Endothelial Interleukin-8: A Novel Inhibitor of Leukocyte-Endothelial Interactions

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Certain inflammatory stimuli render cultured human vascular endothelial cells hyperadhesive for neutrophils. This state is transient and reversible, in part because activated endothelial cells secrete a leukocyte adhesion inhibitor (LAI). LAI was identified as endothelial interleukin-8 (IL-8), the predominant species of which is an extended amino-terminal IL-8 variant. At nanomolar concentrations, purified endothelial IL-8 and recombinant human IL-8 inhibit neutrophil adhesion to cytokine-activated endothelial monolayers and protect these monolayers from neutrophil-mediated damage. These findings suggest that endothelial-derived IL-8 may function to attenuate inflammatory events at the interface between vessel wall and blood.

VASCULAR ENDOTHELIUM ACTIVELY participates in acute and chronic inflammatory reactions, serving as both a target and a source of biological response modifiers (*1*). In particular, the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), as well as bacterial endotoxin (LPS), can act directly on cultured human endothelial cells (HECs) to induce the expression of endothelial-leukocyte adhesion molecules (ELAMs) (*2*) that render the endothelial surface hyperadhesive. Adhesion of leukocytes to the endothelial lining of microvessels in vivo is essential for their emigration into tissues in host defense and, in certain situations, can predispose the endothelium to injury (*3*). However, the role of activated endothelium in leukocyte-endothelial interactions may not be limited to these proinflammatory events. Upon activation with IL-1, TNF, or LPS, HECs also begin to secrete a soluble leukocyte adhesion inhibitor (LAI) that acts on leukocytes to attenuate the hyperadhesive interaction (*4*). The mediator of this potentially anti-inflammatory effect is a de novo synthesized, heat- and acid-stable protein (*4*). We now report the purification of human endothelial-derived LAI. It was a potent inhibitor of neutrophil adhesion to cytokine-activated HECs and acted as a protective agent in neutrophil-mediated endothelial injury.

LAI was partially purified from several liters of medium incubated for 8 hours with IL-1–activated HECs by sequential anion- and cation-exchange chromatography (*5*), by the use of a bioassay for inhibition of neutrophil adhesion to IL-1–activated HEC monolayers (*6, 7*). LAI activity cofractionated with a small protein peak that eluted from a Mono S cation-exchange column at high ionic strength (Fig. 1A). The pool of maximally active fractions (28 to 31) inhibited neutrophil adhesion to IL-1–activated HECs in a concentration-dependent fashion (Fig. 1B). When analyzed by gel electrophoresis (Fig. 1C), this partially purified material contained a prominent 10-kD protein (*8*). Preliminary gel-slicing experiments had indicated that LAI activity migrated at 8 to 10 kD. Pooled peak fractions were subjected to 53 cycles of NH_2-terminal sequencing (*9*). The predominant (>90%) sequence was: NH_2-AVLPRSAKELRCQCIKTYSKPFHP-KFIKELRVIESGPHCANTEIIVKLSDGRE . . . (*10*). This is almost identical to the sequence of interleukin-8 (IL-8) (*11–13*), the 72–amino acid neutrophil-activating polypeptide that is secreted by activated T cells and monocytes (*14*). However, the predominant endothelial-derived polypeptide, here designated $[(Ala)\text{-}IL8]_{77}$, differed from the predominant [70 to 100% (*11–13*)] form of mononuclear leukocyte-derived IL-8, designated $[(Ser)\text{-}IL8]_{72}$, by having a pentapeptide AVLPR extension at the NH_2-terminus. The endothelial cell–derived material contained $[(Ser)\text{-}IL8]_{72}$ as a minor component [7% in 8-hour conditioned medium, 20% in 24-hour conditioned medium; three preparations each]. Two groups have recently shown that endothelial cells activated with LPS or cytokines can secrete IL-8–like material, but sequence data have not been reported (*15, 16*).

Twenty-four–hour conditioned medium was found to be a source of abundant LAI activity and therefore was used for purification of LAI to homogeneity by fast protein liquid chromatography (*5*) and reversed-phase high-performance liquid chromatography (HPLC) (*17*). The resulting 10-kD protein (Fig. 2C, lane 1) potently inhibited the amplified (20 to 62 times) neutrophil adhesion observed with IL-1–activated HECs, with a median effective concentration (EC_{50}) of 0.5 to 1.0 n*M* (threshold, <0.3 n*M*; range of maximum inhibition, 3 to 30 n*M*) (Fig. 2A). Reduced inhibitory activity was noted at high (>50 n*M*) concentrations. In contrast to the inhi-

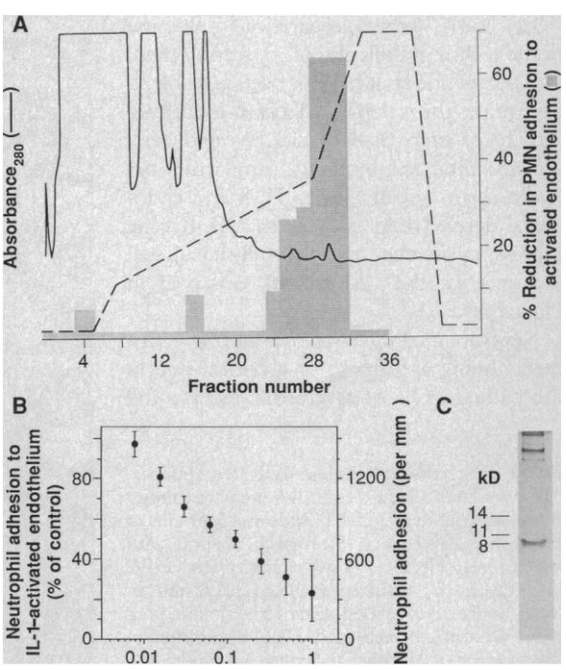

Fig. 1. (**A**) Purification of leukocyte adhesion inhibitor (LAI) activity from medium conditioned by IL-1–activated HECs. Pooled fractions from a Mono S column (*5*) were assayed (*7*) in triplicate at 1:8 final dilution. The dashed line indicates NaCl concentration gradient. One of three representative experiments. (**B**) Concentration-dependent effect (mean ± SD, $n = 3$) of pooled fractions 28 to 31 on neutrophil adhesion to IL-1–activated HECs. One of two representative experiments. (**C**) Silver-stained SDS gel (*8*) of pooled fractions 28 to 31.

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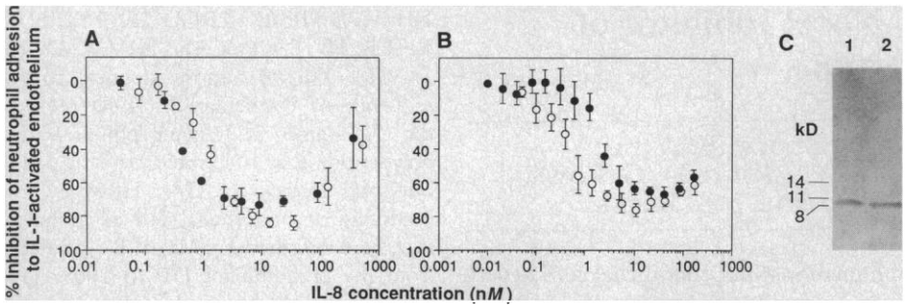

Fig. 2. Inhibition of neutrophil adhesion to activated HEC monolayers by purified endothelial-derived IL-8 (**A**) and recombinant human IL-8. (**B**) Each curve represents a separate preparation (mean ± SD, $n = 3$ to 9). (**C**) Silver-stained SDS gel (*8*) containing endothelial-derived IL-8 (lane 1) and recombinant human IL-8 (lane 2).

bition (up to 80%) observed with cytokine-activated HEC monolayers, the adhesion of unstimulated neutrophils to unactivated monolayers ("basal adhesion," 61 ± 26 neutrophils per square millimeter, mean ± SD, four experiments) was not significantly reduced by LAI, and at very high LAI concentrations was increased (100 n*M*, 2.5-fold; 500 n*M*, 5-fold).

Because of the extensive sequence homology noted between LAI and IL-8, we cloned a human IL-8 cDNA from peripheral blood lymphocyte RNA, inserted it into an expression vector, and transfected a human cell line (*18*). The LAI activity was present in medium conditioned by IL-8 transfected cells but not in medium from mock-transfected cells. Purified recombinant IL-8 [isolated by cation-exchange chromatography (*5*)] was electrophoretically indistinguishable from endothelial-derived LAI (Fig. 2C, lane 2), and exhibited similar LAI-like activity (EC_{50}, 0.4 to 2.0 n*M*; Fig. 2B). As with endothelial-derived LAI, the predominant (~80%) form was $[(Ala)\text{-}IL8]_{77}$; the remainder was $[(Ser)\text{-}IL8]_{72}$ (three preparations). Because of the technical difficulty of separating these two polypeptides, it has not been possible to determine the relative adhesion-inhibitory activity of each IL-8 species. Nonetheless, the structural and biological similarities between recombinant human IL-8 and endothelial-derived LAI indicate that IL-8 gene expression in the human endothelial cell accounts for the LAI activity observed in Figs. 1 and 2A.

Neutrophil adhesion to the vascular endothelial lining appears to be a critical step in the pathogenesis of several life-threatening conditions, including adult respiratory distress syndrome, septic shock, and ischemia-reperfusion injury in the heart and other vital organs (*3*). During our standard (10-min) adhesion assay (*2, 4, 7*), morphologic evidence of injury to the endothelial monolayer was rarely detectable with unstimulated neutrophils. However, upon incubation at higher neutrophil:endothelial ratios (50:1 to 100:1) and for longer time intervals (60 to 120 min), marked damage (cell detachment, monolayer retraction, and cytolysis) was frequently apparent in IL-1–activated, but not unactivated, HEC monolayers (Fig. 3, A and B) (*19*). This enhanced susceptibility of cytokine-activated HECs to neutrophil-mediated damage is consistent with a recent report (*20*), but in our experiments occurred without the addition of neutrophil-directed agonists such as phorbol esters. Endothelial-derived LAI (fractions 27 to 31, Fig. 1; 0.5× final concentration) prevented this neutrophil-mediated injury (Fig. 3C). Similar protection was also observed with more highly purified preparations of natural LAI and recombinant IL-8, at 10 to 50 n*M* concentrations.

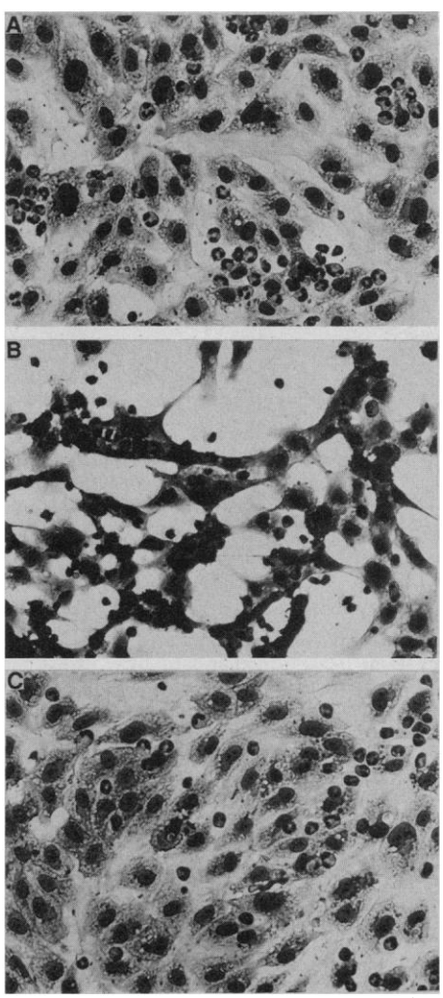

Fig. 3. Protection of cytokine-activated endothelial monolayers from neutrophil-mediated injury by endothelial-derived LAI. Photomicrographs of HEC monolayers after 90-min incubation with neutrophils (50:1) (*19*). (**A**) Unactivated HEC monolayer; (**B**) cytokine-activated HEC monolayer; note extensive monolayer retraction and cell loss; (**C**) cytokine-activated HEC monolayer in the presence of endothelial-derived LAI; note lack of damage manifested in (B).

The identification of LAI as endothelial IL-8, and the demonstration of its novel effects on leukocyte-endothelial interactions, may have important pathophysiological implications. To date, IL-8 has been defined solely as a proinflammatory cytokine, based primarily on its promotion of leukocyte chemotaxis and degranulation in vitro (*11–16*). Our data indicate, however, that in the microenvironment of the "inflamed" endothelial cell, endothelial-derived IL-8 may act to limit the extent of leukocyte–vessel wall adhesion and its potential deleterious consequences. The possibility exists that the chemotactic and adhesion-inhibitory activities of IL-8 are related (*21*). Cytokines and LPS cause sustained upregulation of IL-8 gene expression in HECs (*15, 22*); thus the vascular endothelial lining may have the capacity to exert these potential anti-inflammatory effects on a time scale that extends beyond the initial inflammatory stimulus. Further study of the regulation of production of endothelial IL-8–related proteins and their mechanisms of action in leukocyte-endothelial interactions may provide new insights into "natural" anti-inflammatory mechanisms.

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5. LAI was produced by incubating (37°C, 8 hours) confluent monolayers (second and third passage) of human umbilical vein endothelial cells (*4, 6*) with recombinant human (rh) IL-1β (5 to 10 U/ml) in serum-free RPMI 1640 containing insulin, transfer-

rin, and selenium (ITS, 1 ml/liter Collaborative Research, Cambridge, MA). Conditioned medium was collected in a sterile manner on wet ice, clarified by centrifugation, and stored at −70°C. Upon thawing, 0.2- to 0.5-liter aliquots were adjusted to *p*H 3.0 with trifluoracetic acid (TFA) and, at 4°C, sequentially ultrafiltered through 30-kD and 5-kD YM membranes (Amicon Inc., Danvers, MA). The concentrated (50×) YM-5 retentate was buffer-exchanged with TFA in Milli-Q water (*p*H 3.0), lyophilized, dissolved in anion-exchange column equilibration buffer (10 m*M* tris-HCl, 6*M* urea, and 0.01% Tween 80, *p*H 8.0), and separated on a Mono Q HR 5/5 column. The LAI activity recovered in the unbound material was concentrated by Centricon 10 ultrafiltration (Amicon), diluted with cation-exchange buffer (25 m*M* sodium acetate, 6*M* urea, and 0.01% Tween 80, *p*H 5.0), and separated on a Mono S HR 5/5 column. Bound proteins were eluted with a three-stage linear gradient of NaCl in equilibration buffer (0.15*M* NaCl in 5 min, 0.5*M* NaCl in 40 min, 1*M* NaCl in 50 min; flow rate, 0.5 ml/min). Column fractions (2 ml) were prepared for bioassay by spin dialysis against RPMI with bovine albumin (0.4 mg/ml, Cohn Fraction V). Twenty-four–hour conditioned medium was treated similarly, except that YM-30 filtration and lyophilization were omitted and 0.15*M* NaCl was added to the Mono S equilibration buffer.

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7. The LAI activity was quantified by a modified endothelial-leukocyte adhesion assay (*2, 4*). Confluent HEC monolayers in 96-well microtiter plates were preincubated (37°C, 4 hours) with or without rh IL-1β (5 or 10 U/ml) (*6*) and washed, and LAI or control preparations were added. Human polymorphonuclear leukocytes (>97% neutrophils) labeled with 2′,7′-bis-(2-carboxyethyl)-5 (and -6) carboxyfluorescein, acetoxymethyl ester (BCECF) (Molecular Probes, Eugene, OR) were then added (final concentration, 2×10^5 neutrophils per well, final volume, 0.1 ml). After 10 min at 37°C, plates were sealed, inverted, and centrifuged (250*g*, 5 min), and supernatants were removed. Microscopic monitoring indicated that, at this time interval, the majority of adherent leukocytes were attached to the apical surface of the monolayer (*2, 4*). The number of adherent neutrophils was calculated from monolayer-bound fluorescence read in an automated microtiter plate fluorimeter.
8. Electrophoresis of nonreduced samples in 12% acrylamide gels was performed as described by H. Schagger and G. von Jagow [*Anal. Biochem.* **166**, 368 (1987)].
9. Protein samples were sequenced by a modification of the method of P. Edman and G. Begg [*Eur. J. Biochem.* **1**, 80 (1967)] with 0.1*M* Quadrol (*p*H 10.0), phenylisothiocyanate (Beckman Instruments) and TFA (Applied Biosystems) as reagents. Samples were applied in solution to a reversed-phase sequencing column and washed with water before sequencing. The reversed-phase cartridge was then loaded onto a prototype gas-liquid phase sequencer (EP-257735). The 2-anilino-5-thiazoiinone from each cycle was converted to the phenylthiohydantoin derivatives for identification on a Hewlett-Packard 1090 L liquid chromatograph.
10. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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14. The term "interleukin-8 (IL-8)" [C. G. Larsen, A. O. Anderson, E. Appela, J. J. Oppenheim, K. Matsushima, *Science* **243**, 1464 (1989)] designates the polypeptide produced by stimulated human peripheral blood lymphocytes and monocytes, that has been referred to variously as neutrophil-activating peptide-1 (NAP-1) (C. G. Larsen *et al.*, above), neutrophil chemotactic factor (NCF) [T. Yoshimura, K. Matsushima, J. J. Oppenheim, E. J. Leonard, *J. Immunol.* **139**, 788 (1987)], monocyte-derived neutrophil chemotactic factor (MDNCF) (*11*), neutrophil-activating factor (NAF) [P. Peveri, A. Walz, B. Dewald, M. Baggiolini, *J. Exp. Med.* **167**, 1547 (1988)] (*12*), and monocyte-derived and lymphocyte-derived neutrophil-activating peptide (MONAP/LYNAP) [J.-M. Schröder, U. Mrowietz, E. Christophers, *J. Immunol.* **139**, 3474 (1987)].
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17. Final purification was by reversed-phase HPLC on an Aquapore (C-8) RP-300 guard column equilibrated with 0.1% TFA in water. A linear 0 to 60% gradient of acetonitrile in 0.1% TFA was developed (flow rate, 0.5 ml/min). LAI eluted in ~35% acetonitrile. The purified 10-kD protein was lyophilized before bioassay, NH_2-terminal sequencing, and quantitative amino acid analysis.
18. IL-8 cDNA, identical in coding sequence to that described by J. Schmid and C. Weissmann [*J. Immunol.* **139**, 250 (1987)], was isolated from a phorbol ester–induced human peripheral blood lymphocyte cDNA library [P. W. Gray *et al.*, *Nature* **312**, 721 (1984)] by screening with a synthetic DNA oligonucleotide probe based on the NH_2-terminal amino acid sequence of IL-8. An 800-bp Hpa II–Nhe I fragment spanning the entire coding region of IL-8 was inserted into the mammalian expression vector pRK5 between the Cla I and the Xba I sites in the multiple cloning region downstream from the cytomegalovirus promotor. The resulting plasmid, pRK.hg.8k, was used to transfect human 293 cells by the $CaPO_4$/DNA precipitation method (10 μg of plasmid DNA/100 mm culture dish). Conditioned medium was harvested after 72 hours and centrifuged to remove cell debris before chromatography on S-Sepharose.
19. Neutrophil-mediated damage was assessed by a modification of the monolayer adhesion assay (*7*). Confluent HEC monolayers (control or after 4-hour treatment with rh IL-1β at 10 U/ml) were washed and incubated with unstimulated neutrophils (100:1, 50:1, 20:1, 10:1 neutrophils to endothelial cells) in RPMI with 1% fetal bovine serum (FBS) at 37°C for 10, 30, 60, 90 or 120 min. Purified endothelial-derived LAI or recombinant IL-8 was added with the neutrophil suspension. The assay was terminated by centrifugation, and the contents of each well were fixed and stained for microscopic evaluation.
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23. We thank M. E. Wheeler for her participation in the early phase of this study, K. Case and V. Davis for cell culture, W. Atkinson for photomicroscopy, R. Klein and D. Goeddel for providing the pRK5 expression vector, R. DuBridge and W. Nunes for help with the expression of recombinant protein, and our colleagues for helpful discussions and critical review of this manuscript. This research was supported by National Heart, Lung, and Blood Institute of Health grant P01-HL-36028 and Genentech, Inc.

28 June 1989; accepted 3 October 1989

Conserved Repetitive Epitope Recognized by CD4$^+$ Clones from a Malaria-Immunized Volunteer

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T cell clones obtained from a human volunteer immunized with *Plasmodium falciparum* sporozoites specifically recognized the native circumsporozoite (CS) antigen expressed on *P. falciparum* sporozoites, as well as bacteria- and yeast-derived recombinant falciparum CS proteins. The response of these CD4$^+$CD8$^-$ cells was species-specific, since the clones did not proliferate or secrete gamma interferon when challenged with sporozoites or recombinant CS proteins of other human, simian, or rodent malarias. The epitope recognized by the sporozoite-specific human T cell clones mapped to the 5′ repeat region of the CS protein and was contained in the NANPNVDPNANP sequence.

IMMUNIZATION BY EXPOSURE TO THE bites of irradiated malaria-infected mosquitoes induces protective anti-sporozoite immunity in rodents, monkeys, and human volunteers that involves both humoral and cell-mediated effector mechanisms (*1*). Initial efforts to develop a sporozoite malaria vaccine focused on the induction of antibodies directed against an immunodominant B cell epitope, $(NANP)_3$, located within the repeat region of the CS surface protein of *P. falciparum* (*2*). Immunization of human volunteers with an $(NANP)_3$–tetanus toxoid synthetic peptide vaccine induced a modest level of antibodies to sporozoites in 70% of the recipients and some protection against *P. falciparum* sporozoite challenge (*3*). Studies in rodents (*4*) and humans (*5, 6*), however, have since indicated that T cells of most individuals do not recognize the NANP repeats of the CS

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