

HUMAN INTERLEUKIN 1 INDUCES INTERLEUKIN 1 GENE EXPRESSION IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

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IL-1 is a multipotent inflammatory mediator that may play a central role in vascular pathophysiology. For example, IL-1 promotes the adhesion of all classes of leukocytes to cultured endothelial cells (1-3) and increases their production of procoagulant activity (4), plasminogen activator inhibitor (5, 6), and prostaglandins (7, 8). These actions of IL-1 indicate its importance in mediating disturbances of endothelial function associated with vascular injury and inflammation. However, there is scant information regarding possible effects of IL-1 on vascular smooth muscle cells, the most abundant cell type in most vessels.

Vascular wall cells are not only targets for the action of IL-1, but can also produce IL-1-like molecules. Two distinct genes encode forms of IL-1 with similar biological activities but disparate amino acid sequences: IL-1 α has an isoelectric point of 5, and IL-1 β (the predominant form produced by human monocytes) has an isoelectric point of 7 (9, 10). Recently, we have described the inducible expression of both of these IL-1 genes in endothelial cells and smooth muscle cells cultured from adult human blood vessels (11-13). In these cell types, bacterial endotoxins and recombinant human tumor necrosis factor/cachectin (rTNF)¹ induced the appearance of IL-1 β messenger RNA (mRNA) and release of IL-1. We now report that IL-1 itself is a potent stimulus for IL-1 gene expression in vascular smooth muscle cells from adult humans. The demonstration that smooth muscle is both a source of IL-1 and a target for this cytokine raises the possibility of novel feedback loops in vascular pathophysiology. These findings expand the known responses of vascular smooth muscle cells, and also broaden the scope of the actions of the IL-1 polypeptides on the blood vessel wall.

Materials and Methods

Cell Culture. Human saphenous vein smooth muscle cells (HSVSMC) were isolated from outgrowths of explants of unused portions of veins harvested for coronary artery bypass surgery. The endothelium was removed enzymatically, and the adventitia was

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¹ *Abbreviations used in this paper:* HSVSMC, human saphenous vein smooth muscle cell; TNF, tumor necrosis factor/cachectin.

removed by blunt and sharp dissection before culture of the explants (12). This use of normally discarded tissues was approved by the Human Investigation Review Committee of New England Medical Center, Boston, MA. Human aortic and iliac arterial smooth muscle cells were prepared by enzymatic dissociation from tissues obtained from organ donors, with the cooperation of the New England Organ Bank. The adventitia and abluminal portions of the tunica media were removed before dissociation of the tissue with collagenase (CLS III, 0.2%; Cooper Biomedical, Inc., Malvern, PA). The cells were maintained in DME that contained glucose (5.5 mM), Hepes (25 mM), and FCS (HyClone Laboratories, Logan, UT) (10%). The morphology and growth pattern of the cells determined by phase-contrast microscopy were typical of cultured smooth muscle cells (14, 15). Cultures were used in the third to eighth passage.

Reagents. Phenol-extracted LPS from *Escherichia coli* 0111:B4 was obtained from Sigma Chemical Co., St. Louis, MO, and further purified by enzymatic digestion of contaminating protein and nucleic acid, followed by chromatography on Sepharose 4B, as previously described (16, 17). Recombinant human IL-1 β (rIL-1 β) (18), affinity-purified human monocyte IL-1 (19), and rabbit antisera to semipurified human monocyte IL-1 (20) and to rTNF (21) were provided by Dr. Charles A. Dinarello of Tufts University. Recombinant human IL-1 α (rIL-1 α) (22) was obtained from Dr. Peter T. Lomedico of Hoffmann-LaRoche, Inc., Nutley, NJ. mAb to the monocyte antigen Mo2 (23, 24) was a gift from Dr. Stuart F. Schlossman of the Dana Farber Cancer Institute, Boston, MA. Polymyxin B and indomethacin were purchased from Sigma Chemical Co.

Isolation of RNA and Northern Analysis. Cultured cells were detached by treatment with trypsin (0.25%, TRL III; Cooper Biomedical, Inc.) in EDTA (0.05%) and lysed in guanidine isothiocyanate (4 M). RNA was pelleted by centrifugation through cesium chloride cushions (25, 26). The concentration of RNA was determined from the absorbance at 260 nm, and A_{260}/A_{280} ratios were >2 . RNA was electrophoresed in agarose gels (1.2%) containing formaldehyde (2.2 M) (27). After capillary transfer of the RNA to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL), the RNA was immobilized by shortwave UV illumination for 3 min on each side of the membrane. The membranes were prehybridized for at least 4 h before hybridization with the ^{32}P -labeled probes described below (27). Autoradiography was carried out for 1–5 d using Kodak X-Omat AR-5 film with intensification screens (Eastman Kodak Co., Rochester, NY). Densitometry of autoradiograms was performed with an LKB 2202 Ultrosan laser densitometer and LKB 2220 recording integrator (LKB Produkter AB, Bromma, Sweden).

DNA Probes. IL-1 β was detected using a fragment (positions 278–1,381) of IL-1 β cDNA isolated from pGEM-IL-1 β (11). Drs. Philip E. Auron, C. A. Dinarello, and S. M. Wolff of the Department of Medicine, Tufts University, provided pCD 411 from which pGEM-IL-1 β was constructed (28). β -Tubulin was detected using a fragment (1 kb) of rat cDNA obtained from RBT.3 (29), a gift of Dr. Stephen R. Farmer of the Department of Biochemistry, Boston University. IL-1 α was detected using a synthetic oligonucleotide (positions 298–339) synthesized by Dr. Richard Malavara of Cistron Technology, Inc., Pine Brook, NJ, and a cDNA probe isolated from p Δ 3 IL-1 α , obtained from Dr. Peter T. Lomedico. This construct contains the complete coding region for amino acids 1–132 (cDNA positions 1–432) of the 33-kD IL-1 α precursor. Plasmids were isolated chromatographically (30). Inserts were isolated after restriction endonuclease digestion by low-melt agarose gel electrophoresis or electroelution, and labeled by nick translation with [^{32}P]dCTP and [^{32}P]dGTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) (27). The oligonucleotide was end-labeled with γ -[^{32}P]ATP (27). The specific activity of the probes was $>10^8$ cpm/ μg DNA.

Assay of IL-1 Biological Activity. Medium collected from cultures of HSVSMC (conditioned medium) was centrifuged (10 min at 1,000 g) and stored at -70°C until assay. For determination of intracellular IL-1, cell layers were washed three times with DME, covered with 1 ml of FCS (5%) in RPMI 1640 medium, and frozen at -20°C . After thawing, the plates were scraped with a rubber policeman and the lysate was centrifuged (15 min at 1,000 g), and the supernatants were stored at -70°C until assay. IL-1 activity was

determined by the murine thymocyte costimulation assay (31). At least two dilutions (1:1 and 1:10) of test samples were incubated in 96-well plates (Costar, Inc., Cambridge, MA) with 5×10^5 thymocytes from C3H/HeJ mice (6–8 wk old; The Jackson Laboratory, Bar Harbor, ME) in a final volume of 200 μ l of RPMI 1640 containing FCS (5%), PHA (1 μ g/ml; Burroughs Wellcome, Research Triangle Park, NC), antibiotics, glutamine (2 mM), and 2-ME (5×10^{-5} M). Affinity-purified human monocyte IL-1 was included in each assay as a positive control. After 48 h, [3 H]thymidine (1 μ Ci/well, 20 or 6.7 Ci/mmol; New England Nuclear) was added to the wells, and cells were harvested 18–24 h later onto glass fiber filters using a commercial cell harvester (Cambridge Technology, Cambridge, MA). The filters were counted in Econosolve HP/b scintillation fluid (Beckman Instruments, Inc., Palo Alto, CA), and quench corrections were performed by use of an external standard. The results are reported as the mean \pm standard deviation of disintegrations per minute determined on triplicate cultures. Because conditioned medium contained the rIL-1 used to stimulate smooth muscle cells, as well as IL-1 produced by these cells, both unconditioned and conditioned media were assayed. Smooth muscle cell IL-1 production is the difference between IL-1 activity in conditioned medium (HSVSMC-derived IL-1 + rIL-1) and IL-1 in unconditioned medium (rIL-1 alone).

PGE₂ Assay. HSVSMC were cultured in 24-well dishes (Costar) in 1 ml of DME/10% FCS. The cells were incubated for 1–24 h in the presence or absence of indomethacin (1 μ g/ml) and rIL-1 β (100 ng/ml). The medium was centrifuged (1,000 g for 15 min), acidified with HCl (final pH 3), and extracted with 3 vol of ethyl acetate. The organic phase was dried, and the residue was resuspended in phosphate buffer and assayed for PGE₂ using an RIA kit, according to the manufacturer's instructions (Seragen Inc., Boston, MA).

Endotoxin Determination. Levels of bacterial endotoxin in tissue culture media were determined with the use of the chromogenic limulus lysate assay (QCL 1000, M. A. Bioproducts, Walkersville, MD). This assay is sensitive to 10 pg/ml of bacterial endotoxin. Very low concentrations of bacterial endotoxin (<1 ng/ml) can induce IL-1 β gene product in human vascular smooth muscle cells (12, and data not shown). In some cases we have found IL-1 β mRNA in HSVSMC not deliberately exposed to endotoxin, and have subsequently measured concentrations of endotoxin >100 pg/ml in the tissue culture media. Basal IL-1 production by HSVSMC could be avoided by culturing cells in medium selected for low levels of endotoxin contamination (<40 pg/ml). In addition, the experiments reported here were routinely carried out in the presence of the endotoxin antagonist polymyxin B (10 μ g/ml).

Treatment of Cells with Antimonocyte Antibody and Complement. To test whether IL-1 production by HSVSMC might be due to contamination with blood monocytes, cultures were treated with mAb to the monocyte antigen Mo2 (anti-Mo2) and complement, before stimulation with LPS or rIL-1. The cells were incubated with anti-Mo2 (1:150 dilution in DME/10% heat-inactivated FCS), or medium without the antibody, for 30 min at 4°C. The medium was aspirated and replaced with a 1:4 dilution of baby rabbit complement (Pel-Freez Biologicals, Rogers, AK). After incubation for 1 h at 37°C the cells were washed three times with HBSS and cultured for 24 h in DME/10% FCS. In independent experiments, such treatment completely lysed adherent human peripheral blood mononuclear cells, as judged by phase-contrast microscopy. The HSVSMC were then incubated for 6 h in fresh medium containing LPS (1 μ g/ml), or for 24 h with rIL-1 α (10 ng/ml) or rIL-1 β (100 ng/ml). IL-1 was measured in unconditioned and conditioned media, and IL-1 β mRNA was measured by Northern analysis.

Results

IL-1 Induces IL-1 Gene Expression in Human Vascular Smooth Muscle Cells. Under basal conditions, HSVSMC cultured in low-endotoxin medium (<40 pg/ml) contained no mRNA for IL-1 β . Highly purified IL-1 from human monocytes induced a concentration-dependent increase in IL-1 β mRNA levels (Fig. 1). Monocyte-derived IL-1 concomitantly induced release by HSVSMC of

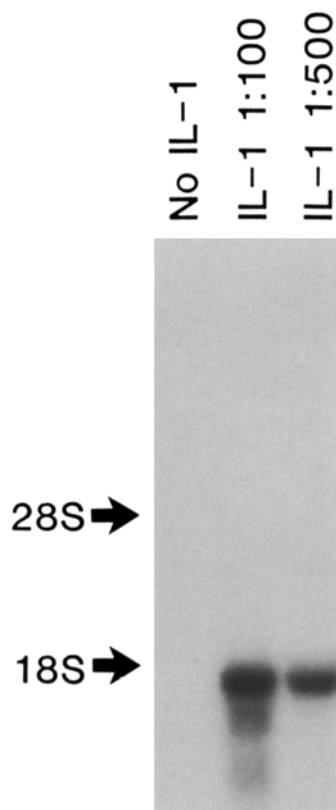


FIGURE 1. Human monocyte-derived IL-1 induces IL-1 β mRNA in human vascular smooth muscle cells. HSVSMC were incubated for 4 h with human monocyte-derived IL-1 diluted 1:100 and 1:500 in DME/10% FCS containing polymyxin B (10 μ g/ml). Northern analysis of total cellular RNA (20 μ g) used a 32 P-labeled IL-1 β cDNA probe. Arrows indicate position of 28S and 18S ribosomal subunits in this and subsequent figures. The activity of the monocyte IL-1 in the thymocyte costimulation assay, measured as [3 H]thymidine incorporation (mean dpm \pm SD of triplicate wells) was 36,823 \pm 2,401 for IL-1 diluted 1:100 and 18,604 \pm 380 for IL-1 diluted 1:500 (both significantly different from PHA background which was 15,571 \pm 515 dpm, $p < 0.01$, Student's t test).

biologically active IL-1, determined as thymocyte costimulation activity (data not shown).

This monocyte-derived IL-1 contained a mixture of both the neutral and acidic IL-1 species (20). rIL-1 α and rIL-1 β are homogeneous preparations that express the biological activities of the corresponding monocyte-derived IL-1 species (18, 22). rIL-1 β (0.01–100 ng/ml) caused a concentration-dependent increase in IL-1 β mRNA levels (Fig. 2A) and secretion of IL-1 (Fig. 2B), with a threshold between 100 pg/ml and 1 ng/ml. The increase in IL-1 mRNA was selective, in that levels of β -tubulin mRNA were not altered substantially by rIL-1 β (Fig. 2B). rIL-1 α (0.01–50 ng/ml) also induced IL-1 β mRNA in these cells as well as the release of thymocyte costimulation activity (data not shown). rIL-1 α was more potent than IL-1 β as an inducer of IL-1 production by HSVSMC, in parallel with an increased potency in the murine thymocyte costimulation assay. This differential potency between the two species of recombinant IL-1 may be due, at least in part, to the sensitivity of rIL-1 β to oxidation of sulfhydryl groups associated with a loss of biological activity. The response of smooth muscle cultures to IL-1 was not limited to venous or explant-derived cells. Arterial smooth muscle cells isolated by enzymatic dissociation from human aorta or iliac artery responded to rIL-1 α and rIL-1 β in the same manner as cells isolated from saphenous vein (data not shown).

HSVSMC treated with rIL-1 α or rIL-1 β did not contain mRNA for IL-1 α ,

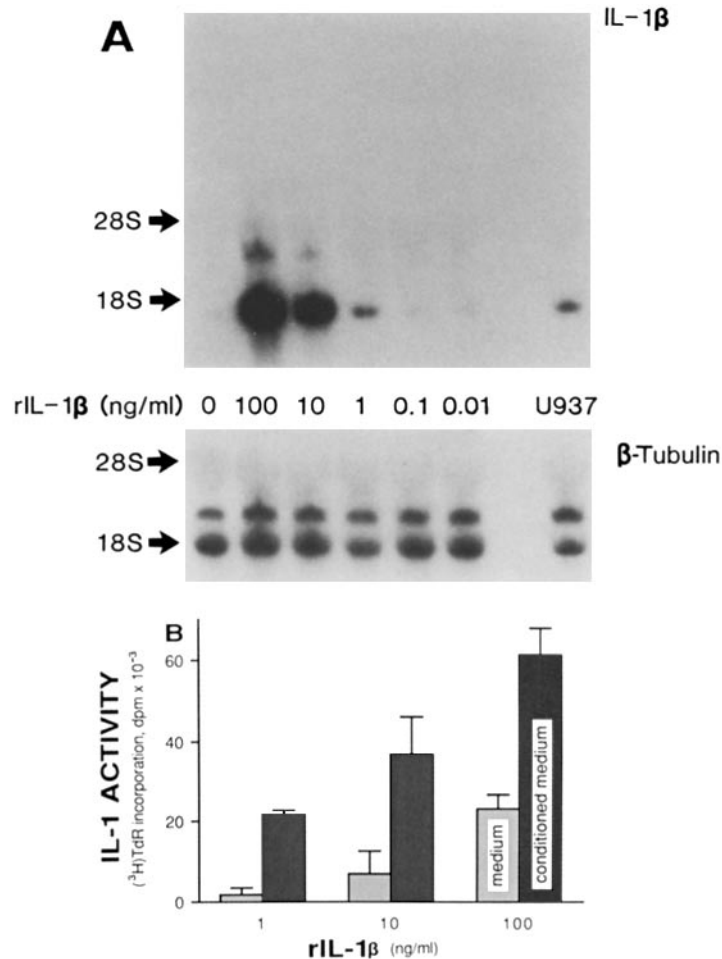


FIGURE 2. Concentration dependence of rIL-1 β -induced production of IL-1 β mRNA and IL-1 activity. HSVSMC were incubated for 4 h with rIL-1 β in DME/10% FCS containing polymyxin B (10 μ g/ml). RNA (20 μ g) isolated from the monocytoid cell line U937 (U937) stimulated for 24 h with LPS (1 μ g/ml) was analyzed simultaneously. The mRNA transcripts from HSVSMC comigrate with U937 IL-1 β mRNA transcripts (1.6 kb). (A) Autoradiogram of Northern analysis. (Top) Northern analysis and hybridization with IL-1 β probe was performed as in Fig. 1. (Bottom) The same blot was rehybridized with ³²P-labeled rat β -tubulin probe, and shows approximately equal amounts of hybridization in each lane, indicating that the RNA in each lane is intact. (B) IL-1 activity. Conditioned (dark bars) and unconditioned (light bars) media from the same experiment shown in A were assayed (1:10 dilution) in the mouse thymocyte costimulation assay. In this and subsequent figures, data are [³H]thymidine incorporation (mean dpm \pm SD, triplicate wells) corrected by subtraction of [³H]thymidine incorporation in the presence of PHA alone (In this experiment, 35,223 \pm 4,441 dpm).

determined by using both an end-labeled ³²P-synthetic oligonucleotide (42-mer) probe and a ³²P-labeled cDNA probe (data not shown). Under the same hybridization conditions, these probes did detect IL-1 α transcripts in RNA from LPS-stimulated human monocytes. We have only observed IL-1 α mRNA in smooth muscle cells treated with cycloheximide alone (1 μ g/ml) or with rIL-1 and cycloheximide (data not shown). This result is in accord with our previous finding

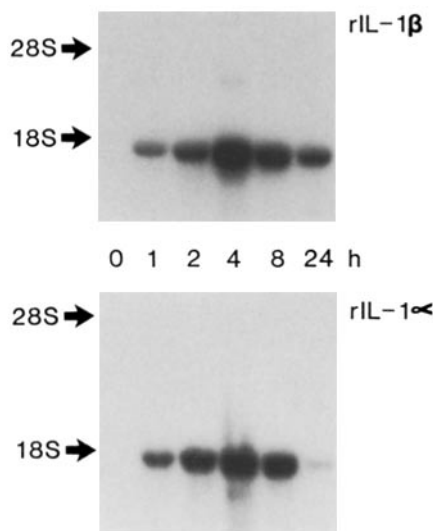


FIGURE 3. Time course of human rIL-1-induced IL-1 β mRNA synthesis in HSVSMC. HSVSMC were incubated in DME/10% FCS containing polymyxin B (10 μ g/ml) with rIL-1 β (100 ng/ml, *top*) or rIL-1 α (10 ng/ml, *bottom*) for the time periods indicated. The incubation with IL-1 α included indomethacin (1 μ g/ml). RNA (20 μ g) was electrophoresed, transferred to a nylon membrane, and hybridized with 32 P-labeled IL-1 β probe.

(12) that IL-1 α mRNA appears in HSVSMC exposed to cycloheximide alone, and that the LPS-induced increase in IL-1 mRNA levels is augmented by this inhibitor. Using less stringent washing conditions (1 \times SSC; 55°C) we have detected IL-1 α mRNA in rIL-1-stimulated HSVSMC (data not shown).

Induction of IL-1 by rIL-1 Is Rapid, Transient, and Dependent on RNA Synthesis. Exposure to rIL-1 β (100 ng/ml) increased IL-1 β mRNA in HSVSMC within 1 h (Fig. 3, *top*). The amount of IL-1 β mRNA was maximal after 4 h of incubation, and declined after 24 h of continued exposure to rIL-1 β . The time course of the appearance of IL-1 β mRNA induced by rIL-1 α (10 ng/ml) paralleled that found in response to rIL-1 β (Fig. 3, *bottom*). In further experiments using shorter incubation times, IL-1 β mRNA appeared as early as 30 min after exposure to rIL-1 β (data not shown). Incubation of HSVSMC for 4 h simultaneously with the RNA synthesis inhibitor actinomycin D (1 μ g/ml) and rIL-1 β (100 ng/ml) inhibited the effect of rIL-1 β on IL-1 β mRNA levels by 99%, measured by scanning densitometry of an autoradiogram of Northern blot analysis. This concentration of actinomycin D inhibited [3 H]uridine incorporation by HSVSMC into material insoluble in perchloric acid (0.2 M) by 93% (data not shown). These results suggest that rIL-1 affects IL-1 β mRNA levels by increasing RNA synthesis.

The synthesis of IL-1 β mRNA resulted in the release of biologically active IL-1 (Fig. 4). Extracellular IL-1 activity, in excess of that due to the stimulus alone, rose after 1 h of exposure to rIL-1 β , continued to increase for up to 8 h, and declined by 24 h (Fig. 4A). This pattern suggested that the smooth muscle cells produce an inhibitor of the thymocyte costimulating ability of IL-1, as do endothelial cells (32). rIL-1 α (10 ng/ml) induced a similar time course of IL-1 release, except that IL-1 activity was not decreased at 24 h (Fig. 4B). The cyclooxygenase inhibitor indomethacin was included in the incubation medium in this latter experiment. Preliminary studies had indicated that indomethacin increased the amount of IL-1 activity measured in conditioned medium from

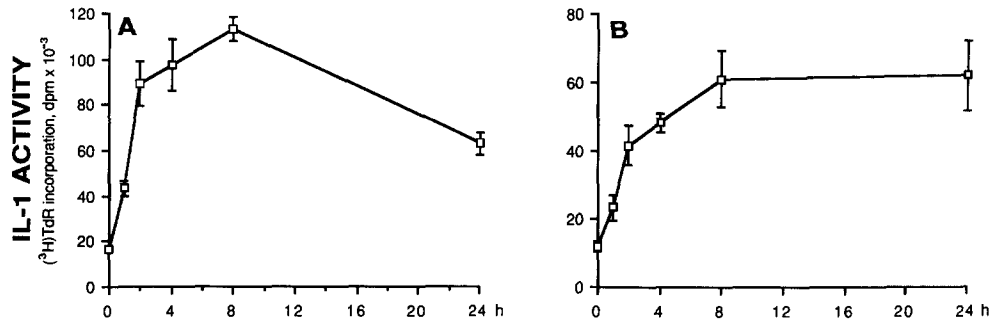


FIGURE 4. Time course of IL-1 release from HSVSMC induced by rIL-1. IL-1 in conditioned media (1:10 dilution) from the same experiments shown in Fig. 3 were measured in the thymocyte costimulation assay. (A) HSVSMC stimulated with rIL-1 β (100 ng/ml). (B) HSVSMC stimulated with rIL-1 α (10 ng/ml), in the presence of indomethacin (1 $\mu\text{g}/\text{ml}$). [^3H]-Thymidine incorporation in response to PHA alone has been subtracted, and was $35,223 \pm 4,441$ dpm for the experiment with rIL-1 β and $14,149 \pm 1,508$ dpm for that with rIL-1 α . Data at zero time represent [^3H]thymidine incorporation in response to unconditioned medium containing rIL-1 alone.

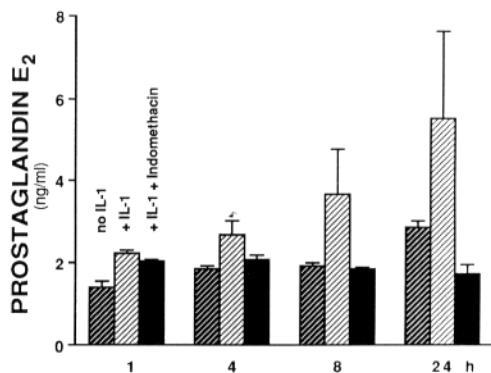


FIGURE 5. PGE₂ production by HSVSMC. Cells were incubated for the indicated times in 24-well dishes containing DME/10% FCS with or without rIL-1 β (100 ng/ml) and indomethacin (1 $\mu\text{g}/\text{ml}$). PGE₂ in the conditioned medium was measured by RIA. Data are mean \pm SD of determinations on triplicate wells.

LPS-stimulated HSVSMC. Prostaglandins, in particular PGE₂, can suppress the response of thymocytes to IL-1 (33), and we postulated that the decreased IL-1 activity in conditioned medium after 24 h was due to accumulated PGE₂.

rIL-1 Stimulates PGE₂ Production by HSVSMC. We therefore determined PGE₂ levels in the supernatants of HSVSMC exposed to medium alone or to rIL-1 β . rIL-1 β (100 ng/ml) stimulated PGE₂ production in a time-dependent manner. Indomethacin (1 $\mu\text{g}/\text{ml}$) prevented this effect (Fig. 5). In addition, conditioned medium from indomethacin-treated cells displayed more IL-1 activity than medium from cells incubated without the inhibitor (Fig. 6). Indomethacin alone did not affect the activity of rIL-1 β in the murine thymocyte costimulation assay (Fig. 6), nor did it affect IL-1 β mRNA levels in HSVSMC. Laser scanning densitometry of the autoradiogram of a Northern blot hybridized with IL-1 β probe yielded 1.28 ± 0.01 arbitrary units (U) for HSVSMC exposed to IL-1 β alone, and 1.20 ± 0.14 U for cells incubated with rIL-1 β and indomethacin (mean \pm SD of three scans at separate positions of each band). These data suggest that rIL-1-stimulated HSVSMC produce PGE₂, which inhibits the responsiveness

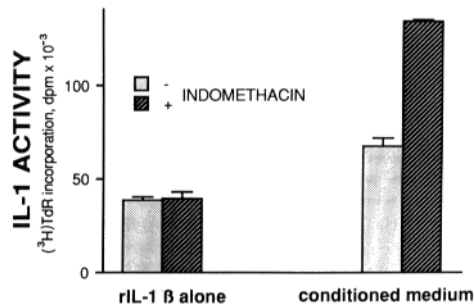


FIGURE 6. Effect of indomethacin on IL-1 production by HSVMC. Cells were incubated for 24 h in DME/10% FCS containing polymyxin B (10 $\mu\text{g}/\text{ml}$) and rIL-1 β (100 ng/ml), with or without indomethacin (1 $\mu\text{g}/\text{ml}$). Unconditioned and conditioned media (1:1 dilution) were assayed for IL-1. [^3H]Thymidine incorporation in response to PHA alone has been subtracted and was $16,257 \pm 2,004$ dpm for medium without indomethacin, and $16,326 \pm 862$ dpm for medium containing indomethacin.

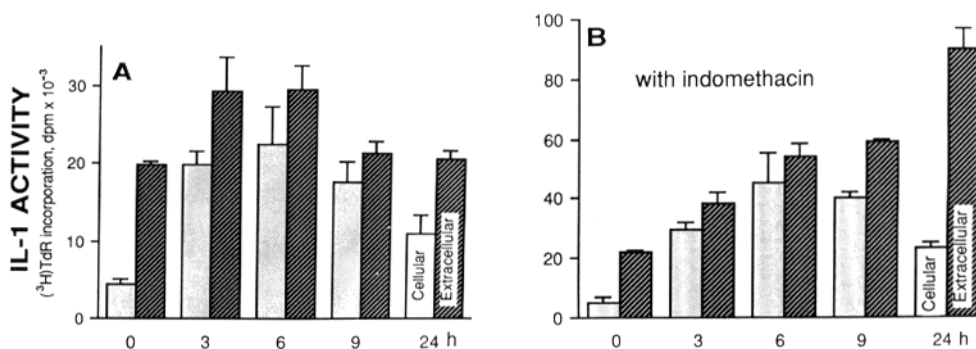


FIGURE 7. Intra- and extracellular IL-1 in HSVMC stimulated with rIL-1 β . Cells were grown in 60-mm culture dishes and stimulated for the indicated time periods with rIL-1 β (100 ng/ml) in 3 ml of DME/10% FCS containing polymyxin B (10 $\mu\text{g}/\text{ml}$), with (B) or without (A) indomethacin (1 $\mu\text{g}/\text{ml}$). Conditioned medium was aspirated and the monolayer was washed with medium without rIL-1 and freeze-thawed in 1 ml of RPMI/5% FCS. Intracellular (dark bars) and extracellular (light bars) IL-1 were determined in the murine thymocyte costimulation assay (1:10 dilution). [^3H]Thymidine incorporation in the presence of PHA alone has been subtracted, and was $10,550 \pm 524$ dpm for medium without indomethacin, and $11,615 \pm 931$ dpm for medium with indomethacin.

of thymocytes to IL-1. Alternatively, indomethacin may decrease the synthesis of a specific IL-1 inhibitor.

rIL-1 Induces Intracellular IL-1 Synthesis and Extracellular IL-1 Release. The intracellular synthesis and extracellular release of IL-1 by phagocytic leukocytes are temporally distinct and stimulus specific (34). We therefore studied the kinetics of the appearance of intra- and extracellular IL-1 in HSVMC exposed to rIL-1 β . In the absence of indomethacin, rIL-1 β (100 ng/ml) produced maximal levels of intracellular IL-1 after 6 h that decreased by 24 h (Fig. 7A). Extracellular IL-1 levels followed a similar time course. In parallel experiments with the same isolate of cells, indomethacin (1 $\mu\text{g}/\text{ml}$) did not alter the pattern of appearance of intracellular IL-1 activity, but caused extracellular IL-1 activity to continue to increase over a 24-h period (Fig. 7B). In addition, indomethacin increased the amount of intra- and extracellular IL-1 activity, as described above (note the difference in the scale of the ordinates in Fig. 7, A and B). These kinetic data suggest that HSVMC respond rapidly to human IL-1 by synthesizing IL-1 β mRNA (peak at 4 h), followed by intracellular IL-1 synthesis (peak at 6 h) and the release of IL-1. rIL-1 also induces release of PGE $_2$ more slowly (peak \geq 24

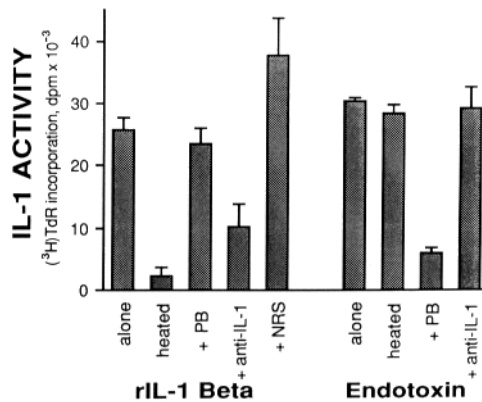


FIGURE 8. Inhibition of rIL-1 β -induced IL-1 production by HSVSMC by anti-IL-1 antiserum and heat treatment, but not by polymyxin B. HSVSMC were incubated for 4 h in DME/10% FCS containing indomethacin (1 μ g/ml) and rIL-1 β (10 ng/ml) or purified bacterial LPS (endotoxin, 10 ng/ml). Polymyxin B (PB, 10 μ g/ml) was included where indicated. Heated endotoxin and rIL-1 were incubated at 95 $^{\circ}$ C for 1 h. Rabbit antiserum to human monocyte-derived IL-1 (anti-IL-1) or nonimmune rabbit serum (NRS) was included at a final dilution of 1:100. Conditioned and unconditioned media (1:10 final dilution) were assayed in the murine thymocyte costimulation assay. Data are [3 H]thymidine incorporation (mean \pm SD, triplicate wells) in response to conditioned media, corrected by subtraction of incorporation due to the corresponding unconditioned medium (13,596 \pm 1,016 dpm for medium alone, and 20,182 \pm 793 dpm for medium containing rIL-1).

h), which masks measurable IL-1 activity in conditioned medium from later time points.

Induction of IL-1 Gene Product by rIL-1 β Is Not Due to Endotoxin Contamination. HSVSMC respond to endotoxin concentrations <1 ng/ml by producing IL-1 β mRNA and releasing IL-1 (12). The terms endotoxin and LPS are not synonymous, although most of the endotoxic properties of Gram-negative bacterial extracts are due to the LPS component (35). Here, we use endotoxin to refer to uncharacterized bacterial products, and LPS to designate a well-characterized, purified preparation. Although the endotoxin content of the rIL-1 used in this study was in the picogram per milligram range (18), we used several approaches to exclude the possibility that rIL-1-induced IL-1 synthesis in HSVSMC might be due to bacterial endotoxin in tissue culture media or IL-1 preparations.

Smooth muscle cells were incubated for 4 h with rIL-1 β or purified LPS (10 ng/ml each), either alone or with the addition of the LPS antagonist polymyxin B (10 μ g/ml), or rabbit anti-IL-1 antiserum (1:100 final dilution). IL-1 release (Fig. 8) and IL-1 β mRNA (data not shown) were measured. Polymyxin B blocked the ability of purified LPS to increase IL-1 β mRNA levels and IL-1 release but did not affect this activity of rIL-1 β . Polymyxin B does not inhibit all endotoxins, however (36), and we used a rabbit antiserum to human monocyte IL-1 to confirm the specificity of the response of HSVSMC to rIL-1 β . Antiserum to monocyte IL-1 blocked the production of IL-1 β mRNA and IL-1 release induced by rIL-1 β but not by LPS. Furthermore, incubating rIL-1 β for 1 h at 95 $^{\circ}$ C destroyed its ability to increase IL-1 β mRNA levels and IL-1 release, whereas LPS was unaffected by such heat treatment. These data indicate that rIL-1-induced IL-1 production by HSVSMC is not an artifact of endotoxin contamination.

IL-1 Production by HSVSMC Is Not Due to Contamination by Blood Monocytes. Blood monocytes, the prototypical source of IL-1, are potential contami-

TABLE I
*Effect of Treatment with Antimonocyte Antibody and Complement on
 IL-1 Production by HSVSMC*

| Exp. | Treatment | IL-1 activity (^3H]TdR incorporation, dpm) | | |
|------|----------------------------------|---|--------------------------------|------------------------|
| | | Unconditioned medium | Conditioned medium | |
| | | | Without anti-Mo2 | With anti-Mo2 |
| 1 | LPS (1 $\mu\text{g}/\text{ml}$) | 19,926 \pm 1,499 | 34,691 \pm 2,754 | 41,332 \pm 3,392* |
| 2 | rIL-1 α (10 ng/ml) | 52,363 \pm 4,872 \ddagger | 130,556 \pm 7,949 \ddagger | 148,525 \pm 13,150** |
| | rIL-1 β (100 ng/ml) | 45,994 \pm 8,547 | 127,322 \pm 3,857 | 148,047 \pm 10,296* |
| | No addition | 12,869 \pm 1,523 | 14,090 \pm 757 | 15,779 \pm 472 |

Cells were treated with to without antibody to Mo2 and then incubated with baby rabbit complement as described in Materials and Methods. IL-1 activity in 1:1 dilutions of unconditioned and conditioned media is reported as mean \pm SD of ^3H]TdR incorporation in triplicate cultures in the thymocyte costimulation assay.

* Not significantly different from cells treated with complement alone, $p \geq 0.08$.

\ddagger Not significantly different from corresponding value for rIL-1 β , $p \geq 0.325$, Student's t test.

nants of blood vessel-derived cell cultures (37). Our primary smooth muscle cell cultures were used in the third or subsequent passage, by which time any surviving monocytes should have acquired macrophage-like characteristics. This differentiation is associated with loss of the ability to secrete IL-1 (35). Nonetheless, we addressed this possibility directly by treating HSVSMC sequentially with mAb to the human monocyte antigen Mo2 (23, 24) and complement. In parallel experiments, this treatment lysed plastic-adherent human peripheral blood mononuclear cells (data not shown). HSVSMC were treated with complement, with or without preincubation with anti-Mo2, and exposed to LPS (1 $\mu\text{g}/\text{ml}$), a potent stimulus for IL-1 production by monocytes. Exposure to anti-Mo2 and complement did not impair the ability of HSVSMC to produce IL-1 in response to LPS when compared with the ability of cells exposed to complement alone (Table I). Similar results were found with rIL-1 α or rIL-1 β as IL-1 secretagogues (Table I). IL-1 β mRNA levels were similar in both groups of cells (data not shown). The data in Table I also show that concentrations of rIL-1 α or rIL-1 β that are equipotent in the thymocyte costimulation assay induce the release of approximately equal amounts of IL-1 activity from HSVSMC.

rIL-1-induced IL-1 Production Is Not Mediated via TNF Release. TNF induces the production of IL-1 in vascular smooth muscle cells (13), and IL-1 may also induce the release of TNF in some cell types. We therefore addressed the possibility that rIL-1-induced IL-1 production by HSVSMC was mediated by the induction of TNF release. Cells were incubated with rIL-1 α (10 ng/ml) or rIL-1 β (100 ng/ml) for 24 h in the presence of rabbit anti-TNF antiserum or nonimmune serum (both at 1:100 dilution). This anti-TNF antiserum neutralized the cytotoxicity of rTNF for the murine fibroblast line L929 at a 1:400 dilution (Ikejima, T., and C. A. Dinarello, unpublished data), and a 1:200 dilution detected 1 ng of rTNF in Western blots (data not shown). rIL-1-induced IL-1 release was not inhibited by the anti-TNF antiserum (Fig. 9). In addition, the induction of IL-1 β mRNA in HSVSMC incubated for 4 h with rIL-1 β (100 ng/ml) was unaffected by anti-TNF antiserum at a concentration (1:100 dilution)

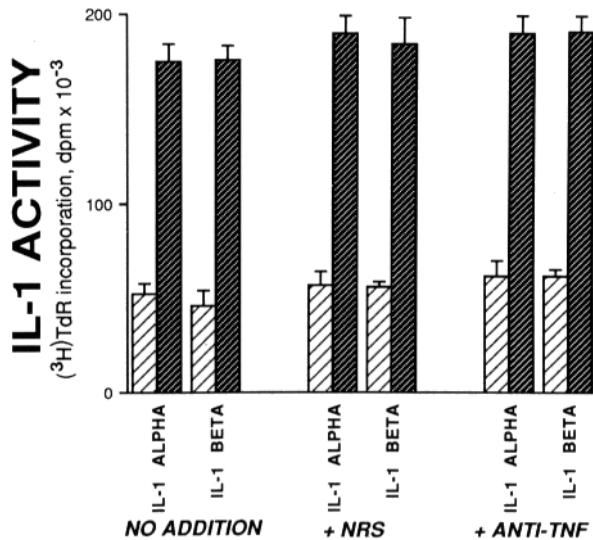


FIGURE 9. Anti-TNF antiserum does not inhibit rIL-1-induced IL-1 release from HSVSMC. Cells were incubated for 24 h in DME/10% FCS containing indomethacin (1 μ g/ml) and rIL-1 α (10 ng/ml) or rIL-1 β (100 ng/ml) alone, or with the addition of either anti-TNF antiserum or non-immune serum (1:100 dilution). After 24 h, IL-1 activity in unconditioned (*light bars*) and conditioned media (*dark bars*) (1:1 dilution) was measured. [3 H]Thymidine incorporation in the presence of PHA alone was $12,869 \pm 1,523$ dpm, and has not been subtracted from the data.

that blocked TNF-induced IL-1 β mRNA production (data not shown). These data indicate that rIL-1-induced IL-1 production is not mediated via the release of TNF.

Discussion

The manifold effects of IL-1 on vascular wall cells suggest an important role for this mediator in blood vessel pathology. IL-1 induces morphologic changes in cultured endothelial cells (38) and the production of procoagulant activity (4), plasminogen activator inhibitor (5, 6), prostaglandins (7, 8), and platelet-activating factor (39). In addition, IL-1 enhances adhesion to endothelial cells of all classes of leukocytes studied (1–3). Although the repertoire of endothelial cell responses to IL-1 has been widely studied, the effect of this mediator on human vascular smooth muscle cells has received scant attention. We report here the surprising observation that IL-1 induces IL-1 gene expression in human vascular smooth muscle. To our knowledge, this is the first definitive demonstration that IL-1 may regulate its own synthesis and release in any cell type.

The threshold concentration for the production of IL-1 in HSVSMC by rIL-1 β was <1 ng/ml, which is at the low range of concentrations that elicit PGE $_2$ production by human dermal fibroblasts and IL-2 generation from human T cells (18). Concentrations of rIL-1 α and rIL-1 β that were equipotent in the thymocyte costimulation assay induced similar amounts of IL-1 release (Table I and Fig. 9). rIL-1-induced IL-1 mRNA production and the extracellular release of IL-1 began within 1 h of stimulation. Experiments with an antiserum that neutralized the biological activity of rTNF excluded a role for extracellular TNF in the mediation of rIL-1-induced IL-1 production.

Our finding that smooth muscle cells exposed to monocyte-derived IL-1 for short periods in vivo could respond by releasing further IL-1 may have important implications for the pathogenesis of atherosclerosis and vasculitis. Human atherosclerotic plaques contain smooth muscle cells, macrophages, and T cells in a

specific spatial distribution (40, 41). Smooth muscle cells in these plaques, but not in normal arteries, express class II transplantation antigens, an indicator of immune activation (42). Many of the T lymphocytes in plaques express HLA/DR antigens (40). These considerations indicate that the complicated human atherosclerosis is not a static accumulation of lipid, calcium, and extracellular matrix, but a site of active inflammatory and immunologic reaction. Our present findings suggest novel humoral interactions between these cells that may contribute to the formation of this localized lesion.

In cholesterol-fed animals, monocytes adhere to vascular endothelium, and migrate into the developing atherosclerotic plaque (43–45). Vascular smooth muscle cells and endothelial cells produce factors that are chemotactic for monocytes (46, 47). Indeed, IL-1 itself is a potent monocyte chemoattractant (9), and IL-1 secreted by vessel wall cells could recruit monocytes to areas of local derangement. IL-1 produced locally by recruited monocytes, endothelial cells, or smooth muscle cells could stimulate further IL-1 and PGE₂ production by vascular smooth muscle cells, with concomitant alterations of lymphocyte, smooth muscle, and endothelial cell functions. IL-1 causes T cells to produce IL-2 and IFN- γ , cytokines that activate T cells themselves and induce the expression of IL-2 receptors and class II antigens on their surface (48). Activated T cells and IFN- γ also induce class II antigen expression on cultured endothelial cells and smooth muscle cells (37, 49). Thus, local production of IL-1 in the vessel wall may account for the expression of class II antigens on plaque cells observed in vivo (40, 42). This study also shows that rIL-1 β stimulates PGE₂ release by HSVSMC, in accordance with a previous report (8) that monocyte-derived IL-1 increased prostaglandin synthesis in human arterial smooth muscle cells. In addition to modulating platelet function and vascular tone, prostaglandins regulate smooth muscle cell cholesterol metabolism (50), and may be chemoattractant for leukocytes (46).

Our observation that IL-1 induces further IL-1 production from smooth muscle cells also suggests a mechanism for the amplification and perpetuation of vasculitis. Vascular smooth muscle cells from MRL/*lpr* mice that spontaneously develop a genetically determined autoimmune vasculitis, express class II antigens, and produce an IL-1-like factor (51). The vasculitic lesion is characterized by perivascular cuffing and infiltration of mononuclear cells. Moyer and Reinisch (51) suggested that smooth muscle cell-derived IL-1 stimulated the influx of monocytes into these lesions. The potential role of IL-1 in human vascular inflammation in vivo is illustrated by recent studies (52) using an mAb, H4/18, raised against IL-1-treated endothelial cells. IL-1 or TNF/cachectin induce H4/18 binding sites on endothelial cells, but not on other cells tested. H4/18 does not bind normal endothelium in skin or other tissues. However, in human delayed hypersensitivity reactions and other lesions associated with the presence of activated lymphocytes and macrophages, microvascular endothelium does bind H4/18 (53).

These examples show how IL-1 could be involved in the pathogenesis of important pathologic processes involving the blood vessel wall. The common ability of both vascular endothelial cells and smooth muscle cells to secrete IL-1, and the stimulation of IL-1 synthesis and release by IL-1 itself reported here

may thus be a crucial factor in the initiation, maintenance, and propagation of these pathologic processes in vivo.

Summary

The recognition that cells of the vascular wall can secrete cytokines such as IL-1 suggests new mechanisms for initiating or sustaining inflammatory responses in blood vessels. We report that purified human monocyte-derived IL-1 or recombinant human IL-1 (rIL-1 β and rIL-1 α) induce cultured human smooth muscle cells derived from veins or arteries to synthesize IL-1 β mRNA and produce and release biologically active IL-1. rIL-1 β also stimulated the production of PGE₂ by smooth muscle cells. Exposure to rIL-1 β (1–100 ng/ml), or rIL-1 α (0.01–10 ng/ml) increased IL-1 β mRNA levels within 30 min. Actinomycin D (1 μ g/ml) prevented the induction of IL-1 β mRNA by rIL-1. IL-1 α mRNA was detected in SMC treated with cycloheximide (1 μ g/ml) and rIL-1 β , or cycloheximide alone. rIL-1 α and rIL-1 β produced maximal levels of IL-1 β mRNA after 4 h, and intracellular IL-1 biological activity after 6 h of exposure. Release of IL-1 activity in the extracellular medium began after 1 h of incubation with rIL-1 β or rIL-1 α , and continued for up to 24 h. Anti-TNF antiserum that neutralized the biological activity of rTNF did not affect rIL-1-induced production of IL-1 β mRNA or IL-1 release, suggesting that the release of TNF does not mediate these processes. Several experimental approaches indicated that the release of IL-1 by smooth muscle cells was not due to endotoxin contamination of the IL-1 preparations. Anti-IL-1 antiserum blocked the induction of smooth muscle cell IL-1 gene expression by rIL-1 β . Polymyxin B did not prevent IL-1-induced IL-1 expression by these cells, but blocked the effect of endotoxin. Heat treatment destroyed the stimulatory capacity of rIL-1 β , but did not affect the ability of bacterial endotoxin to induce IL-1 expression. The production of IL-1 by human vascular smooth muscle cells was not due to contamination of the cell cultures with blood monocytes, inasmuch as treatment with an antimonocyte antibody (anti-Mo2) and complement did not alter IL-1 β mRNA content or the amount of IL-1 released from the cells in response to endotoxin, rIL-1 α , or rIL-1 β . IL-1 production by smooth muscle cells, the most abundant cell type in the blood vessel wall, may amplify and sustain local inflammatory responses in vasculitis, allograft rejection, atherosclerosis, and vascular responses to injury or pathogens in general.

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