and kindly provided to us by Dr Esmail Zanjani. This recombinant protein also was found not to contain endotoxin. Recombinant human β-IF with a specific activity of 1×10^8 U/mg protein was obtained from Triton Biosciences (Alameda, CA).

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The reported biologic activities of the cytokines were confirmed by the appropriate assays before use in these experiments. All cytokines were diluted and stored at −70°C in serum-free tissue culture medium. Frozen preparations were thawed and discarded after each use.

Endothelial Cell Isolation and Culture

Human umbilical vein endothelial cells (HuECs) were isolated from umbilical cords obtained after normal vaginal delivery and cultured as described previously.[16] Briefly, the umbilical vein in undamaged segments of an umbilical cord was identified and cannulated and the endothelial cell layer was isolated by digestion with collagenase (0.1% wt/vol, Type 1 from *Clostridium histolyticum;* Sigma Chemical, St Louis, MO). The cells were cultured in growth media consisting of Iscove's modified Dulbecco's medium (IMDM) containing heat-inactivated human serum (10% vol/vol) and fetal bovine serum (10% vol/vol; Whittaker Bioproducts, Walkersville, MD), catalase (5 U/mL, from bovine liver), heparin (3.75 U/mL, grade 1, sodium salt from porcine intestine), transferrin (1 μg/mL, human), glutamine (4 mmol/L), 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 150 μg/mL endothelial cell growth supplement (E-0760, from bovine pituitary glands; Sigma Chemical) on a growth surface previously coated with gelatin (1% wt/vol, porcine skin, type II, 175 bloom). Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2, and the medium was removed and replenished with medium of the same composition every 3 days. Cells were grown to near confluence and then subcultured at a ratio of 1:3 after harvesting the culture with trypsin-EDTA (0.05%:0.02% wt/vol) at 37°C. Cells were used for experimentation only after 2 to 4 passages. The endothelial identity of the cells was confirmed by visual observation of the "cobblestone" growth pattern and the presence of factor VIII–associated antigen.

BAEC Isolation and Culture

BAECs were obtained from cows that had been slaughtered within the past 6 hours. The endothelial cells were then harvested and cultured as described for HuECs except that the medium (BAEC culture medium) consisted of IMDM with 10% (vol/vol) fetal bovine serum and penicillin, streptomycin, and amphotericin B at the concentrations already described for the culture of HuECs. These cells were kindly provided by Dr G. Vercellotti of the University of Minnesota School of Medicine. Cells were used only between passages 4 and 8.

Lipoprotein Isolation

Blood was collected in EDTA (1 mg/mL blood) from normal subjects after a 12- to 14-hour fast. The LDL fraction (1.019 < density [d] < 1.063 g/mL) was isolated from the plasma after preliminary ultracentrifugation of plasma at d = 1.019 g/mL. The floating lipoprotein fraction was discarded and the plasma solvent density was increased to 1.063 g/mL. LDL was isolated by tube-slicing after ultracentrifugation in a 60 Ti rotor at 60,000 rpm at 10°C for 22 hours. The isolated LDL solution was then washed and concentrated by ultracentrifugation. All buffers used for LDL isolation contained EDTA (0.01% wt/vol) and were saturated with nitrogen to prohibit LDL modification. The washed LDL was dialyzed against saline (0.9% NaCl and 0.01% wt/vol EDTA, pH 7.4), sterilized by passage through a 0.2-μm filter (Gelman Sciences, Ann Arbor, MI), and stored under a nitrogen atmosphere at 4°C. The salt solutions used during ultracentrifugation also contained 0.01% (wt/vol) EDTA, pH 7.4. Ac-LDL was prepared as described previously.[17] Aliquots of LDL or Ac-LDL were radiolabeled with ^{125}I as described previously[18] and stored under a nitrogen atmosphere for up to 7 days until they were incubated with cells. A different batch of radiolabeled LDL was prepared for each experiment, but the same preparation of radiolabeled LDL was incubated with both human and bovine endothelial cells.

Protocol

For each experiment, HuECs were obtained from an individual donor and BAECs were pooled cultures. To conduct these experiments, the endothelial cells were inoculated into 24-well cluster culture plates and incubated until the cultures were confluent as determined by visual observation. The cultures were then maintained for an additional 2 days before the experiments began. Cells to be used for the determination of ^{14}C-oleate incorporation into cholesteryl esters were inoculated into 6-well cluster culture plates and incubated in the same manner. On the day of an experiment, the medium was removed from each culture and the endothelial cells were then incubated in HuEC culture medium or, where appropriate, BAEC culture medium, containing the inflammatory cytokine at the indicated concentration for the specified period. The medium containing the inflammatory cytokine was then removed and replaced with medium containing IMDM, 5 U/mL catalase, 3 mmol/L glutamine, 1 μg/mL transferrin, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 35 mg/dL fetal bovine lipoprotein-deficient serum ([LPDS] inoculation media). The rates of degradation and accumulation of ^{125}I-LDL and incorporation of ^{14}C-oleate into cellular cholesteryl esters were then determined.

LDL Degradation and Accumulation Studies

Confluent HuECs or BAECs were incubated with the inflammatory cytokine for the indicated period. The media containing the cytokine were removed, and the cultures were washed with IMDM and then incubated with inoculation media containing ^{125}I-LDL (10 μg/mL) or ^{125}I-Ac-LDL (10 μg/mL) with or without nonradiolabeled LDL (250 μg/mL) or Ac-LDL (250 μmg/mL). The proteolytic degradation of ^{125}I-labeled lipoproteins by endothelial cells was measured by determining the amount of ^{125}I-trichloroacetic acid–soluble (noniodide) material formed by the cells and excreted into the culture medium.[18] The rates of total degradation were determined in incubations containing only ^{125}I-LDL or ^{125}I-Ac-LDL, and rates of nonspecific degradation were determined in parallel incubations containing a 25-fold excess of nonradiolabeled LDL. High-affinity degradation rates were calculated as the difference between total and nonspecific degradation rates. Corrections were made for the small amount of ^{125}I-labeled, acid-soluble material found in parallel incubations without cells. The cellular accumulation of ^{125}I-LDL was determined after removing the medium and washing each culture with three 1-mL aliquots of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and with three 1-ml aliquots of PBS. The cells were then lysed and solubilized in 1 mL 0.2N NaOH, and the amount of ^{125}I radioactivity associated with the cells was determined. These data are measures of ^{125}I-LDL that is both surface-bound and internalized. An aliquot of the cells was taken for determinations of cellular protein.[19]

Immunoblotting of LDL Receptor

For blotting experiments, HuECs were seeded into 100-mm culture dishes and the cells grown to confluence as described before. The cultures were then incubated with growth media or growth media containing IL-1 at 50 or 100 U/mL (final concentration) for 24 hours. A control culture was also incubated with IMDM containing LPDS (5% vol/vol) for 24 hours. After the incubation, the culture medium was removed, the cultures were washed with PBS, and 0.5 mL lysis buffer (10 mmol/L HEPES, pH 7.4, containing 200 mmol/L NaCl, 2 mmol/L $CaCl_2$, 2.5 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride and 1.5% Triton X-100) was added to each culture. An aliquot of the cell lysate (20 μg protein) was dissolved in a reducing sample buffer (25 mmol/L Tris hydrochloride, pH 6.8, 1% sodium dodecyl

sulfate [SDS], 5% glycerol, and 2.5% 2-mercaptoethanol) and boiled for 5 minutes. This lysate preparation was subjected to SDS-polyacrylamide gel electrophoresis (8% resolving gel) using the Laemmli buffer system as described previously.[16] After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane in a solution containing 15 mmol/L Tris-glycine, pH 8.3, and 20% (vol/vol) methanol. The membrane was incubated with blocking buffer (20 mmol/L Tris hydrochloride, pH 7.6, 130 mmol/L NaCl, 0.1% Tween 20 [TBS-T] containing 5% nonfat dry milk) for 1 hour at room temperature and then incubated overnight at 4°C in TBS-T that contained the anti–LDL receptor peptide antibody peptide antibody prepared as described before, or nonimmune serum. After the incubation, the membrane was washed 5 times with TBS-T buffer and incubated an additional hour at room temperature with the same buffer containing a 1:5,000 dilution of sheep anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. After this incubation, the membrane was washed 5 times with TBS-T buffer and the immunoreactive proteins were detected using a light-emitting nonradioactive method of detection (ECL; Amersham, Arlington Heights, IL) and X-Omat film (Kodak Chemical, Rahway, NJ). The relative LDL receptor content in each lane was estimated by scanning densitometry analysis (National Institutes of Health Image software) of the film.

To prepare anti–LDL receptor antibody, a peptide consisting of the 16 C-terminal amino acids of the LDL receptor (NH_3-Cys-Gly-Tyr-Pro-Ser-Arg-Gln-Met-Val-Ser-Leu-Glu-Asp-Asp-Val-Ala-COOH)[20] was synthesized in the Peptide Synthesis Laboratory at the Medical University of South Carolina and coupled to keyhole limpet hemocyanin using *M*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester[21] as described previously.[22] New Zealand white rabbits were immunized with the coupled peptide, and the serum was collected and stored at −70°C until analysis.

Incorporation of [1-^{14}C]-Oleate Into Cholesteryl Esters

The rate of cholesteryl ester synthesis was determined in HuECs grown to confluence and incubated with the indicated cytokine as described before. The medium containing the cytokine was removed, and the cultures were washed with sterile PBS and then incubated in 1 mL serum-free medium containing 0.2 mmol ^{14}C-oleate, 2.5 mg bovine serum albumin, and 100 μg LDL per 1 mL media. The cells were incubated for 20 hours at 37°C and washed as described before. Cellular lipids were extracted with hexane/isopropanol (3:2 vol/vol), and cholesteryl-[^{14}C]-oleate was isolated by thin-layer chromatography of the lipid extracts on silica gel plates developed in a solvent system of petroleum ether/ethyl ether/acetic acid (80:20:2 vol/vol/vol).[18] The lipids were visualized with I_2 vapor, and the spots that comigrated with a cholesteryl oleate standard were marked and scraped into scintillation vials after the total disappearance of color. The samples were analyzed in a liquid scintillation counter, and correction of procedural losses was made by adding ^{3}H-cholesteryl oleate as an internal standard to the extraction mixture. After the lipid extraction, the cell residue was solubilized with 0.2N NaOH and the protein content was determined as already described.

Monocyte Binding to HuECs

To perform an experiment, human monocytes were isolated from normal donors according to the method of Recalde as described previously.[7] The purity of the monocyte preparations averaged 82% as determined by cell morphology on Wright's-stained cytocentrifuge preparations, with the major contaminating cell population being lymphocytes. The freshly isolated human mononuclear leukocyte preparation (1×10^7 cells) was stained with a vital dye (PKH 26; Sigma Chemical) exactly as described by the supplier, and the stained monocytes were diluted in inoculation medium at 5×10^5 cells/mL). Cell viability after staining as determined by trypan blue exclusion averaged 95.7%. Confluent HuECs cultured in 6-well cluster plates were stained using a nucleus-staining vital dye (Hoechst 33342; Molecular Probes, Eugene, OR) by removing serum containing media from each HuEC culture well, washing the well twice with IMDM, and incubating the cultures at 37°C for 60 minutes in IMDM containing the Hoechst dye at 450 ng/mL. After incubation, the HuEC cultures were washed twice with IMDM.

To quantify mononuclear cell binding to the HuEC layer, the stained HuEC cultures were incubated with 1 mL stained human monocytes for 1 hour at 37°C. After the incubation, the medium was aspirated from each well, each culture was washed vigorously 2 times using 1 mL IMDM at 37°C, and 1 mL 37°C growth media was added to each well. Each culture well was analyzed using a Zeiss IM35 inverted microscope (50W HBO mercury light source, standard rhodamine and fluorescein filter sets, and 50× N.A. Zeiss Neo-fluor objective; Zeiss, Oberkichen, Germany). Fluorescence was transmitted to a video camera (DAGE/MCI series 725 high-CCD camera) controlled by a computerized image analysis system (Zeiss/Kon IBAS 2000). The number of endothelial cells was determined by quantifying the spherical 75- to 150-μg diameter images delimited using the fluorescein filter set in 5 fields per well of 5 replicate cultures, and the cell number averaged 100 to 136 cells per field. Monocyte binding to endothelial cell layers was determined by quantifying the number of 15- to 50-μm diameter images delimited using the rhodamine filter set in the same fields examined for endothelial cells. Only fluorescent images coincident with areas corresponding to endothelial cells were quantified as endothelial cell–bound monocytes. Monocyte binding to endothelial cells is reported as monocytes bound per 100 endothelial cells.

Statistical Analyses

Statistical analysis with the SigmaStat statistical package (Jandel Scientific, San Rafael, CA) was performed using the Wilcoxon signed rank test to compare the differences between paired data. Data for monocyte binding to endothelial cell layers were analyzed using 2-way ANOVA with time (replication of experiments) and cytokine treatment as the factors. Post hoc analysis of the ANOVA was made using the Student-Newman-Keuls test to determine which treatments were significantly different. All results are expressed as the mean ± SEM.

RESULTS

We performed preliminary studies to determine the cytokine concentrations that would lead to maximal rates of ^{125}I-LDL degradation by HuECs. These results are shown in Fig 1. IL-1 at concentrations greater than 100 U/mL resulted in maximal rates of degradation of ^{125}I-LDL. In contrast, γ-IF, β-IF, or TNF at a concentration greater than 0.1 U/mL failed to stimulate the degradation of ^{125}I-LDL.

The stimulation of the degradation of ^{125}I-LDL by HuECs preincubated with IL-1 occurred rapidly. Preincubation of HuECs with IL-1 for only 6 hours increased the rate of degradation of ^{125}I-LDL (Fig 2). Incubation of HuECs with IL-1 for up to 72 hours resulted in increasing rates of degradation of ^{125}I-LDL.

The rate of high-affinity, receptor-mediated degradation of ^{125}I-LDL by HuECs was significantly increased ($P < .02$) in HuECs preincubated with IL-1 (100 U/mL) compared with control incubations without the cytokine, and was 2,254 ± 800 versus 1,597 ± 600 ng ^{125}I-LDL degraded/mg cell protein/20 h respectively (Fig 3A). In contrast, the rate of high-affinity, receptor-mediated degradation of ^{125}I-LDL did not differ significantly from the rate in control cultures when HuECs were preincubated with γ-IF (100 U/mL), β-IF (100 U/mL), or TNF

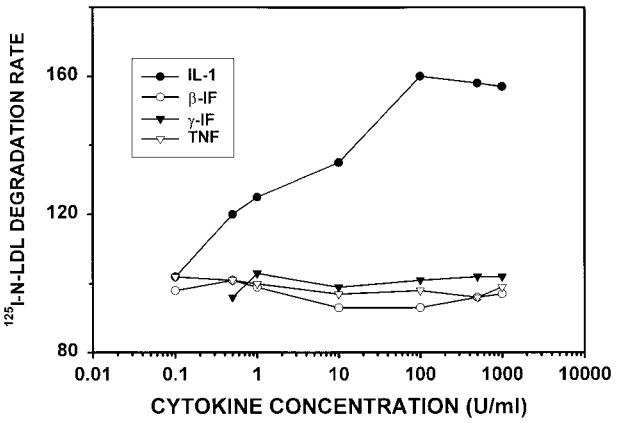

Fig 1. Effect of increasing cytokine concentration on the rate of degradation of ^{125}I-LDL. HuECs were incubated with IL-1, γ-IF, β-IF, or TNF at the indicated concentration for 20 hours and the rate of degradation of ^{125}I-LDL was determined. The degradation rate is expressed as a percentage of the degradation rate of ^{125}I-LDL incubated in control cultures without cytokine. The rate of high-affinity, receptor-mediated degradation of ^{125}I-LDL in control cultures was 1,483 ng/mg cell protein/20 h for 2 experiments in triplicate and is expressed as the 100% value.

(100 U/mL), the rate of high-affinity lipoprotein degradation by receptor-mediated pathways was 1,582 ± 697, 1,500 ± 700, and 1,550 ± 900 ng ^{125}I-LDL degraded/mg cell protein/20 h, respectively. There were no significant differences in the rate of nonspecific degradation of ^{125}I-LDL by HuECs preincubated with the cytokine compared with the control cultures (Fig 3A). The rate of accumulation of ^{125}I-LDL by HuEC high-affinity, receptor-mediated pathways was significantly greater ($P < .05$) in HuECs preincubated with IL-1 compared with control cultures, and was 747 ± 200 versus 599 ± 170 ng/mg cell

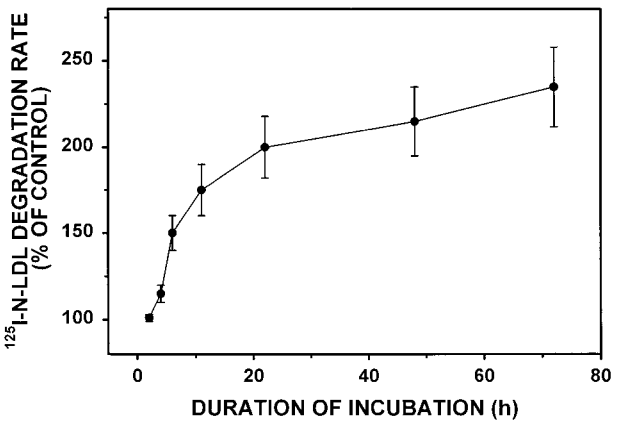

Fig 2. Effect of the duration of incubation of HuECs with IL-1 (100 U/mL) on the receptor-mediated degradation of ^{125}I-LDL. HuECs were incubated with IL-1 for the indicated period and the rate of degradation of ^{125}I-LDL was determined as described in Fig 1. The degradation rate is expressed as a percentage of the degradation rate of ^{125}I-LDL incubated in control cultures not preincubated with cytokine. The rate of high-affinity, receptor-mediated degradation of ^{125}I-LDL in control cultures was 1,502 ng/mg cell protein/20 h for 3 experiments in duplicate and is expressed as the 100% value.

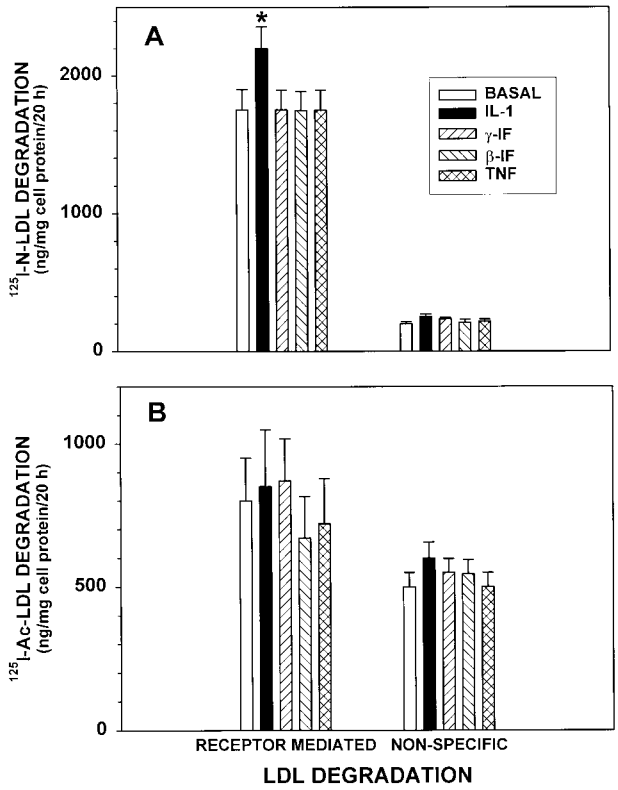

Fig 3. Influence of cytokines on the degradation by HuECs of (A) ^{125}I-LDL or (B) ^{125}I-Ac-LDL. HuECs were grown to confluence and incubated with the cytokines IL-1 (100 U/mL), γ-IF (100 U/mL), β-IF (100 U/mL), or TNF (100 U/mL) for 24 hours. The medium containing the cytokine was removed; the cultures were washed and then incubated with the radiolabeled lipoprotein for 20 hours and the rate of degradation of ^{125}I-lipoprotein was determined. The rates of degradation in control cultures not incubated with cytokine (basal) are shown for reference. Data are the mean ± SEM of 9 experiments with HuECs incubated with cytokines in duplicate or triplicate. *$P < .02$ *v* basal.

protein/20 h, respectively. The rate of accumulation of native-LDL in HuECs preincubated with γ-IF, β-IF, or TNF did not differ significantly from the rate in control cultures, and was 548 ± 129, 499 ± 138, and 369 ± 173 ng ^{125}I-LDL accumulated/mg cell protein/20 h, respectively.

The effect of each cytokine on the scavenger receptor activity in HuECs was also determined. The rates of high-affinity, receptor-mediated degradation of ^{125}I-Ac-LDL did not differ statistically and were 795 ± 197, 862 ± 338, 900 ± 247, 498 ± 231, and 604 ± 336 ng ^{125}I-Ac-LDL degraded/mg cell protein/20 h for control incubations and cultures preincubated with IL-1, γ-IF, β-IF, or TNF, respectively (Fig 3B). Similarly, there also were no significant differences in the rate of nonspecific degradation of ^{125}I-Ac-LDL by HuECs (Fig 3B). The rate of accumulation of ^{125}I-Ac-LDL also did not differ significantly between control cultures and cells preincubated with IL-1, γ-IF, β-IF, or TNF, and was 1,272 ± 531, 1,365 ± 530, 1,013 ± 317, 912 ± 268, and 826 ± 120 ng/mg cell protein/20 h, respectively. Although the rates of catabolism of ^{125}I-Ac-LDL were not significantly different, both β-IF and TNF dem-

onstrated a trend for reduced levels of degradation and accumulation.

To determine the mechanism of the increased degradation of LDL by HuECs incubated with IL-1, we examined the expression of receptors for native LDL in treated and nontreated HuECs as shown by the immunoblot presented in Fig 4. HuECs were incubated for 24 hours in growth media or growth media supplemented with 100 U/mL IL-1. As a positive control, cultures were also incubated with inoculation media containing 5% LPDS to upregulate LDL receptors. IL-1 (100 U/mL) increased the expression of LDL receptor in HuECs by 38% compared with cells incubated in media without IL-1. LDL receptor expression was increased an additional 31% after incubating the cells in LPDS.

The increased rates of degradation and accumulation of ^{125}I-LDL by HuECs were accompanied by changes in the metabolism of cholesterol by HuECs preincubated with the cytokines. The rate of cholesteryl ester synthesis in HuECs incubated with IL-1 was significantly increased compared with the rate in control cells, and was 646 ± 83 versus 534 ± 36 pmol ^{14}C-cholesteryl oleate/mg cell protein/20 h, respectively. The cholesteryl ester synthesis rate in HuECs preincubated with γ-IF, β-IF, or TNF was not significantly different from the rate in control incubations, and was 586 ± 49, 504 ± 52, and 521 ± 30 nmol ^{14}C-cholesteryl oleate/mg cell protein/20 h, respectively.

To determine if the stimulatory effect of IL-1 on the rate of degradation of ^{125}I-LDL is specific for endothelial cells of human origin, we performed similar studies using BAECs. We conducted preliminary studies to determine the concentration

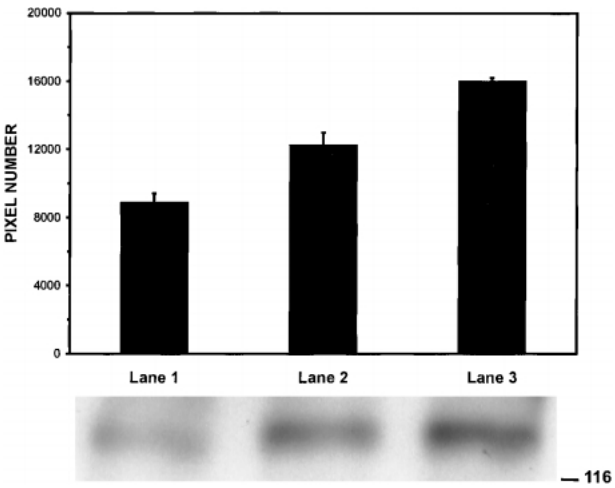

Fig 4. Immunoblot of LDL receptor expression in HuECs incubated with serum-free medium ([SFM] lane 1), SFM + IL-1 100 U/mL (lane 2), and SFM + 5% LPDS (lane 3). A cell lysate was prepared from incubated cultures. The samples (20 μg protein) were applied to each well of an 8% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred onto a nitrocellulose filter. The filter was incubated with rabbit anti–LDL receptor peptide. The bands were visualized using horseradish peroxidase–conjugated sheep anti-rabbit IgG. Blots were developed using chemiluminescence. The graph depicts the pixel number of the band corresponding to LDL receptor in each lane. The mean + SD of duplicate determinations is shown.

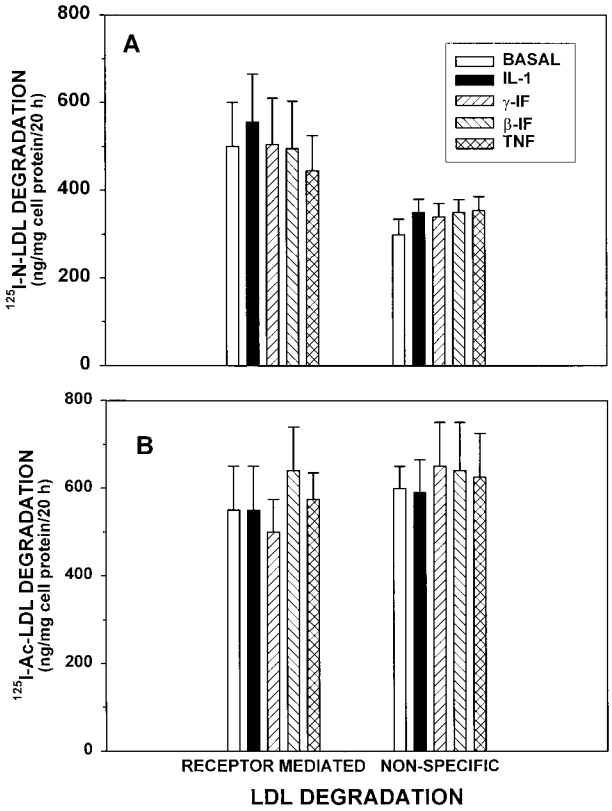

Fig 5. Influence of cytokines on BAEC degradation of (A) ^{125}I-LDL or (B) ^{125}I-Ac-LDL. BAECs were grown and incubated with the indicated cytokine at the same concentration as described for HuECs in Fig 3. Data are the mean ± SEM of 3 experiments with BAECs incubated with the cytokine in duplicate.

of the cytokines that would lead to maximal rates of ^{125}I-LDL degradation by BAECs. Preliminary studies indicated no significant differences in the rate of high-affinity, receptor-mediated degradation of ^{125}I-LDL in control incubations compared with BAECs preincubated with the cytokines IL-1, γ-IF, β-IF, or TNF at a concentration in the medium up to 1,000 U/mL. The results for studies with cytokines incubated at 100 U/mL are summarized in Fig 5. The rate of degradation of ^{125}I-LDL (Fig 5A) did not differ between control cultures and BAECs preincubated with cytokines, and was 496 ± 200, 579 ± 229, 491 ± 218, 469 ± 240, and 381 ± 125 ng/mg cell protein/20 h in control cultures and in BAECs preincubated with IL-1 (100 U/mL), γ-IF (100 U/mL), β-IF (100 U/mL), or TNF (100 U/mL), respectively. The rate of receptor-mediated accumulation of ^{125}I-LDL also did not differ significantly in control incubations compared with BAECs preincubated with cytokine; the rate of accumulation was 314 ± 81, 309 ± 84, 257 ± 91, 291 ± 64, and 183 ± 43 ng/mg cell protein/20 h in control cultures compared with BAECs preincubated with IL-1, γ-IF, β-IF, or TNF, respectively.

Similarly, preincubation of BAECs with cytokines did not influence the rates of degradation or accumulation of ^{125}I-Ac-LDL. The degradation rate of ^{125}I-Ac-LDL via high-affinity, receptor-mediated pathways was 578 ± 219, 578 ± 227, 316 ±

176, 761 ± 376, and 654 ± 225 ng/mg cell protein/20 h in control cultures compared with BAECs preincubated with IL-1, γ-IF, β-IF, or TNF, respectively. These data are shown in Fig 5B. The rate of accumulation of ^{125}I-Ac-LDL in the same experiments was 835 ± 316, 904 ± 379, 886 ± 324, 774 ± 342, and 667 ± 195 ng/mg cell protein/20 h, respectively.

Cytokines have been shown to alter the binding of monocytes to endothelial cells. We wished to determine if the binding of monocytes is altered when, in addition, the cholesterol and lipoprotein metabolism of endothelial cells is altered by inflammatory cytokines. Endothelial cell cultures were preincubated with LDL (100 μg/mL), IL-1 (100 U/mL), or LDL plus IL-1 for 16 hours. Cells were also incubated with inoculation media or lipopolysaccharide (*Escherichia coli* serotype 055: B5; Sigma Chemical) as negative and positive controls, respectively. A control incubation using growth media alone was also included in each experiment. After the incubation period, the medium was removed from each culture, the culture was washed twice with PBS, and the binding of monocytes to the endothelial cell layer was determined as detailed earlier.

There was a significant increase in the binding of monocytes to endothelial cells previously incubated with IL-1 ($P < .05$) compared with endothelial cells incubated with inoculation media (Fig 6). LDL combined with IL-1 significantly increased the binding of monocytes to endothelial cells ($P < .05$) versus that observed with inoculation media, but did not increase monocyte binding above that observed in cultures incubated with IL-1 alone. There were no significant differences between the binding of monocytes to cultures incubated with lipopolysaccharide, IL-1, or IL-1 plus LDL. Similarly, inoculation media alone did not alter the binding of monocytes when compared with endothelial cells incubated in the usual growth media.

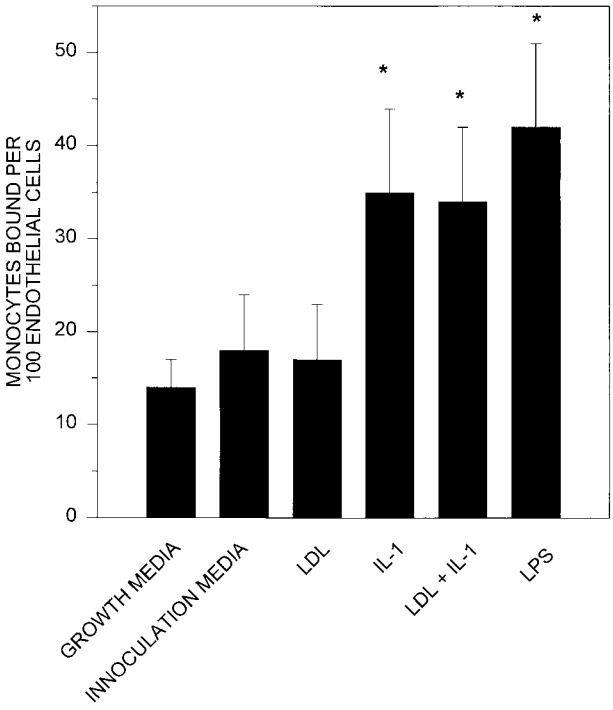

Fig 6. Effect of IL-1, LDL, and IL-1 + LDL on monocyte adherence to human endothelial cells. Confluent human endothelial cells were incubated for 16 hours with LDL (100 μg/mL), IL-1 (100 U/mL), or LDL + IL-1 for 16 hours. The medium was then removed and monocyte adherence to the endothelial cell layer was quantified. Control incubations with complete media routinely used for cell growth (growth media), serum-free media (inoculation media), and media containing lipopolysaccharide (LPS) are shown for reference. Results are the mean ± SEM of 8 studies in sextuplicate. *$P < .05$ *v* inoculation media.

DISCUSSION

We have demonstrated that preincubation of human endothelial cells with IL-1 significantly increased the rates of high-affinity, receptor-mediated accumulation and degradation of ^{125}I-native LDL compared with control cells incubated with medium without inflammatory cytokines or cells preincubated with medium containing TNF, β-IF, or γ-IF. This increase in catabolism resulted from increased expression of LDL receptor protein as evidenced by the immunoblot in Fig 4. The increased LDL catabolism also was accompanied by a significant increase in the rate of cholesteryl ester synthesis in the endothelial cells. Preincubation of endothelial cells with inflammatory cytokines did not alter the endothelial cell metabolism of AcLDL. The increased metabolism of LDL by endothelial cells incubated with IL-1 did not significantly increase monocyte adherence to the cell beyond that observed with IL-1 incubation alone. Native LDL alone did not influence monocyte adherence to endothelial cells.

The precise mechanism whereby IL-1 may contribute to atherogenesis is uncertain. IL-1 has been shown to increase LDL receptor expression and activity in HepG2 cells[23,24] and human omental microvascular cells.[25] We now show for the first time that IL-1 increases the degradation and accumulation of native LDL by human endothelial cells (Figs 1 to 3) as a result of increased LDL receptor activity (Fig 4). Most importantly, the increased catabolism of LDL was accompanied by alterations in cholesterol metabolism in the cells, as significant increases in endothelial cell cholesteryl ester synthesis were also observed.

According to one traditional theory, vascular disease is associated with injury to the endothelium and this injury may range in severity from the overt to the subtle.[26] We believe that more subtle endothelial injury may be more significant in the development of atherosclerosis. Factors that induce subtle alterations in endothelial cell metabolism, such as increased LDL catabolism resulting from exposure of the cell to inflammatory cytokines, may be of fundamental importance in the pathogenesis of atherosclerosis because endothelial cells are important modulators of intravascular homeostasis.

One potential mechanism whereby vascular endothelial cells can regulate vascular homeostasis is by modulating the binding of monocytes to the cell.[9] It is the increased monocyte adherence to the endothelial cell layer that is considered to be the first step in the development of an atheromatous lesion. Our group (Fig 6) and others[9] have shown that IL-1 significantly increases the binding of monocytes to the endothelial cell layer. However, we wished to determine if the altered cellular lipoprotein and cholesterol metabolism induced by IL-1 could

further increase monocyte binding to the endothelial cell layer. Incubation of endothelial cells with both IL-1 and LDL failed to increase the binding of monocytes to the endothelial cell layer beyond that found with IL-1 alone (Fig 6). Studies of the effect of cytokines combined with lipoproteins on adhesion molecule expression in endothelial cells are limited. In human aortic endothelial cells previously incubated with TNF,[27] oxidized LDL, but not native LDL, enhanced the expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), but not E-selectin. The results of our studies confirm and extend those reported by Khan et al.[27] While the inflammatory cytokines used in the two studies differed, both studies demonstrate the ability of inflammatory cytokines to influence monocyte binding to endothelial cells, but also the inability of native LDL to enhance the degree of monocyte binding.

In contrast to the results obtained when native LDL was used in the incubations, there were no differences in the accumulation or degradation of Ac-LDL by endothelial cells preincubated with IL-1 (Fig 2). This suggests that IL-1, at least with the experimental conditions described herein, does not affect the expression of the scavenger receptors known to recognize Ac-LDL (class A receptors).

The results of our studies examining the metabolism of LDL by endothelial cells of bovine origin when exposed to inflammatory cytokines were not similar to those observed with human cells. It is likely that the bovine cells did not respond to IL-1 due to differences in responsiveness between species. Alternatively, the lack of response may be related to considerations of species specificity, since the cytokines used to stimulate bovine cells were of human origin. Additionally, there may be a difference in the responsiveness of cells as a function of their stage of development or site of origin.[27] The human endothelial cells used in these studies were isolated from umbilical cords, while the bovine cells were isolated from aorta.

In conclusion, we have provided additional evidence supporting the role of cytokines in atherogenesis. Autoantibodies against epitopes of oxidized LDL have been identified in sera obtained from human subjects and animal models and in atherosclerotic lesions.[1-4,28-31] Ingestion of LDL immune complex by the macrophage not only leads to foam cell formation but also induces cytokine release from the macrophage.[7] Thus, cytokines released by macrophages activated by LDL containing immune complex may therefore indirectly upregulate the expression of adhesion molecules on endothelial cells. In addition, we now demonstrate that the release of cytokines, specifically IL-1, from these activated macrophages may also alter LDL metabolism by endothelial cells and endothelial cell cholesterol metabolism. It should be noted that LDL immune complex adsorbed to red blood cells also activates human macrophages.[6] Therefore, it seems likely that LDL containing immune complex adsorbed to red blood cells may stimulate cytokine release and thus stimulate monocyte adhesion to endothelial cells and alter endothelial cell lipoprotein and cholesterol metabolism. In conclusion, the body of evidence supporting the significance of immune mechanisms in the pathogenesis of atherogenesis continues to grow and is further supported by the data presented herein.

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