Endothelial Cell Gene Expression of a Neutrophil Chemotactic Factor by TNF-α, LPS, and IL-1β

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Human endothelial cells produced a neutrophil chemotactic factor (NCF) upon stimulation with tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β), or lipopolysaccharide (LPS). The expression of endothelial cell–derived NCF messenger RNA and biological activity was both time- and concentration-dependent. Maximal NCF mRNA expression occurred at 10 and at 2 nanograms per milliliter for TNF and IL-1β, respectively; mRNA expression was first observed 1 hour after stimulation and was maintained for at least 24 hours. In situ hybridization analysis showed that NCF mRNA peaked in treated cells by 24 hours, whereas unstimulated cells were negative. These studies demonstrated that endothelial cells may participate in neutrophil-mediated inflammation by synthesizing a chemotactic factor in response to specific monokines and LPS.

VASCULAR ENDOTHELIAL CELLS PARTICIPATE in acute inflammatory reactions (*1*), a marked feature of which is the selective early influx of neutrophils from the peripheral blood (*2*). Here we show that endothelial cells can secrete a neutrophil chemotactic factor (NCF) with molecular and physical characteristics consistent with monocyte-derived NCF (*3*).

The human recombinant TNF-α (20 unit/ng) used in these investigations was expressed and purified as described (*4*). The steady state of NCF gene expression by TNF-α–treated human umbilical vein endothelial cells was measured by RNA blot analysis and was both concentration- and time-dependent (Figs. 1 and 2). There was maximal NCF mRNA expression at a TNF-α concentration of 10 ng/ml, and detectable expression at 0.5 ng/ml (Fig. 1A). We detected little or no endothelial cell–derived NCF mRNA in resting cells, with 50% of the maximum response at a TNF-α concentration of 2 ng/ml (quantitated by laser densitometry). Recombinant interleukin-1β (IL-1β) and lipopolysaccharide (LPS) could also induce endothelial cells to transcribe NCF mRNA. The possibility that LPS contamination may have been responsible for the effect observed with TNF-α was addressed by subjecting the TNF-α preparation to either neutralizing polyclonal antibody treatment or heat inactivation. Both treatments inhibited the induction of NCF mRNA (*5*).

We observed NCF activity 1 hour after challenge with TNF-α, IL-1β, or LPS, and this activity was present for at least 24 hours (Fig. 2). The kinetics of NCF mRNA expression also showed an induction of NCF transcripts 1 hour after challenge. A peak in NCF mRNA occurred between 4 and 8 hours; expression was maintained for 24 hours (Fig. 2). The expression of mRNA at this latter time point corresponded with maximal expression of biological activity. Neither TNF-α, IL-1β, nor LPS altered the expression of β-actin in any of the above investigations. Endothelial cell–derived NCF mRNA migration was indistinguishable from monocyte-derived NCF at approximately 1.8 kb (*3*).

The human umbilical vein endothelial cell cultures used for these studies were judged to be uncontaminated by other cell types as determined by morphology under phase contrast microscopy, positive immunofluorescence for von Willebrand factor, and positive staining with *Ulex europaeus* 1 lectin. Concurrent cultures were also examined for NCF mRNA by in situ hybridization analysis (Fig. 3, A and B). We used a ^{35}S-labeled oligonucleotide, 3′ end-labeled with terminal transferase, and found that most of the endothelial cells were positive for NCF mRNA after treatment with TNF-α. Thus, even minimal monocyte contamination of the endothelial cultures could not account for the observed results. TNF-α also induced NCF mRNA in human alveolar macrophages (Fig. 3, C and D). A similar expression pattern was observed in TNF-α–treated human monocytes (*5*).

Both resting and stimulated endothelial cells can produce a range of chemotactic factors (*6, 7*). To demonstrate that the NCF mRNA is associated with the translation of a molecule consistent with that for monocyte-derived NCF (*3*), we subjected TNF-A–stimulated endothelial cell supernatants to gel exclusion by high-performance liquid chromatography (HPLC). Neutrophil chemotactic activity in individual fractions was determined in modified Boyden chambers (*8*) (Fig. 4). Fractionated supernatants from endothelial cells treated with TNF-A (20 ng/ml) expressed significant levels of NCF. Most of the activity eluted between phenol red and

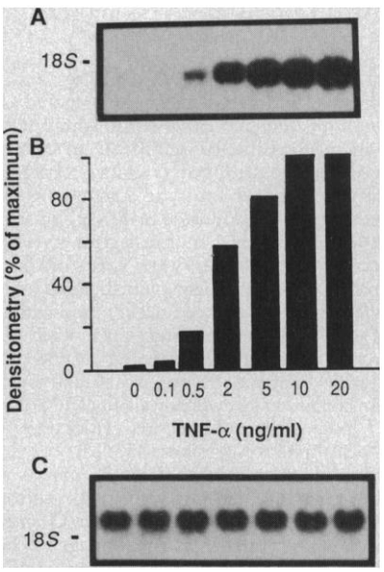

Fig. 1. Dose-dependent induction of NCF mRNA by TNF-α. Endothelial cells were extracted from human umbilical veins (*18*) and cultured in 20% fetal bovine serum in medium 199 supplemented with endothelial cell growth supplement (ECGS; 100 μg/ml) (Collaborative Research, Bedford, Massachusetts) and bovine lung heparin (100 μg/ml). Cells used in assays were derived from the second to fourth passages. The day before stimulation, cells were washed and cultured in fresh medium without growth factors. The following day, the cells were placed in fresh medium and TNF-α was added. After 16 hours of incubation at 37°C, the medium was removed and total cellular RNA was extracted by a modification of the method of Chirgwin *et al.* (*19*) and Jonas *et al.* (*20*). A 30-nucleotide probe for human monocyte–derived chemotactic factor was synthesized with a cDNA sequence that was complementary to nucleotides 262 to 291 and had the sequence 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′ (*3*). The β-actin probe (*21*) used to demonstrate uniform RNA loading was 5′-GGC-TGG-GGT-GTT-GAA-GGT-CTC-AAA-CAT-GAT-CTG-GGT-CAT-CTT-3′. Both probes were 5′end-labeled with [γ-^{32}P]adenosine 5′-triphosphate (ICN). The extracted RNA was separated by electrophoresis, transblotted to nitrocellulose, hybridized with ^{32}P-labeled probe, and washed in 6× saline sodium citrate (SSC) and 0.5% sodium pyrophosphate at 57°C for 1 hour. The blots were subjected to autoradiography and quantitated by laser densitometry. (**A**) RNA blot analysis of mRNA from cells stimulated with increasing doses of TNF-α and probed for NCF; (**B**) the relative density of the signals as determined by laser densitometry; and (**C**) the same blot re-probed for β-actin mRNA. The RNA blot data in this figure are representative of four individual studies.

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cytochrome c, with a molecular size of 7.5 kD, consistent with monocyte-derived NCF (*3*). Fractionated supernatants from unstimulated endothelial cells had no NCF activity in this range. Neither unfractionated nor fractionated TNF-A–treated endothelial cell supernatants exhibited chemotactic activity for isolated peripheral blood monocytes (*9*). Media alone with TNF-A, LPS, or IL-1B also failed to demonstrate significant chemotactic activity (*5*).

Vascular endothelial cells form the inner lining of the entire cardiovascular system. They are active participants in pathophysiological reactions and, in inflammation, they use their strategic position to influence interactions of circulating leukocytes and the tissues. TNF-A is responsible for altering endothelial function leading to the active participation of this cell type in inflammation (*1*). For example, TNF-A–stimulated endothelial cells can produce biologically active lipids (*10*), tissue factor (*11*), IL-1 (*12, 13*), platelet-derived growth factor (*14*), major histocompatibility complex class I gene, and surface antigen expression (*15*). In addition, TNF-A–treated endothelial cells can express freshly synthesized surface proteins that are adherent for neutrophils (*16, 17*). Although these proteins may be a mechanism for trapping granulocytes in a localized area of tissue injury or inflammation, other molecules such as NCF may

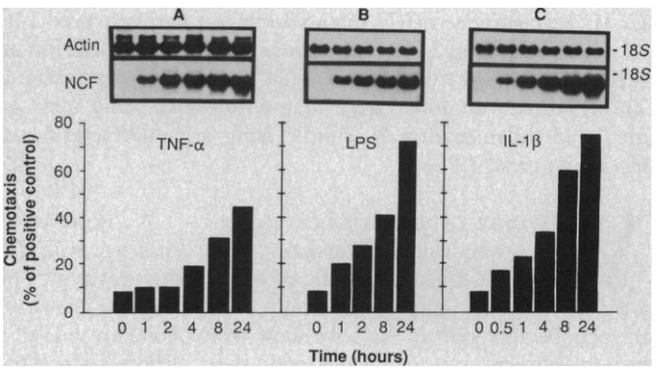

Fig. 2. Time course of TNF-α-, LPS-, and IL-1β–induced endothelial NCF expression. Endothelial cells were prepared as in Fig. 1. The cells were placed in fresh medium and either left unstimulated or treated with TNF-α or IL-β (20 ng/ml) or LPS (10 μg/ml). At indicated time points the culture medium was collected, centrifuged to remove particulate material, and stored at −70°C until neutrophil chemotactic activity was determined. RNA was extracted from the cells and evaluated by RNA blot analysis for transcripts for NCF and β-actin, as described in the legend to Fig. 1. For the determination of bioactivity, neutrophils were prepared from peripheral blood by Ficoll-Hypaque density gradient centrifugation and hypotonic lysis and were resuspended in Hanks balanced salt solution with calcium and magnesium (HBSS) at 2×10^6 cells per milliliter. Chemotaxis was performed in blind multiwell Boyden chemotactic chambers (*8*). HBSS (225 μl) was placed in the bottom well with either HBSS alone, a 50% dilution of culture supernatant, or $10^{-7}M$ formylmethionylleucylphenylalanine (FMLP, Sigma). A 3-μm polycarbonate filter (polyvinylpyrrolidone-free, Nuclepore) was placed in the assembly, and the neutrophil suspension (333 μl) was added to the top chamber. The neutrophils were incubated for 30 min at 37°C in a humidified chamber. The filters were removed and stained with Diff-Quik (Baxter Healthcare). Neutrophils that had migrated to the bottom of the filter were counted in ten high-power fields. These chemotactic studies are representative of four individual experiments. (**A**), (**B**), and (**C**) show mRNA for endothelial cell–derived β-actin and NCF treated with either TNF-α, LPS, or IL-1β, respectively. Bioactivity is expressed as a percentage of chemotactic activity induced by FMLP ($10^{-7}M$). An unstimulated culture was also assayed after 24 hours, and neither mRNA nor chemotactic activity could be detected (*5*). The RNA blot analyses are representative of four individual studies.

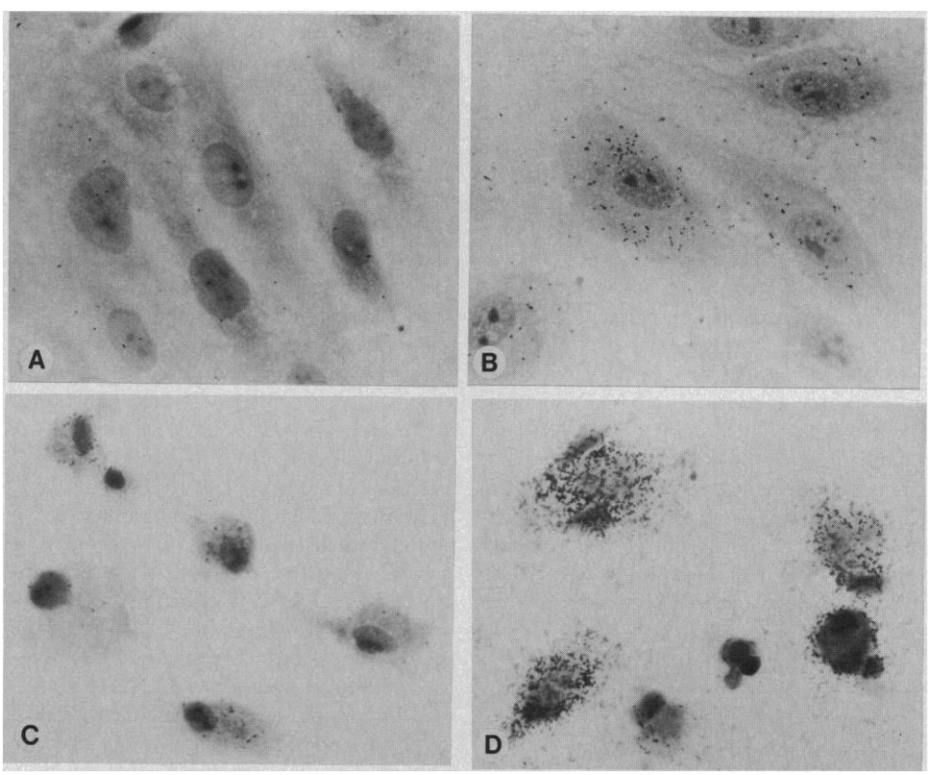

Fig. 3. In situ hybridization of human endothelial cells and human alveolar macrophages with a 3′ ^{35}S-labeled oligonucleotide probe. Endothelial cells in (**A**) the absence or (**B**) presence of TNF-α. Alveolar macrophages in (**C**) the absence or (**D**) presence of TNF-α. Endothelial cells were prepared as in Fig. 1 and cultured at 2×10^4 cells per well in eight-well Lab Tek (Miles) tissue culture slides for 48 hours before stimulation. Human alveolar macrophages (10^5 per well) were placed in Lab Tek tissue culture slides and allowed to adhere for 2 hours in RPMI 1640 medium. Cells were unstimulated or treated with TNF-α (20 ng/ml) for 24 hours. After incubation, the slides were placed in 4% paraformaldehyde (Eastman Kodak) in phosphate-buffered saline for 15 min at room temperature and then rinsed three times in ice-cold 70% ethanol prepared with diethyl pyrocarbonate–treated water. The slides were fixed in 4% paraformaldehyde for 10 min at room temperature, then rinsed in 0.5× SSC for 10 min at room temperature and covered with prehybridization solution [10% deionized formamide, 5× SSC, 10% dextran sulfate, salmon sperm DNA (100 μg/ml), transfer RNA (100 μg/ml), 1× Denhardt's, and 10 m*M* dithiothreitol]. Slides were incubated for 2 hours at 42°C in a sealed container. The prehybridization solution was shaken off and replaced with the same solution (100 μl) containing 10^6 cpm of the labeled probe. After 16 hours at 42°C, the slides were rinsed twice in 2× SSC for 10 min at room temperature, once in 1× SSC for 60 min at 42°C, then dehydrated in graded ethanols with 0.3*M* NH_4 acetate, coated with NTB-2 emulsion (Eastman Kodak), air-dried, and placed in a lightproof box with desiccant at 4°C. After 7 days the slides were developed with Kodak D-19, fixed with rapid fix, and counterstained with hematoxylin and eosin. The NCF probe, as described in the legend to Fig. 1, was 3′ end-labeled with [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate (Du Pont Biotechnology Systems) with the use of a terminal deoxynucleotidyltransferase kit (Bethesda Research Laboratories).

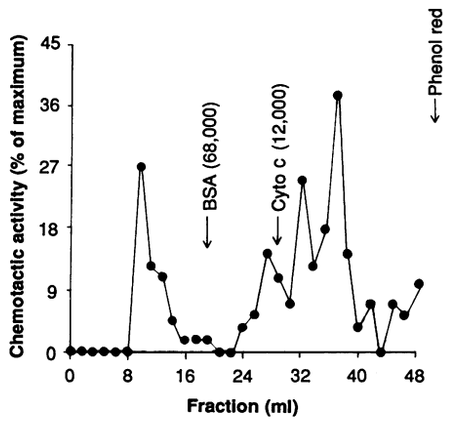

Fig. 4. Molecular size profile (in daltons) of TNF-α–induced endothelial cell supernatant chemotactic activity. Endothelial culture media were collected after 24 hours of treatment with TNF-α (20 ng/ml) or from unstimulated cultures. Particulate material was removed by centrifugation, concentrated fivefold, and fractionated by HPLC in a TSK-3000 SW (Varian Instruments) column. Fractions (1.6 ml) were collected and chemotactic activity was determined, as described in the legend to Fig. 2. The results are expressed as a percentage of the chemotactic activity (minus unstimulated control) induced by FMLP ($10^{-7}M$) and are representative of four individual studies. Abbreviations: BSA, bovine serum albumin; and cyto c, cytochrome c.

provide a signal to elicit selective diapedesis of neutrophils into the extravascular space.

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Identification of a Platelet Membrane Glycoprotein as a Falciparum Malaria Sequestration Receptor

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Infections with the human malaria parasite *Plasmodium falciparum* are characterized by sequestration of erythrocytes infected with mature forms of the parasite. Sequestration of infected erythrocytes appears to be critical for survival of the parasite and to mediate immunopathological abnormalities in severe malaria. A leukocyte differentiation antigen (CD36) was previously suggested to have a role in sequestration of malaria-infected erythrocytes. CD36 was purified from platelets, where it is known as GPIV, and was shown to be a receptor for binding of infected erythrocytes. Infected erythrocytes adhered to CD36 immobilized on plastic; purified CD36 exhibited saturable, specific binding to infected erythrocytes; and purified CD36 or antibodies to CD36 inhibited and reversed binding of infected erythrocytes to cultured endothelial cells and melanoma cells in vitro. The portion of the CD36 molecule that reverses cytoadherence may be useful therapeutically for rapid reversal of sequestration in cerebral malaria.

ERYTHROCYTES INFECTED WITH MATURE stages of the human malaria parasite *P. falciparum* are found sequestered along venular endothelium and not in the peripheral circulation of patients with malaria (*1*). Sequestration appears to contribute to the immunopathology of malaria (*2, 3*) and to the survival of the parasite by protecting nondeformable infected erythrocytes from destruction in the spleen (*4*). Assays of infected erythrocyte cytoadherence to cultured endothelial cells or C32 melanoma cells have been developed as in vitro correlates of sequestration (*5, 6*). Electron-dense knobs that develop on the surface of infected erythrocytes as the parasite matures are necessary for cytoadherence and are the points of attachment to endothelial cells in vivo (7) and to endothelial cells (*5*), melanoma cells (*6*), and monocytes (*3*) in vitro. An 88-kD leukocyte differentiation antigen (CD36), present on endothelial cells, melanoma cells, monocytes, and platelets and recognized by monoclonal antibody (MAb) OKM5, was shown indirectly to have a role in cytoadherence of malaria-infected erythrocytes (*8–10*). Malaria-infected cells can bind to all these cell types but not to cells lacking CD36. This glycoprotein was purified from platelets, where it is known as glycoprotein IV (GPIV) (*11*). We now present direct evidence that CD36 is a receptor for malaria-infected erythrocytes.

When a suspension containing 6% malaria-infected erythrocytes and 94% uninfected erythrocytes was incubated with immobilized CD36, selective binding of infected erythrocytes to CD36 is observed (Fig. 1). Binding to CD36 was observed with human and *Aotus* monkey erythrocytes infected with knobby (K+) *P. falciparum* isolates

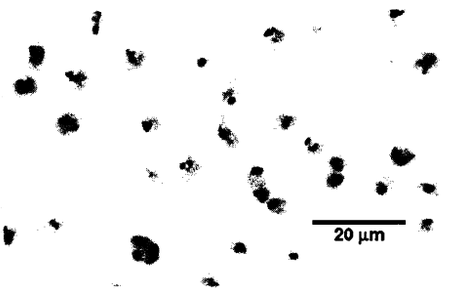

Fig. 1. Binding of *P. falciparum*–infected erythrocytes to CD36. Human erythrocytes infected with the K+ Brazilian ItG strain of *P. falciparum* (*12*) were cultured to the mature trophozoite stage of parasite development. Infected erythrocytes (2% suspension, 6% parasitemia) were washed, resuspended in RPMI 1640 medium, and a 2-ml portion was added to a plastic petri dish containing immobilized CD36 (*21*). After incubation at 37°C for 60 min, the dish was rinsed with RPMI 1640 to remove unattached erythrocytes, fixed with 2% glutaraldehyde, and stained with 1% Giemsa.

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