PAPER

Proinflammatory Mediators Stimulate Neutrophil-Directed Angiogenesis

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Background: Vascular endothelial growth factor (VEGF; vascular permeability factor) is one of the most potent proangiogenic cytokines, and it plays a central role in mediating the process of angiogenesis or new blood vessel formation. Neutrophils (PMNs) recently have been shown to produce VEGF.

Hypothesis: The acute inflammatory response is a potent stimulus for PMN-directed angiogenesis.

Methods: Neutrophils were isolated from healthy volunteers and stimulated with lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and anti-human Fas monoclonal antibody. Culture supernatants were assayed for VEGF using enzyme-linked immunosorbent assays. Culture supernatants from LPS- and TNF-α–stimulated PMNs were then added to human umbilical vein endothelial cells and human microvessel endothelial cells and assessed for endothelial cell proliferation using 5-bromodeoxyuridine labeling. Tubule formation was also assessed on MATRIGEL basement membrane matrix. Neutrophils were lysed to measure total VEGF release, and VEGF expression was detected using Western blot analysis.

Results: Lipopolysaccharide and TNF-α stimulation resulted in significantly increased release of PMN VEGF (532 ± 49 and 484 ± 80 pg/mL, respectively; for all, presented as mean ± SEM) compared with control experiments (32 ± 4 pg/mL). Interleukin 6 and Fas had no effect. Culture supernatants from LPS- and TNF-α–stimulated PMNs also resulted in significant increases ($P<.005$) in macrovascular and microvascular endothelial cell proliferation and tubule formation. Adding anti–human VEGF-neutralizing polyclonal antibody to stimulated PMN supernatant inhibited these effects. Total VEGF release following cell lysis and Western blot analysis suggests that the VEGF is released from an intracellular store.

Conclusion: Activated human PMNs are directly angiogenic by releasing VEGF, and this has important implications for inflammation, capillary leak syndrome, wound healing, and tumor growth.

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THE NEUTROPHIL, or polymorphonuclear leukocyte (PMN), is the most abundant circulating leukocyte in the bloodstream and plays an essential role in the host immune response. Activated PMNs have the important ability to leave the circulation via endothelial cell transmigration and hence accumulate in host tissues. Their classical function is to phagocytose, but they are also known to secrete various cytokines, including tumor necrosis factor α (TNF-α),[1] growth-related oncogene α,[2] platelet activating factor,[3] interleukin (IL) 1,[4] IL-6,[5] and IL-8.[2] Neutrophils recently have been shown to secrete vascular endothelial growth factor (VEGF), also known as vascular permeability factor.[6-8]

Vascular endothelial growth factor is an endothelial cell–specific growth factor and plays an essential role in the process of angiogenesis, a process that is fundamental to reproduction, development, and repair and is now established as playing a pivotal role in solid tumor growth and metastasis.[9] Vascular endothelial growth factor is the most potent proangiogenic cytokine and can increase vascular permeability.[10]

We hypothesized that acute inflammatory mediators, lipopolysaccharide (LPS), TNF-α, IL-6, and Fas could stimulate PMN-directed VEGF release and subsequent angiogenesis. Lipopolysaccharide, or endotoxin, is a bacterial cell-wall product derived from most gram-negative and some gram-positive bacteria. It is a potent inflammatory mediator. Tumor necrosis factor α and IL-6 are proinflammatory cytokines. Fas is a membrane-bound glycoprotein receptor that mediates

MATERIALS AND METHODS

REAGENTS

Lipopolysaccharide (*Escherichia coli* O55:B5) was purchased from Sigma-Aldrich Corporation (St Louis, Mo). Tumor necrosis factor α, IL-6, and goat anti–human VEGF-neutralizing polyclonal antibody were from R&D Systems (Minneapolis, Minn). Anti-human Fas monoclonal antibody (clone CH-11) and MATRIGEL basement membrane matrix were obtained from Coulter Immunotech (Miami, Fla) and Becton Dickinson (Bedford, Mass), respectively.

PMN ISOLATION AND CULTURE

Whole venous blood was collected from healthy adult volunteers using lithium heparin–coated bottles (Greiner Labortechnik, Kremsmünster, Austria). The PMNs were isolated using dextran sedimentation (6% dextran in 0.9% sodium chloride) followed by Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) gradient centrifugation. Contaminating erythrocytes were removed by centrifugation through an 81% isotonic colloidal suspension of silica gradient (Percoll; Sigma-Aldrich Corporation). The granulocyte layer at the interface was collected, washed in RPMI 1640 (GibcoBRL, Paisley, Scotland), and resuspended in complete RPMI 1640 containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin sulfate (100 µg/mL), amphotericin B (Fungizone) (0.25 µg/mL), and levoglutamide (2 mmol/L). The PMNs were counted, and cell viability as determined using trypan blue exclusion was more than 98%. Cell purity was more than 97% as determined by a triple stain for rapid Romanowsky staining using Rapi-Diff II stain (DiaCheM, Lancashire, England) on cytocentrifuged samples.

The PMNs (1×10^7 cells/mL) were then cultured in 24-well plates (Falcon, Lincoln Park, NJ) (3×10^6 cells/well) in the presence of TNF-α (0.1, 0.5, 1.0, 2.5, 5, 25, and 50 ng/mL), LPS (0.1, 0.5, 1.0, 5, 10, 50, and 100 ng/mL), IL-6 (0.5, 1.0, 1.5, 5, 10, and 15 ng/mL), and Fas (50, 100, and 200 ng/mL) and incubated at 37°C in 5% carbon dioxide (CO_2) conditions for different time points. Unstimulated PMNs were used as control cells. At 2- and 6-hour incubation time points, the supernatants were harvested from PMN cultures and stored at –80°C for further VEGF quantification.

ENDOTHELIAL CELL CULTURE

Human umbilical vein endothelial cells (HUVEC) were isolated using collagenase treatment of umbilical veins and cultured on 2% gelatin-coated culture flasks (Falcon) in complete Medium 199 (GibcoBRL) supplemented with 20% fetal calf serum, penicillin (100 U/mL), streptomycin sulfate (100 µg/mL), amphotericin B (0.25 µg/mL), heparin (16 U/mL), endothelial cell growth supplement (75 µg/mL), and levoglutamide (2 mmol/L) as previously described.[12] Cells were grown at 37°C in humidified 5% CO_2 conditions and subcultured by trypsinization with 0.05% trypsin–0.02% EDTA when confluent monolayers were reached. Endothelial cells were identified by typical phase contrast "cobblestone" shape and by the presence of von Willebrand factor antigen using the immunofluorescence technique. Human umbilical vein endothelial cells were used between passages 3 and 5.

Human dermal microvascular endothelial cells (HMVEC) at passage 3 were purchased from Clonetics Corporation (San Diego, Calif) and cultured in endothelial cell basal medium (Clonetics Corp) supplemented with Bulletkit (Clonetics Corp), including 5% fetal calf serum, human epidermal growth factor (10 ng/mL), bovine brain extract (12 µg/mL), hydrocortisone (1.0 µg/mL), gentamycin (50 µg/mL), and amphotericin B (50 ng/mL). Cells were grown at 37°C in humidified 5% CO_2 conditions and split once a week. Human dermal microvascular endothelial cells were used until passage 8.

ENZYME IMMUNOASSAY FOR VEGF

Vascular endothelial growth factor was assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) on a microtiter plate reader (Dynex Technologies Inc, Chantilly, Va) according to the

apoptosis or programmed cell death for several cell types, including PMNs. Inflammatory angiogenesis induced by agonistic anti-Fas monoclonal antibody in a murine model has been described recently.[11] Therefore, the aim of our study was to evaluate the effect of LPS, TNF-α, IL-6, and Fas on PMN VEGF release and PMN-dependent angiogenesis as assessed by endothelial cell proliferation and capillarylike tubule formation.

RESULTS

Lipopolysaccharide and TNF-α stimulation resulted in significantly increased release of PMN VEGF compared with controls. As shown in **Figure 1**, TNF-α stimulation followed by 2- and 6-hour incubation times resulted in a dose-dependent increase in VEGF release compared with control values. The minimal effective dose was measured at 2.5 ng/mL, with a maximal effect observed at a concentration of 5 ng/mL (484 ± 80 vs 32 ± 4 pg/mL at 2 hours and 794 ± 131 vs 95 ± 18 pg/mL at 6 hours; $P = .006$). Similarly, LPS stimulation resulted in a dose-dependent increase in VEGF release, with a minimal effective dose measured at 5 ng/mL, peaking at 10 ng/mL (532 ± 49 vs 32 ± 4 pg/mL after 2 hours' incubation and 774 ± 107 vs 95 ± 18 pg/mL after 6 hours' incubation; $P = .004$) as shown in **Figure 2**.

The effect of TNF-α and LPS together on PMN VEGF release also was examined. Following TNF-α (5 ng/mL) and LPS (10 ng/mL) stimulation for 2 hours, VEGF release was measured at 700 ± 88 pg/mL (n = 5). Compared with VEGF release of 484 ± 80 pg/mL after TNF-α stimulation alone and 532 ± 49 pg/mL after LPS stimulation alone, this indicated an additive effect of both proinflammatory mediators.

At the concentrations of IL-6 we studied (0.1, 0.5, 1.5, 5, 10, and 15 ng/mL), no effect on VEGF release could

manufacturer's recommended protocol. The sensitivity of this ELISA kit was less than 5.0 pg/mL. Concentrations of VEGF in the samples were calculated by extrapolation from the VEGF standard curve.

WESTERN BLOT ANALYSIS FOR VEGF PROTEIN

The PMNs (total cells per group, 1×10^7) were incubated with culture medium alone as the control group or with TNF-α (5 ng/mL) or LPS (10 ng/mL) for 2 hours at 37°C in 5% CO_2 conditions. The PMNs were then lysed using the liquid nitrogen freeze-thaw technique. The supernatant was then harvested following centrifugation at 3000 rpm at 4°C for 20 minutes. Protein concentrations in the supernatant were quantified using a micro bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill). The proteins were denatured at 100°C for 10 minutes in loading buffer (60 mmol/L Tris hydrochloride, 2.5% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 0.01% bromophenol blue). Aliquots containing equal amounts of proteins from each sample were electrophoresed on a 12% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were blotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and blocked with 6% skimmed milk powder in a combination of phosphate-buffered saline solution and 0.05% polysorbate (Tween; GibcoBRL). After the primary mouse anti–human VEGF monoclonal antibody (R&D Systems) and the secondary alkaline phosphatase–conjugated goat anti–mouse monoclonal antibody (Promega, Madison, Wis) were incubated with the membranes, the protein was visualized using BCIP/NBT color development substrate (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium; Promega).

ASSAY FOR ENDOTHELIAL CELL PROLIFERATION

Human umbilical vein endothelial cells and HMVEC were cultured in 96-well plates (Falcon) (1×10^4 cells/well) for 12 hours at 37°C in 5% CO_2 conditions. Following replacement of respective culture medium with fresh culture medium, supernatants harvested from unstimulated PMNs (control experiments) and TNF-α (5 ng/mL)– and LPS-stimulated (10 ng/mL)–PMNs were added to the cultured endothelial cells in 96-well plates. In addition, supernatants harvested from TNF-α– and LPS-stimulated PMN cultures were cocultured with goat anti–human VEGF-neutralizing polyclonal antibody (10 µg/mL) at 4°C for 2 hours to fully neutralize VEGF present in the supernatants. The neutralized supernatants were also added to the cultured endothelial cells. Human umbilical vein endothelial cells and HMVEC were incubated for a further 24 hours at 37°C in 5% CO_2 conditions. Endothelial cell proliferation was then assessed using a commercially available 5-bromodeoxyuridine labeling and detection kit (kit III; Boehringer Mannheim, Mannheim, Germany) as per manufacturer's protocols.

ASSESSMENT OF CAPILLARY TUBE FORMATION ON MATRIGEL

Human umbilical vein endothelial cells capillary tube formation was evaluated as previously described.[13] Briefly, the precooled chamber slide system (Lab-Tek; Nalge Nunc, Naperville, Ill) was coated with MATRIGEL (250 µL/well) used at 4°C, which was then allowed to polymerize at 37°C for at least 1 hour. Human umbilical vein endothelial cells (4×10^4 cells/well) in a final volume of 0.5-mL HUVEC culture medium containing the supernatants from unstimulated PMNs and TNF-α (5 ng/mL)– and LPS stimulated (10 ng/mL)–PMNs and the supernatants neutralized with VEGF-neutralizing polyclonal antibody were plated on the MATRIGEL substratum. After an 18-hour incubation, the medium was aspirated, and the adherent cells were fixed and stained using Rapi-Diff II. Tube formation was examined visually, and the total tubelike structures per well were counted under light microscopy. The assay was performed in duplicate.

STATISTICAL ANALYSIS

All data are presented as mean ± SEM. Statistical analysis was performed using analysis of variance. Differences were judged statistically significant when the *P* value was less than .05.

be demonstrated (data not shown). Fas stimulation (50, 100, and 200 ng/mL) also resulted in no significant increase in PMN VEGF release (data not shown).

Following the maximal stimulatory effect of TNF-α (5 ng/mL) and LPS (10 ng/mL) on PMN VEGF release, we then added the culture supernatants from TNF-α– and LPS-stimulated PMNs to HUVEC and HMVEC and assessed endothelial cell proliferation using 5-bromodeoxyuridine labeling. As shown in **Figure 3**, addition of both supernatants resulted in significant increases in macrovascular and microvascular endothelial cell proliferation.

To confirm that the VEGF in the stimulated PMN supernatants was playing a major role in this proliferative response, we added anti–human VEGF-neutralizing polyclonal antibody to the culture supernatants from LPS- and TNF-α–stimulated PMNs. As shown in **Figure 4**, endothelial cell proliferation was blocked by the VEGF-neutralizing polyclonal antibody.

Culture supernatants from LPS- and TNF-α–stimulated PMNs also demonstrated significant increases in capillarylike tubule formation compared with controls ($P<.05$) (**Figure 5**, A-C). Again, adding anti–human VEGF-neutralizing polyclonal antibody to the supernatants blocked this tubule formation (Figure 5, D).

Western blot analysis was used to detect $VEGF_{165}$ protein expression in control PMNs (unstimulated) and LPS- and TNF-α–stimulated PMNs. There appeared to be no difference in protein expression between the different PMN-stimulated groups as shown in **Figure 6**.

Finally, PMNs were lysed using a liquid nitrogen freeze-thaw technique to quantify total VEGF concentration in the PMNs. Concentration of VEGF was measured using ELISA. Total VEGF level (mean total VEGF release, 2049 ± 209 pg/mL [n = 5]) was greater than twice the maximum mean level released by the PMNs

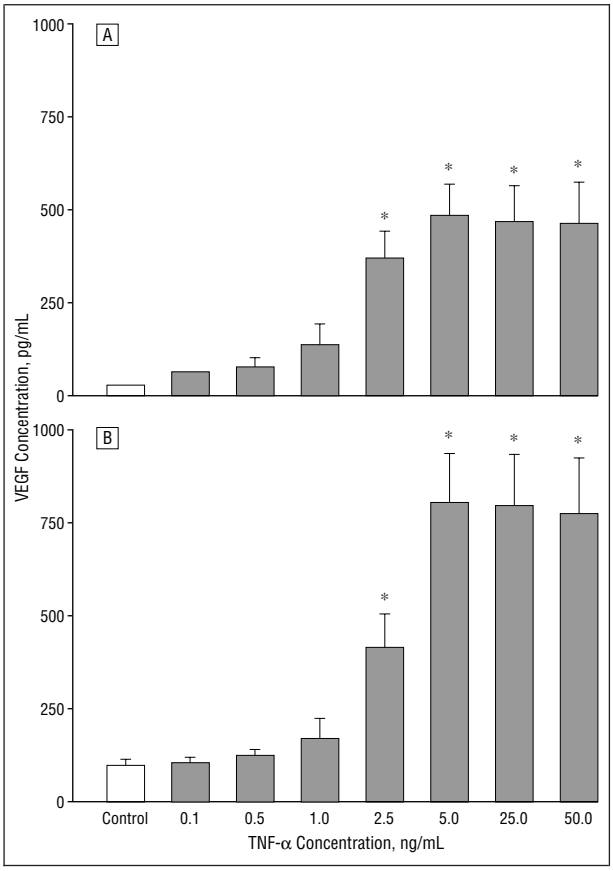

Figure 1. *Vascular endothelial growth factor (VEGF) release by neutrophils following tumor necrosis factor α (TNF-α) stimulation. Neutrophils (3 × 10⁶ cells/well) were incubated in culture medium at 37°C in 5% carbon dioxide conditions with 0.1, 0.5, 1.0, 2.5, 5, 25, and 50 ng/mL TNF-α for 2 (A) and 6 hours (B). Release of VEGF was quantified using enzyme-linked immunosorbent assay. Data are expressed as mean ± SEM and represent 5 separate experiments. Asterisk indicates* P<.05 *vs culture medium as control experiment.*

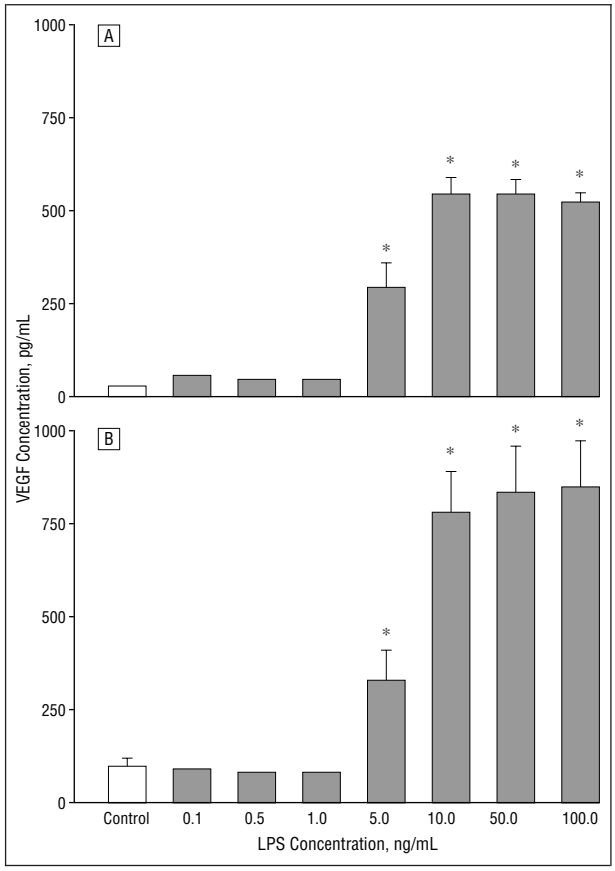

Figure 2. *Vascular endothelial growth factor (VEGF) release by neutrophils following lipopolysaccharide (LPS) stimulation. Neutrophils (3 × 10⁶ cells/well) were incubated in culture medium at 37°C in 5% carbon dioxide conditions with 0.1, 0.5, 1.0, 5, 10, 50, and 100 ng/mL LPS for 2 (A) and 6 hours (B). Release of VEGF was quantified using enzyme-linked immunosorbent assay. Data are expressed as mean ± SEM and represent 5 separate experiments. Asterisk indicates* P<.05 *vs culture medium as control experiment.*

following LPS and TNF-α stimulation (774 and 794 pg/mL, respectively).

COMMENT

Angiogenesis is a complicated and highly regulated multistep biological process consisting of the following 3 stages: initiation of proliferation, migration of endothelial cells, and lumen formation. This process is under tight control and is mediated by a balance between proangiogenic and antiangiogenic growth factors and cytokines. Vascular endothelial growth factor is a very potent proangiogenic and endothelial cell specific cytokine. Numerous cell types can secrete VEGF, including tumor cells,[14] macrophages,[15] fibroblasts,[16] smooth muscle cells,[17] keratinocytes,[18] and platelets.[19] Tumor necrosis factor α has been shown to stimulate PMN VEGF release.[7,8] We wished to look at the effect of other inflammatory cytokines in addition to TNF-α, and, to our knowledge, have shown for the first time that LPS is a potent mediator in stimulating PMN VEGF release. Lipopolysaccharide binds to PMN via a CD14 receptor, and it has been shown that TNF-α enhances LPS binding to PMNs.[20] We also looked, therefore, at the effect of stimulating the PMNs with LPS and TNF-α together, and found an additive effect on VEGF release.

Interleukin 6 and Fas had no effect on PMN VEGF release. Interleukin 6 is a pleiotropic cytokine and plays an integral role in the acute-phase response to injury and infection. Interleukin 6 can augment PMN cytotoxic potential[21] and can delay PMN apoptosis.[22] It has also been shown to induce the expression of VEGF in various cell lines.[23] We could not demonstrate an IL-6–induced PMN VEGF release at the concentrations we studied.

We also investigated whether Fas could stimulate PMN VEGF release on the current understanding that the Fas receptor-ligand interaction, in addition to mediating apoptosis, could also mediate cell activation signals. It is known that the PMNs express Fas and its ligand; however, we could not demonstrate any effect of Fas on PMN VEGF release in our assay system.

We have shown that activated human PMNs are directly angiogenic by stimulating macrovascular and microvascular endothelial cell proliferation and promoting tubule formation in a well-established model for in vitro angiogenesis using MATRIGEL. The VEGF released from these PMNs plays the major role in this stimu-

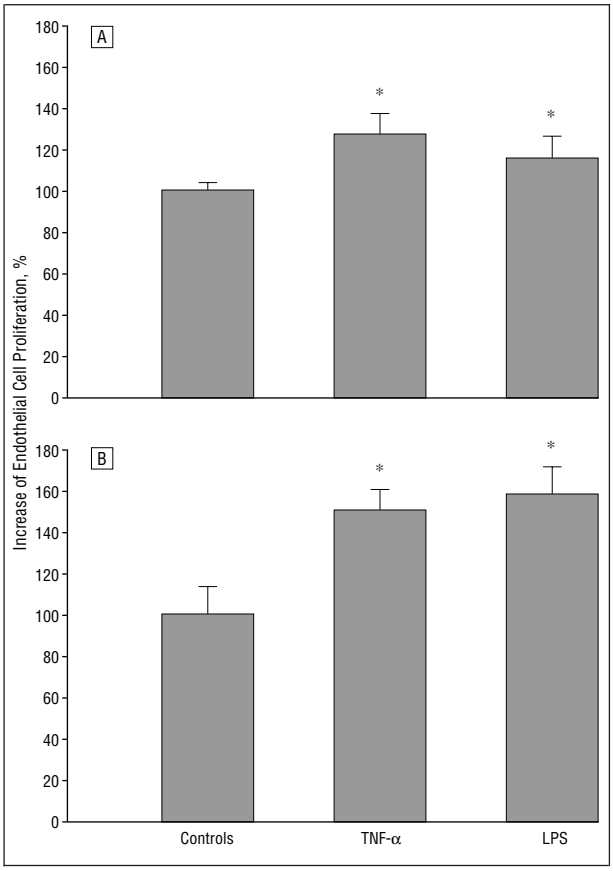

Figure 3. *Percentage of increase in endothelial cell proliferation after culture with the supernatant from lipopolysaccharide (LPS)– and tumor necrosis factor α (TNF-α)–stimulated neutrophils (PMNs). Human umbilical vein endothelial cells (A) and human microvessel endothelial cells (B) were treated with the supernatant from LPS- and TNF-α–stimulated PMNs and incubated at 37°C in 5% carbon dioxide conditions for 24 hours. Endothelial cell proliferation in the control group was taken as 100%. Data are expressed as mean ± SEM and represent 5 separate experiments. Asterisk indicates* $P<.05$ *vs control experiment.*

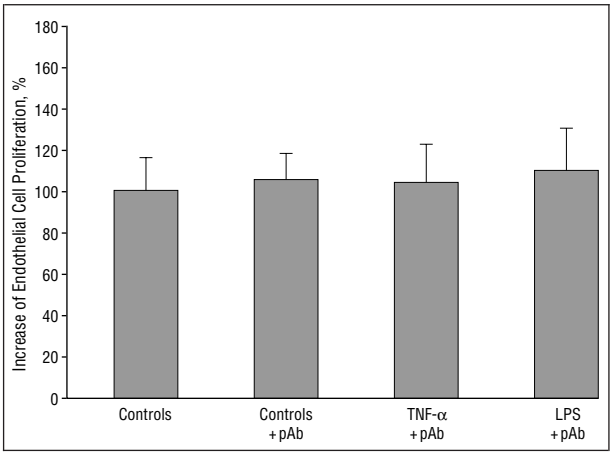

Figure 4. *Effect of adding anti–human vascular endothelial growth factor (VEGF)–neutralizing polyclonal antibody (pAb) to the supernatant from lipopolysaccharide (LPS)– and tumor necrosis factor α (TNF-α)–stimulated neutrophils on endothelial cell proliferation. Endothelial cell proliferation in the control group was taken as 100%. Data are expressed as mean ± SEM and represent 5 separate experiments.*

lation as evidenced by the blocking effect on endothelial cell proliferation and tubule formation when anti–human VEGF-neutralizing polyclonal antibody was added to the stimulated PMN supernatants. The PMNs can also produce a number of other proangiogenic cytokines, including TNF-α (in vivo),[1] IL-1,[4] IL-6,[5] and IL-8.[4] These cytokines may also play a role in the PMN-directed angiogenic process, but obviously not as significant as that played by VEGF, as our results indicate. Neutrophils also can release antiangiogenic factors, including TNF-α (in vitro)[24] and transforming growth factor β_1.[25] These factors could attenuate the stimulatory effect of PMN VEGF on endothelial cell proliferation. Further studies stimulating endothelial cells with recombinant human VEGF at equivalent doses to those released by PMNs could show optimal endothelial cell proliferation and tubule formation.

Next, we wished to ascertain if PMNs synthesize VEGF in response to certain stimuli or whether VEGF is released from a presynthesized intracellular pool. Lysed PMNs released more than double the VEGF concentration compared with maximal VEGF release following LPS and TNF-α stimulation, indicating that there is a preformed pool of VEGF intracellularly.

Vascular endothelial growth factor consists of 4 main isoforms produced by alternate splicing of messenger RNA, resulting in $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. The mature form of VEGF, $VEGF_{165}$, is the most expressed homodimer in human tissues. Western blot analysis was used to detect the $VEGF_{165}$ homodimer expression in the PMNs and to compare this level of expression between LPS- and TNF-α–stimulated PMNs. There appeared to be no difference in this expression between the different PMN-stimulated groups, further indicating that the VEGF is stored in its mature form awaiting the appropriate stimulation for release.

The findings that PMNs can stimulate angiogenesis directly have important implications in physiological and pathologic processes where PMNs play a major role. Angiogenesis is a characteristic feature of wound healing. Neutrophils accumulate at the wound site within hours of the initial injury. In addition to phagocytosing, PMNs may play an active role in the early stages of wound repair by releasing their VEGF stores into the wound microenvironment. Lipopolysaccharide and TNF-α are present in the wound in these early stages, and we have shown their stimulatory effects on PMN VEGF release. Vascular endothelial growth factor has been demonstrated at marked biological levels in surgical wound fluid in animal models[26] and in surgical patients.[27] The levels of VEGF released by PMNs appear to contribute substantially to total VEGF levels seen in these wounds. It has been shown that levels of basic fibroblast growth factor (bFGF), which is another proangiogenic cytokine, peak in wound fluid on day 0 following surgery and decline rapidly on subsequent days.[28] A synergistic effect exists between bFGF and VEGF, thus enhancing endothelial cell mitogenic effects.[29] A similar effect may exist in wounds.

Adult respiratory distress syndrome is associated with increased alveolar capillary permeability. Neutrophil sequestration and migration within the lung are histological hallmarks. Vascular endothelial growth factor, which also demonstrates vascular permeabilizing capabilities,

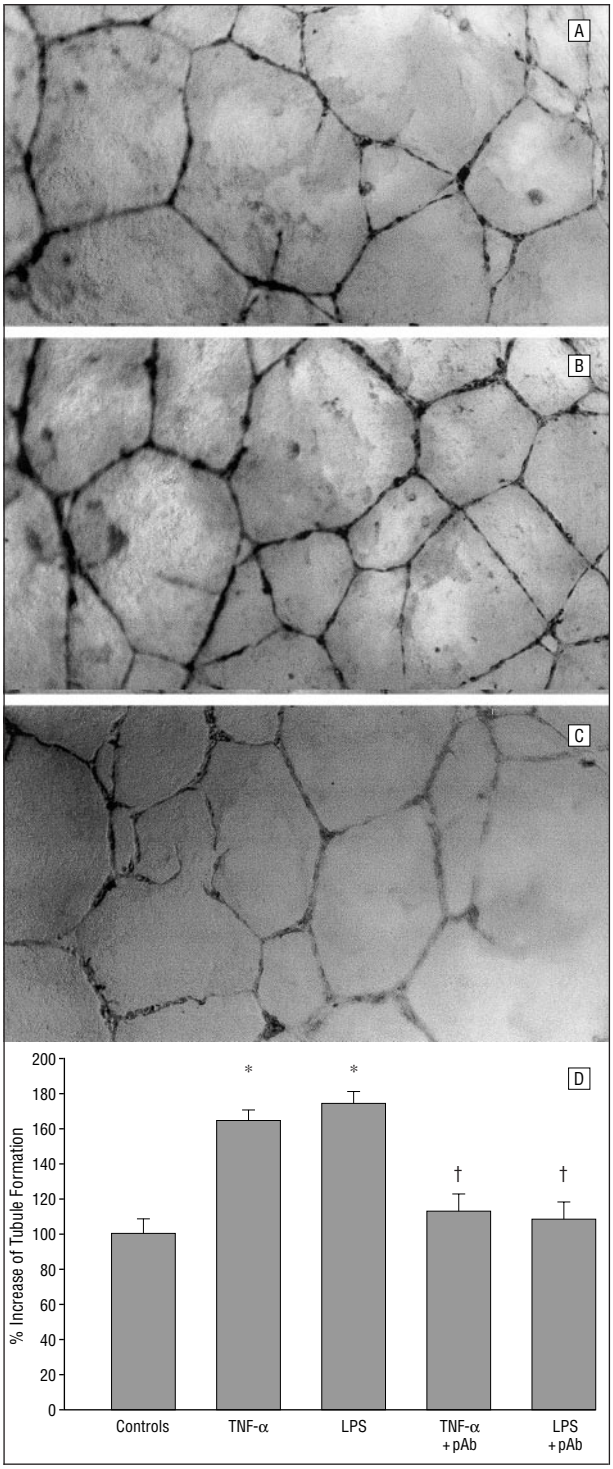

Figure 5. *Human umbilical vein endothelial cell (HUVEC) tubule formation assessed on MATRIGEL. A, Human umbilical vein endothelial cells form capillarylike tubules in the presence of culture medium alone (control experiment). B, Increased tubule formation when supernatant from lipopolysaccharide (LPS)-stimulated neutrophils (PMNs) is added to the cells. C, Adding anti–human vascular endothelial growth factor (VEGF)–neutralizing polyclonal antibody (pAb) to the supernatant blocked this increased tubule formation. D, Increase in HUVEC tubule formation after culture with the supernatant from LPS- and tumor necrosis factor α (TNF-α)–stimulated PMNs followed by addition of anti–human VEGF-neutralizing pAb to these supernatants in a second experiment before culturing the cells. Tubule formation in the control group was taken as 100%. Data are expressed as mean ± SEM and represent 5 separate experiments. Asterisk indicates P<.05 vs culture medium–treated endothelial cells; dagger, P<.05 vs LPS- and TNF-α–stimulated PMN supernatant-treated endothelial cells.*

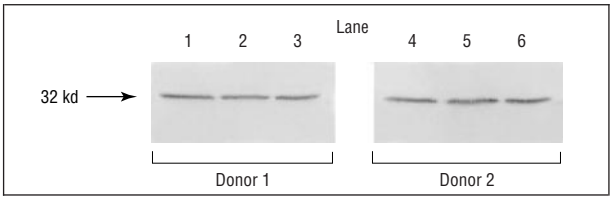

Figure 6. *Vascular endothelial growth factor (VEGF) protein was detected in neutrophils (PMNs) isolated from 2 donors using Western blot analysis. Protein bands in the 32-kd range corresponding to $VEGF_{165}$ were detected in control PMNs and in lipopolysaccharide (LPS)– and tumor necrosis factor α (TNF-α)–stimulated PMNs. Lanes 1 and 4 contain protein from control PMNs; lanes 2 and 5, protein from LPS-stimulated PMNs; and lanes 3 and 6, protein from TNF-α–stimulated PMNs.*

Statement of Clinical Relevance

In this study, we demonstrate that proinflammatory mediators can stimulate PMNs to release VEGF and that this VEGF is directly angiogenic in vitro by stimulating endothelial cell proliferation and tubule formation. This has important implications in physiological and pathologic angiogenic processes where PMNs play a dominant role. Angiogenesis is fundamental to wound healing, and thus the PMN has the potential to mediate the early angiogenic response. Release of PMN VEGF could potentiate the inflammatory response by increasing vascular permeability, resulting in fluid sequestration in a variety of organs, as seen during the systemic inflammatory response syndrome. Finally, PMNs may play a role in promoting the growth of residual dormant tumor cells by releasing VEGF into the surrounding stroma following endothelial cell transmigration.

therefore could be associated with the increased vascular permeability and capillary leak syndrome seen in adult respiratory distress syndrome.

Solid tumor growth and metastases are dependent on angiogenesis. This vasculature provides the necessary oxygen and nutrients for the proliferating cells and also provides access to the circulation for disseminating tumor cells and hence increases their metastatic potential. Surgical removal remains the primary treatment modality for most solid tumors, but current thinking suggests that in the perioperative period, an environment conducive to promoting tumor growth and metastases may exist. Tumor growth is promoted in healing wounds.[30] A marked leukocytosis develops after surgery and peaks at 24 hours.[31] These PMNs easily can accumulate in host tissues via endothelial transmigration and be stimulated to promote angiogenesis by inflammatory mediators like LPS, which is found ubiquitously in air and so can enter surgical wounds. Laparotomy can also induce a transient state of portal endotoxemia via translocation across the intestinal wall.[32] This PMN-directed angiogenesis has the potential, therefore, to promote the growth and metastasis of residual dormant tumor cells.

In conclusion, we provide further evidence that activated human PMNs release VEGF and are angiogenic in vitro. Neutrophils appear to release VEGF by a process of degranulation rather than actively synthesizing

it following activation. Lipopolysaccharide and TNF-α are potent mediators of VEGF release.

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DISCUSSION

Walter L. Biffl, MD, Denver, Colo: The authors hypothesized that acute inflammatory mediators stimulate angiogenesis via neutrophil release of VEGF. Their data support this hypothesis, at least as far as TNF and LPS are concerned. I have a couple of questions regarding the data.

First, the dose-response curves for both TNF and LPS are very narrow and sigmoidal; they go from essentially no effect to maximal effect within 1 log dose range. In contrast, there was no effect with IL-6, but the entire dose-response curve essentially covered just 1 log dose range. Have you looked at any higher doses? While higher concentrations may not seem clinically relevant as far as circulating concentrations of IL-6 go, we know that tissue levels of cytokines can be 2 or 3 orders of magnitude higher than circulating concentrations.

Your antibody data suggest that it is VEGF and not the leftover TNF or LPS in the supernatant that mediates the effect. It would be reassuring, however, if you showed that a lower dose, for example, the 1-ng/mL dose of either TNF or LPS, which did not increase the VEGF, also would not increase endothelial proliferation in your model.

There is a saying that the importance of a hypothesis is not in its correctness but in how much good it generates. In other words, assuming your hypothesis is correct, does it pass the "so-what" test? I think in this case it does, and you have understated the clinical relevance of your work, but I would like to get your thoughts on a couple of things.

You discuss the potential implications of this work in 3 contexts: wound healing, vascular hyperpermeability states, and tumor growth and metastasis.

First, wound healing. You state in the discussion that LPS is found ubiquitously in air and so can enter surgical wounds. You are not suggesting that Galen's hypothesis is correct and that pus is essential to wound healing, are you? What do you think is the real role of this process in wound healing, and what other mediators do you suspect might be involved?

Regarding vascular hyperpermeability states such as ARDS [adult respiratory distress syndrome], I personally don't believe that all ARDS is associated with TNF and LPS. How about IL-8, neutrophil-activating peptide? Does it stimulate PMN VEGF release?

As you mentioned in the manuscript, tumor cell growth is enhanced in healing wounds. Clinical observations suggest that surgical extirpation of a tumor is occasionally followed by the early appearance of metastatic disease. Do you believe this is related to increased angiogenesis stimulated by the surgical procedure?

Professor Redmond and his colleagues recently demonstrated that laparotomy, compared with laparoscopy, is associated with accelerated tumor growth. Laparotomy stimulates neutrophils and prolongs their life span and increases the inflammatory cytokine response to a greater degree than laparoscopy. Have you got any animal data to corroborate your findings in laparotomy vs laparoscopy with regard to angiogenesis? Should surgeons be attempting more tumor resections with minimally invasive techniques, given these theoretical considerations?

Getting back to the question of whether this is a laboratory phenomenon unique to TNF and LPS, GM-CSF [granulocyte-monocyte colony-stimulating factor] stimulates PMN inflammatory function, and if angiogenesis was related to this function, I would expect increased angiogenesis with GM-CSF. Have you looked at GM-CSF in this model? Dr Redmond and colleagues showed a couple of years ago that GM-CSF inhibits tumor growth in the postoperative period, which would suggest that it also inhibits angiogenesis. If you haven't looked at GM-CSF, I would like your thoughts regarding those apparent discrepant timings.

Dr McCourt: Thank you very much for those comments, Dr Biffl. To start off with, actually I agree that the dose-response curve isn't very startling, and indeed, when you look at it, we actually see no effect and then a small effect at 2.5 ng/mL and a peaking effect at 5 ng/mL. You may be right. As we know, TNF-α is a degranulating agent like PMA [phorbol-12-myristate 13-acetate], and perhaps what we are actually seeing is, at a concentration of 5 ng/mL, a strong degranulating effect.

And as regards looking at the effects of higher doses of IL-6, I would agree, the concentrations we used ranged from 1 to 10 ng/mL, and a number of papers would say that in trauma patients the circulating levels in blood of IL-6 could range from 1 to 3 ng. Obviously in patients with more severe trauma or burns, you can get increasing levels and for longer periods of time, and we did not look at those higher levels. But as you say, with higher levels present in local tissues, perhaps we should be looking at those.

And relevance of VEGF and wound healing, it is a little difficult to comment on. As we know, PMNs in wounds accumulate in the wound after platelets arrive, and we know that platelets can release VEGF. And the PMNs are present perhaps in the wound for 5 or 6 days. We know that the most intense period of angiogenesis occurs after the PMNs have arrived and departed, but perhaps in the early phase of wound angiogenesis, the PMNs may play a role. As we know, the first cytokine in wound healing is bFGF, and there is a synergistic effect between bFGF and VEGF in stimulating endothelial cell mitogen capabilities.

As regards looking at the effect of a number of other cytokines on VEGF release, yes, we have looked at IL-6 and IL-2, TNF-α. There are other such inflammatory cytokines which may be worth looking at, including IL-1β.

As regards looking at the chemotactic peptide IL-8, no, we haven't looked at that. And, again, work shown by Professor Redmond, looking at the difference in tumor growth between the laparotomy vs laparoscopy group, we found that in the laparotomy group we were finding increased tumor growth. We believe this is due to a local stimulatory effect of LPS present in the air and stimulating cytokines in the intraperitoneum, and we know that the amount of LPS present in the air is about 1 $\mu g/m^3$. So at the time of surgery, it is likely that LPS can enter the peritoneal cavity both in the air but also by bacterial translocation from the gut.

And with regards to GS-CMF, no, we haven't looked at that effect on neutrophil VEGF release. Perhaps we should look at that.

Alfred Ayala, MD, Providence, RI: A very brief question. Since your granulocytes are in the wound environment, and the wound environment is thought to be classically hypoxia, have you considered looking at hypoxia as a stimulus for this response? I would suggest to you this is probably the stronger stimulant for VEGF.

Dr McCourt: I would agree that hypoxia is probably one of most potent stimulators for angiogenesis, but in studies—shown in I think it is the *Annals of Surgery* sometime last year—they looked at normoxic wounds and actually saw marked increase in VEGF in the wound fluid. So certainly hypoxia does play a role, but I think the cytokine milieu also present in wounds—LPS and TNF-α, as we have shown—also play a very important role in stimulating local angiogenesis.