RAPID COMMUNICATION

Endotoxin and Tumor Necrosis Factor Induce Interleukin-1 Gene Expression in Adult Human Vascular Endothelial Cells

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Interleukin 1 (IL-1) can induce potentially pathogenic functions of vascular endothelial cells. This mediator was formerly thought to be produced primarily by activated macrophages. We report here that bacterial endotoxin and recombinant human tumor necrosis factor cause accumulation of IL-1β mRNA in adult human vascular endothelial cells. IL-1α mRNA was also detected when endothelial cells were exposed to endotoxin under "superinduction" conditions in the presence of cycloheximide. Metabolic labeling of these cells during endotoxin stimulation demonstrated increased synthesis and secretion of immunoprecipitable IL-1 protein that comigrated electrophoretically with the predominant monocyte species. In parallel with increased IL-1 mRNA and protein, endothelial cells exposed to endotoxin also released biologically active IL-1 that was neutralized by anti-IL-1-antibody. Because bloodborne agents must traverse the endothelium before entering tissues, endothelial IL-1 production induced by microbial products or other injurious stimuli could initiate local responses to invasion. Endothelial cells are both a source of and target for IL-1; accordingly, this novel autocrine mechanism might play an early role in the pathogenesis of vasculitis, allograft rejection, and arteriosclerosis. (Am J Pathol 1986, 124: 179–185)

INTERLEUKIN-1 (IL-1) is an important mediator of generalized host responses to tissue injury or infection. This protein hormone signals many of the classic systemic components of inflammation, including fever, neutrophilia, lymphocyte activation, and hepatic synthesis of acute phase proteins.[1] Changes in blood vessels are also hallmarks of inflammation, and it is now apparent that IL-1 may mediate some of the local vascular effects of this response as well. Endothelial cells form the inner lining of all blood vessels and normally resist coagulation.[2] However, IL-1 causes endothelial cells to express procoagulant activity that actually promotes blood clotting.[3] IL-1 also increases adhesion of leukocytes,[4] affects prostanoid synthesis,[5,6] and induces shape changes in vascular endothelial cells.[7] Such alterations in endothelial function produced by IL-1 may play important roles in inflammation and wound healing as well as the pathogenesis of vascular diseases.

Until recently, activated mononuclear phagocytes were considered the unique source of IL-1.[1] Human blood monocytes can express at least two genes that encode molecules with IL-1 activity.[8,9] The product of the IL-1β gene that is the predominant form produced by human monocytes has an isoelectric point of 7, whereas the less abundant species encoded by the IL-1α gene has an isoelectric point of 5. Vascular endothelial cells themselves appear to secrete a substance that shares with IL-1 the ability to augment proliferation of murine thymocytes exposed to mitogenic lectins.[10–13] However, the study of IL-1-like substances secreted by endothelial cells based on biologic activity alone is limited because these cells may also produce an inhibitor of lymphocyte activation.[13] Furthermore,

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such studies cannot establish whether this activity is actually due to expression of a specific IL-1 gene or genes, or whether elaboration of this activity from endothelium is regulated at the level of transcription, translation, or release of preformed mediator.

The issue of IL-1 production by endothelium is important because this cell type, strategically located at the interface between the blood and tissues, would be the first tissue cell to encounter bloodborne injurious stimuli such as infectious agents or antigen–antibody complexes. Microbial pathogens must traverse the endothelium to gain access to tissues and disseminate. Thus, endogenous IL-1 production by vascular endothelial cells could provide an early warning system that signals local tissue invasion or injury. For these reasons, we studied IL-1 gene expression in cultures of endothelial cells isolated from adult human blood vessels.

Materials and Methods

Cell Culture and Bioassay of IL-1 Activity

Endothelial cell cultures were prepared and cultured from human saphenous veins.[14] These experiments used cells in Passage 3–6, at least 3 weeks after original isolation. Cells prepared in this manner exhibit several characteristics of human vascular endothelium *in situ*, including morphology, prostacyclin production, expression of receptors for *Ulex europaeus* agglutinin I and of von Willebrand factor (vWF) protein, and mRNA.

Assay for IL-1 biologic activity used thymocytes from C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) incubated in RPMI 1640 medium supplemented with fetal calf serum (10%) and phytohemagglutinin (1 μg/ml) in the presence or absence of the test specimen. After 48 hours tritiated thymidine (6 Ci/mmol, 10 μCi/ml) was added for 18 hours, and incorporated radioactivity was determined by liquid scintillation spectroscopy. Each sample was assayed in triplicate.

Nucleic Acid Hybridization Studies

A number of plasmids were constructed in our laboratory for these studies: pGEM-IL1β contains a 1.1 kB fragment (FnudII [position 278] to AhaIII [position 1381]) of a full-length cDNA clone of the IL-1β precursor (pcD-415)[8] subcloned into the Sma I site of the polylinker of pGEM-2 (Promega Biotech, Madison, Wis). pSP-6 RBT.3 contains a highly conserved 1 kb segment (Pst I to Bam HI) of a rat β-tubulin gene that is constitutively expressed in many tissues.[15] Plasmids were isolated chromatographically,[16] and inserts were labeled by nick translation with ^{32}P-dGTP and ^{32}P-dCTP.[17] The specific activity of such probes was generally >10^8 cpm/μg DNA. An oligonucleotide probe for IL-1α (positions 298-339)[9] was end-labeled with the use of ^{32}P-dATP.[17]

For northern analysis, cellular RNA was isolated by the guanidine isothiocyanate/CsCl method,[17] electrophoresed through agarose (1.2%) that contained formaldehyde, transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, Il), fixed by ultraviolet irradiation, and prehybridized and then hybridized with ^{32}P-labeled probes.[17]

Metabolic Labeling and Immunoprecipitation

Newly synthesized endothelial cell proteins were labeled by incubating cultures in the presence of ^{35}S-methionine (0.1 mCi/ml) added to methionine-free medium. After 48 hours the media were removed, clarified at 1600*g*, ultrafiltered and concentrated (molecular weight cut-off, 10,000 daltons; Centricon-10 apparatus, Amicon Corp., Danvers, Mass), and equilibrated with immunoprecipitation buffer (NaCl, 150 mM; Na phosphate, 10 mM, pH 7.4; Na desoxycholate, 0.5%; Nonidet NP-40, 0.5%; phenylmethylsulfonyl fluoride, 0.1 mM; soybean trypsin inhibitor, 20 μg/ml; and the additional protease inhibitors leupeptin and antipain at 5 μM). After two preliminary absorptions with normal rabbit serum (20 μl/ml), polyclonal rabbit antihuman IL-1 antiserum or normal rabbit serum was added (20 μl/ml). After 1 day at 4 C, antibodies were absorbed with protein-A-agarose (8 mg/ml, P-1406, Sigma Chemical Co., St. Louis, Mo). After 2 hours, the pellets were washed 6 times, extracted with Laemmli sample solvent at 100 C for 10 minutes, and electrophoresed on linear discontinuous SDS polyacrylamide gels (12.5%). After fixation, the gel was treated with Fluoro-hance (RPI, Mount Prospect, Ill) and exposed to Kodak AR film at −70 C for 4 days.

Results

Induction of IL-1 mRNA in Human Endothelial Cells

Under the usual culture conditions, endothelial cells cultured from saphenous veins of human adults express little or no IL-1β mRNA (Figure 1A). Bacterial endotoxins are potent inducers of IL-1 production by human monocytes.[1,8] Furthermore, endotoxins increase the production of tissue factor, colony-stimulating factors, plasminogen activator, and IL-1-like biologic activities by human endothelial cells.[11–13,18–20] We tested whether endotoxin also stimulates IL-1 gene expression by these cells. Human endothelial cells exposed to *Escherichia coli* endotoxin (055:B5, Sigma L-2880, 0.1–10

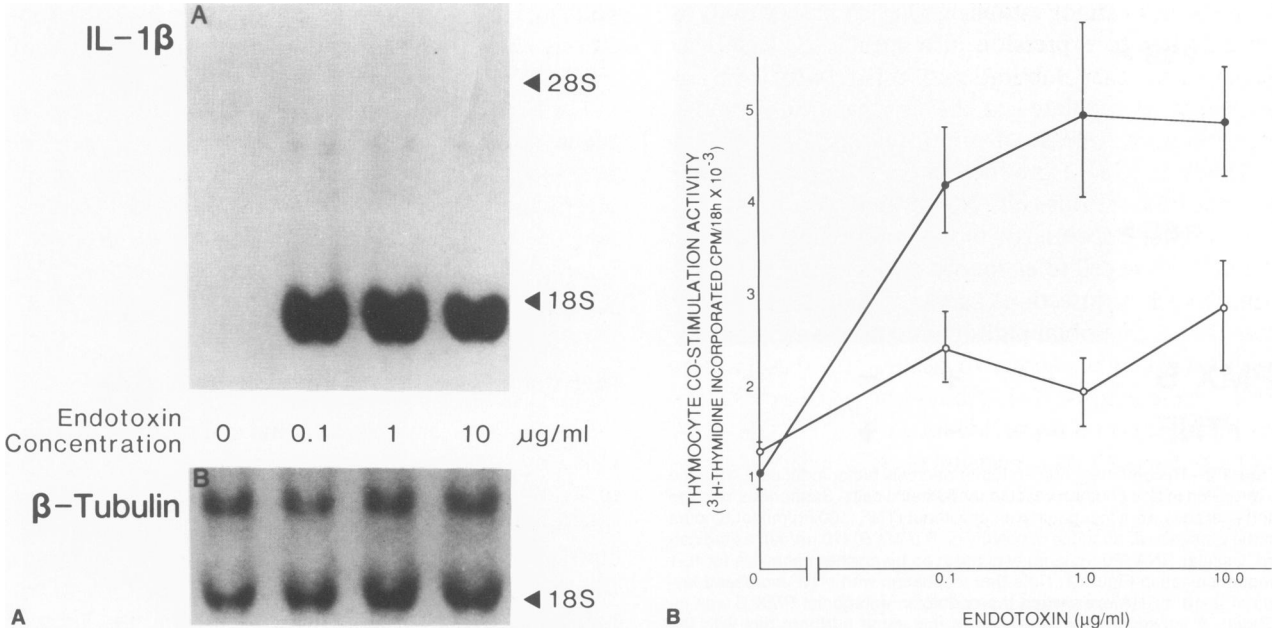

Figure 1A—Endotoxin induces IL-1 expression by human endothelial cells. *Upper panel*: RNA was extracted from human saphenous vein endothelial cells incubated for 5 hours in the indicated concentrations of *E coli* endotoxin (055:B5, Sigma). Northern analysis of RNA (20 μg/lane) used as probe the 1.1 kb insert of human IL-1β cDNA sequences contained in pGEM-IL1B. Note the marked increase in level of IL-β mRNA (1.6 kb) in the endotoxin-stimulated cells, compared with the unstimulated control. The positions of 28 S and 18 S ribosomal RNA species are indicated by *arrows*. *Lower panel*: This panel shows a rehybridization of the same blot with a probe derived from a constitutively expressed β-tubulin gene. Two different lengths of transcript are generally found with this probe (2.9 and 1.8 kb).[15] Note the similar levels of β-tubulin mRNA in all conditions, an indication of selectivity of the induction of IL-1β gene expression by endotoxin. **B**—In parallel with the cultures analyzed for mRNA in **A**, replicate cultures of human endothelial cells were incubated with the indicated concentrations of *E coli* endotoxin (055:B5) for 24 hours in the presence (*open circles*) or absence (*closed circles*) of polymyxin B (10 μg/ml), an inhibitor of endotoxin activity. The media were then assayed for thymocyte costimulation activity. The final concentration of polymyxin B in the conditioned media was adjusted to the same level after exposure to endothelial cells, but before the thymocyte stimulation assay. Data are the mean ± SD of triplicate determinations on duplicate samples (*closed circles*) or a single sample (*open circles*).

μg/ml) for 18 hours contained much greater amounts of IL-1 mRNA than did unstimulated cells (Figure 1A).

Analysis of this and other northern blots established that the size of this message was approximately 1.6 kb, and that it co-migrated with IL-1β mRNA extracted from human peripheral blood monocytes or the human monocytoid cell line U-937 after endotoxin stimulation (data not shown). This endotoxin-induced increase in IL-1β mRNA was not due to a generalized effect on gene expression, because the level of β-tubulin mRNA did not change appreciably in stimulated endothelial cells (Figure 1A). Other experiments showed that briefer exposures (120 minutes) also markedly increased levels of IL-1β mRNA in human endothelial cells (data not shown). Similar induction of IL-1β mRNA was produced by all other bacterial endotoxins tested (*E coli* 0111:B4 [Sigma L-2630], *Salmonella minnesota* [Sigma L-6261], and *S typhosa* [Sigma L-6386]) (data not shown).

Levels of mRNA for inducible genes often increase when the antibiotic cycloheximide is present during stimulation, a phenomenon known as superinduction.[21,22] In the absence of cycloheximide, we never detected IL-α mRNA in endothelial cells exposed to endotoxin. However, in two independent experiments, when cycloheximide (10 μg/ml) was included during endotoxin stimulation, we readily detected IL-1α mRNA (approximately 2.2 kb), as well as increased levels of IL-1β mRNA (data not shown). Thus, human endothelial cells can express both known IL-1 genes.

Although bacterial endotoxin is an exogenous stimulus of obvious pathologic importance, we also wished to determine whether an endogenous mediator might regulate IL-1 expression in endothelial cells. The recently described human tumor necrosis factor (TNF),[23] also called cachectin, stimulates IL-1 release from human mononuclear phagocytes and alters a number of properties of endothelial cells.[24-27] We therefore tested whether recombinant human TNF (rTNF) could influence IL-1 gene expression in human endothelial cells. In three independent experiments, rTNF increased IL-1β mRNA levels in these cells (Figure 2). The rTNF used in these experiments was free of chemically detectable endotoxin contamination.[28] Addition of the endotoxin antagonist polymyxin B did not affect the induction of IL-1β mRNA by rTNF, a further indication that this effect was not due to endotoxin contamination (Figure 2). The concentration of TNF used in these experiments (100 ng/ml) was chosen because it is the

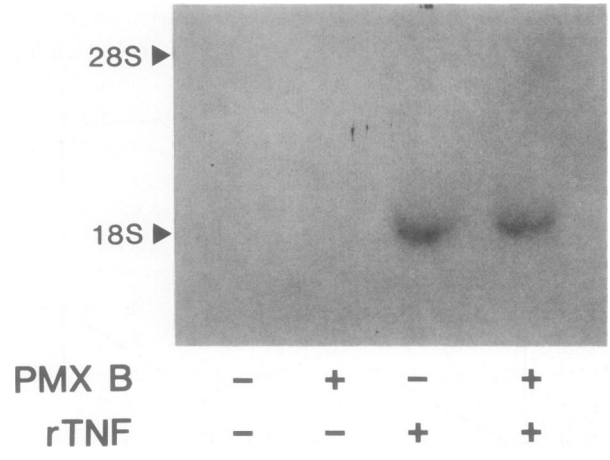

Figure 2—Recombinant human tumor necrosis factor induces IL-1β gene expression in adult human vascular endothelial cells. Saphenous vein endothelial cells were incubated with or without rTNF (100 ng/ml) for 6 hours in the presence or absence of polymyxin B (*PMX B*) (10 μg/ml), as indicated. Cellular RNA (20 μg/lane) was assayed by northern analysis for IL-1 sequences as in Figure 1. Note that incubation with rTNF increased levels of IL-1β mRNA even when the endotoxin antagonist PMX B was included. A subsequent hybridization of this same northern blot with the β-tubulin probe (not shown) showed approximately equal amounts of hybridization in each condition as in the bottom panel of Figure 1A.

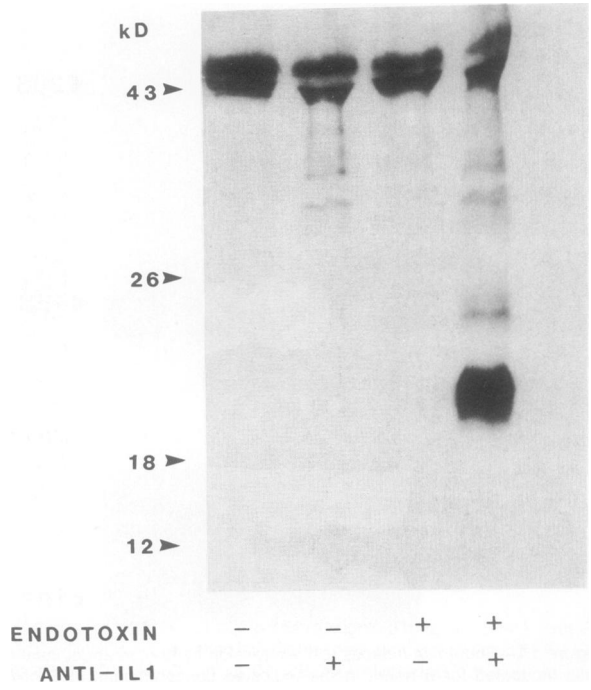

Figure 3—Endotoxin increases the *de novo* synthesis and secretion of interleukin-1 protein by adult human vascular endothelial cells. Human saphenous vein endothelial cells were metabolically labeled with ^{35}S-methionine in the presence or absence of *E coli* endotoxin (055:B5, 10 μg/ml) as indicated. The secreted proteins were precipitated with normal rabbit serum (−) or anti IL-1 (+), separated by electrophoresis, and visualized by fluorography. An independent experiment yielded similar results (data not shown).

minimum concentration that induced IL-1 release from human monocytes.[28]

Endothelial Cells Can Synthesize and Secrete Immunoprecipitable IL-1

Without exposure to endotoxin, adult human endothelial cells secreted negligible amounts of metabolically labeled IL-1 detected by immunoprecipitation (Figure 3). Exposure to endotoxin consistently increased the level of secreted IL-1 manyfold (Figure 3). This effect of endotoxin was selective, because the levels of metabolically labeled proteins nonspecifically precipitated by this polyclonal anti-IL-1 serum did not change substantially (Figure 3). The principal endothelial cell product recognized by this antiserum comigrated with the major IL-1 species secreted by endotoxin-stimulated human monocytes (data not shown) and yielded a molecular weight under these electrophoresis conditions of about 22 kD. The molecular weight obtained in these experiments for both monocyte and endothelial cell-derived IL-1 may be higher than the 17 kD obtained under other conditions because of the broad spectrum of protease inhibitors present during analysis. This electrophoretic migration pattern of human monocyte IL-1 on SDS-polyacrylamide gels has been reported previously.[29]

Endothelial Cells Can Secrete Biologically Active IL-1

We also wished to determine whether exposure to endotoxin increased the release of functional IL-1 from endothelial cells. Of the many biologic activities of IL-1, one of the most characteristic is augmentation of the mitogenic effect of certain lectins on T lymphocytes, a property known as lymphocyte activation or thymocyte costimulation.[1] Exposure to endotoxin consistently increased the amount of this activity found in endothelial cell culture medium (Figures 1B and 4). Maximal release of IL-1 activity was produced by the lowest concentration of endotoxin studied (0.1 μg/ml) (Figure 1B). This result agrees closely with the mRNA levels measured in the same experiment (Figure 1A). Polymyxin B, a selective antagonist of endotoxin actions mediated by the lipid A moiety, inhibited production of IL-1 activity by endotoxin-stimulated endothelial cells (Figure 1B). Anti-IL-1 antiserum neutralized the thymocyte activation activity in medium from endotoxin-stimulated endothelial cells (Figure 4), a further indication that this activity is due to material immunologically similar to monocyte-derived IL-1.

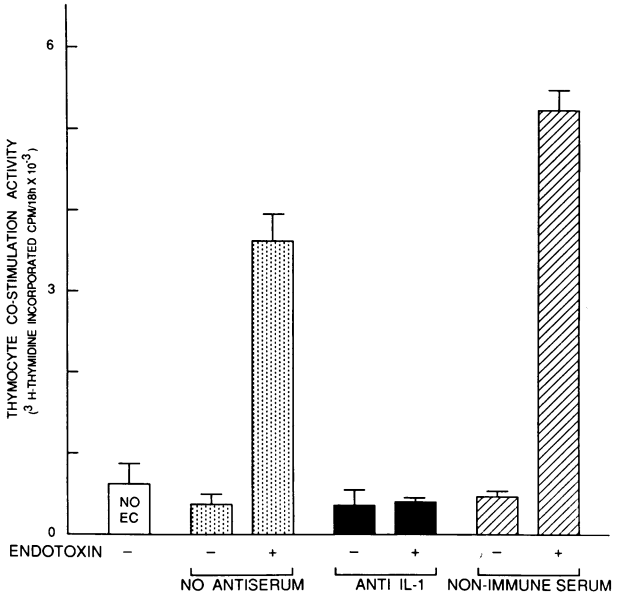

Figure 4—Secretion of biologically active IL-1 by adult human endothelial cells. Human saphenous vein endothelial cells were incubated with (+) or without *E coli* endotoxin (055:B5, 10 μg/ml, 24 hours). Thymocyte costimulation activity was measured in the media without antisera (*stippled bars*), with rabbit anti-human blood monocyte IL-1 antiserum (1:100) (*solid bars*), or with nonimmune rabbit serum (1:100) (*hatched bars*). Data are the mean ± SD of triplicate measurements.

Discussion

These findings establish that adult human vascular endothelial cells can accumulate IL-1 mRNA and synthesize *de novo* and secrete biologically active IL-1 protein product in an inducible manner. Exposure to endotoxin can regulate other endothelial cell functions such as production of tissue factor, colony-stimulating factor, and plasminogen activator.[18-20] IL-1 or TNF can cause endothelial cells to express some of these same activities, including procoagulant synthesis and increased leukocyte adhesion.[3,4,24-27] Some of these effects of endotoxin or TNF on endothelium may be indirect, in that they might be mediated by IL-1 produced by the endothelium itself. Although endotoxin and TNF are cytolytic or cytostatic to many types of cultured cells,[23] phase-contrast microscopy revealed no morphologic signs of injury to human endothelial cells due to either substance in these experiments, nor did endotoxin (*E coli* 055:B5, 10 μg/ml, 24 hours) reduce cell number or DNA content of endothelial cultures (data not shown).

In the past mononuclear phagocytes were considered the sole source of IL-1; however, it is now appreciated that certain other cells can produce similar activity (eg, keratinocytes, B lymphocytes, astrocytes, and corneal epithelial cells).[1,30] Our data indicate that the majority of this activity from both endothelial cells and blood monocytes is due to products of the same or highly related genes. Vascular endothelium and mononuclear phagocytes can share a number of other specialized functions. For example, endothelial cells can express Class II histocompatibility antigens in response to immune interferon, and function as antigen-presenting cells.[31] Endothelial cells and monocyte-derived cells (but few others) express receptors for modified low-density lipoprotein.[32] These two cell types are also the only nontransformed cells other than platelets (or megakaryocytes) currently known to elaborate platelet-derived growth factor-like molecules.[33-35] Like macrophages, endothelial cells can ingest particles made of synthetic polymers.[36] These various findings show that despite their distinct origins, vascular endothelial cells and the bone-marrow-derived phagocytic leukocytes exhibit an unexpected functional kinship.

A possible alternative explanation for our results could be contamination of cultures of endothelial cells with mononuclear phagocytes. We consider this explanation unlikely for a number of reasons. Examination of these cultures by phase-contrast microscopy and histochemical staining for *Ulex europeaus* lectin receptors (selective for endothelium and group 0 substance in man)[37]showed no morphologic evidence for leukocyte contamination. These cultures are derived from small inocula and undergo at least 13 population doublings in culture before study. Although leukocytes can contaminate primary unpassaged fetal endothelial cell cultures,[38] it is unlikely that blood monocytes would remain numerous, viable, or capable of IL-1 production after three to six passages and at least 3 weeks in monolayer culture.[30] Miossec et al recently presented several lines of experimental evidence that monocytes are not the source of thymocyte-activating activity from human umbilical vein endothelial cell cultures.[13] Moreover, endotoxin induces expression of both TNF and IL-1 in monocytes, but we detected no TNF production by endotoxin-stimulated endothelial cell cultures by radioimmunoassay or radioimmunoprecipitation (data not shown). In addition, the minimum concentration that induced IL-1 gene expression in our endothelial cell experiments (100 ng/ml) was 10^2 times higher than required to stimulate maximal IL-1 production by monocytes. Finally, although we readily detected IL-1α mRNA in endotoxin-stimulated human monocytes, we only found IL-1α transcripts in endothelial cell cultures under "superinduction" conditions.

The recent recognition of inducible endothelial functions has greatly expanded the concepts of the participation of this cell type in health and disease. Activated endothelial cells may play a central role in the host re-

sponse to injury as part of the inflammatory and immune responses and in wound healing. When certain inducible functions of endothelium such as IL-1 production are expressed inappropriately, they may contribute to the development of vascular diseases. For example, adhesion of monocytes to arterial endothelium is one of the earliest morphologic changes in animals fed diets rich in cholesterol.[39] Brief exposure of endothelial cells to IL-1 can induce the adhesion of monocytes and granulocytes, and IL-1 is chemotactic for leukocytes.[1,4] Thus, initial local production of IL-1 by endothelial cells may recruit granulocytes and monocytes that, in turn, can secrete more IL-1 and other mediators of host defenses. Such amplification loops are common in other pathways of biologic responses to injury (eg, complement, thrombosis). In this manner, IL-1 production by endothelium, a likely early response to injury, could contribute to an important way to the initiation of inflammation in hematogenous infection, allograft rejection, arteriosclerosis, and the vasculitides.

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Note Added in Proof

Since submission of this manuscript, Nawroth et al have reported that tumor necrosis factor/cachectin induces release of thymocyte costimulation activity from human umbilical vein endothelial cells (Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D: Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 1986, 163:1363–1375).