Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8

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Schruefer, Ruth, Nicola Lutze, Jürgen Schymeinsky, and Barbara Walzog. Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving interleukin-8. Am J Physiol Heart Circ Physiol 288: H1186–H1192, 2005. First published October 21, 2004; doi:10.1152/ajpheart.00237.2004.—Neovascularization by sprouting angiogenesis is critical for inflammation-mediated tissue remodeling and wound healing. We report here that human polymorphonuclear neutrophils (PMN) stimulated for 1 h with 100 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP) released a proangiogenic entity that induced sprouting of capillary-like structures in an in vitro angiogenesis assay. The effect was comparable to the response obtained on stimulation with 100 ng/ml basic FGF. The PMN-mediated response was inhibited by neutralizing antibodies against VEGF or IL-8. As measured by ELISA technique, we found that fMLP-activated PMN (5 × 10⁶/ml) released 78 pg/ml IL-8 and 39 pg/ml VEGF within 1 h after stimulation. IL-8 release was blocked by actinomycin D or cycloheximide, but the inhibitors had no effect on VEGF release, suggesting that IL-8 secretion required de novo synthesis whereas VEGF was secreted from preformed stores. Accordingly, RT-PCR analysis revealed that IL-8 mRNA was upregulated on PMN stimulation, whereas the expression of VEGF mRNA was not affected. Moreover, supernatant derived from activated PMN induced upregulation of endothelial IL-8 mRNA expression, suggesting that release of VEGF and IL-8 from activated PMN may activate a paracrine feedforward mechanism involving endothelial IL-8. Moreover, VEGF-induced upregulation of endothelial IL-8 expression as well as spraying of capillary-like structures was inhibited by a neutralizing anti-IL-8 antibody. These findings suggest that bacteria-derived tripeptides stimulate human PMN to release VEGF and IL-8, which activate endothelial cells and induce angiogenesis by a paracrine feedforward mechanism involving endothelial IL-8 upregulation.

inflammation; vascular endothelial growth factor; tissue repair

HUMAN POLYMORPHONUCLEAR NEUTROPHILS (PMN) play an important role in host defense and inflammation. However, patients suffering from leukocyte adhesion deficiency type I not only show compromised PMN recruitment and host defense due to the absence of adhesion molecules of the β₂-integrin family but also suffer from impaired wound healing (2). This suggests that the inflammatory PMN infiltration may be involved in wound repair, which critically involves angiogenesis. Growing evidence indicates that angiogenesis can be initiated by inflammatory cytokines (6). Human PMN release a variety of proinflammatory cytokines, including IL-8 (9), which was originally identified as a potent activator of human PMN (4). Subsequently, IL-8 was shown to stimulate angiogenesis in the rat cornea model by promoting proliferation and chemotaxis of endothelial cells (15). Moreover, IL-8 inhibits endothelial cell apoptosis and induces the upregulation of endothelial matrix metalloproteinase-2 and -9 (17), which play an important role in angiogenesis (21). The proangiogenic effect of IL-8 and other Glu-Leu-Arg (ELR)⁺ members of the CXC chemokine family is mediated by endothelial CXC receptor 2 (CXCR2) as demonstrated by the finding that the neovascularization induced by ELR⁺ CXC chemokines was inhibited in the presence of neutralizing antibodies against CXCR2 or in CXCR2-deficient mice (1). Evidence for the possible role of PMN in inflammation-mediated angiogenesis and wound repair was provided by the finding that CXCR2-deficient mice, which lacked PMN infiltration in thioglycollate-induced peritonitis (7), showed delayed angiogenesis and, moreover, impaired cutaneous wound healing (11). Thus the linkage of inflammation, angiogenesis, and wound healing may result from the multifunctional role of proinflammatory cytokines, e.g., IL-8, which bind to CXCR2 on endothelial cells. However, different cell types produce IL-8, including macrophages and PMN (9). Evidence for the fact that PMN play an important role in IL-8-mediated angiogenesis in vivo was provided by the finding that the angiogenic response on IL-8 treatment was absent in neutropenic animals (5). Moreover, it has been reported that PMN secrete VEGF (12), which plays a fundamental role in angiogenesis (19). Thus there is a body of evidence suggesting that human PMN may be involved in the induction of angiogenesis. However, whether PMN have the ability to induce angiogenesis directly or whether PMN recruit or activate additional cell types that then induce the angiogenic response is still an unresolved question. In the present study, we addressed the question of whether human PMN directly induce neovascularization. Therefore, we used an in vitro angiogenesis assay in which we studied the effect of supernatant obtained from activated human PMN. Subsequently, the molecular mechanisms underlying the observed response were elucidated.

MATERIALS AND METHODS

Reagents. Collagenase, FCS, fibronectin, Hanks’ balanced salt (HBSS) solution, HEPES, MCDB 131 medium, medium 199, penicillin, PBS, sodium hydrogen carbonate, streptomycin, and trypsin were obtained from Biochrom (Berlin, Germany). Recombinant human basic FGF (bFGF) and recombinant human VEGF were purchased from Biomol (Hamburg, Germany). Recombinant human IL-8 was obtained from Biosource Europe (Nivelles, Belgium). Agarose was provided by GIBCO-BRL (Eggenstein, Germany). DNA markers

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After erythrocyte sedimentation and informed consent, according to the institutional guidelines of Ludwig-Maximilians-Universität. Heparinized blood (10 IU/ml) of healthy adult volunteers who gave written consent was centrifuged at 600 g. PMN were resuspended in PBS supplemented with 0.1% glucose, 0.25% BSA, and fibronectin-coated (10 μg/ml) flasks in isolation medium (isolation medium was replaced by culture medium (isolation medium supplied pack for endothelial cell growth medium under gentle rotation). Subsequently, HUVEC were grown to confluence on the microbeads by incubation under static cell culture conditions. The in vitro angiogenesis assay was performed in duplicate under sterile conditions with 48-well Transwell microtaxis chambers with 8-μm-pore polyvinylpyrrolidone-free filters (Neuroprobe, Gaithersburg, MD) coated with fibronectin (50 μg/ml) and gelatin (1 mg/ml). HUVEC (2 × 10⁴/50 μl) in medium 199 supplemented with 1% FCS were loaded into the upper chamber. The stimulus was applied to the lower chamber. After incubation for 4 h at 37°C and 5% CO₂, the capillary-like structures were counted after addition of the supernatant from activated PMN. PMN viability was assessed by the Trypan blue exclusion test; purity was determined with a Nikon microscope (DM510) with an epifluorescence adapter.

**Isolation and stimulation of human PMN.** PMN were isolated from heparinized blood (10 IU/ml) of healthy adult volunteers who gave informed consent, according to the institutional guidelines of Ludwig-Maximilians-Universität. After erythrocyte sedimentation in the presence of 40% (vol/vol) autologous plasma, the leukocyte-rich plasma was layered onto a discontinuous Percoll gradient as described previously (14) and centrifuged at 600 g for 20 min. The PMN-containing band, washed twice with HBSS, and resuspended in PBS, PMN were resuspended in PBS supplemented with 0.1% glucose, 0.25% BSA, 1.2 mM Ca²⁺, and 1 mM Mg²⁺. PMN viability was >99% as assessed by the Trypan blue exclusion test; purity was >99% as analyzed by microscopy with Haemacolor staining (Merck). After stimulation of PMN (5 × 10⁶/ml) for indicated times with 100 nM fMLP, supernatant of activated PMN was harvested by centrifugation and stored at −80°C.

**Isolation of human umbilical vein endothelial cells.** Human umbilical veins were rinsed with HBSS and filled with 0.2% collagenase type II in HBSS. After incubation for 15 min at 37°C, human umbilical vein endothelial cells (HUVEC) were eluted by two rinses with HBSS. After being washed twice with HBSS, HUVEC were cultured in fibronectin-coated (10 μg/ml) flasks in isolation medium (MCDB 131 medium containing a supplement for endothelial cell growth medium and 50 μg/ml amphotericin B). After 2 days, the isolation medium was replaced by culture medium (isolation medium without amphotericin B). For all cell experiments, only primary cells were used. Confluent primary HUVEC were harvested by trypsinization, washed twice with HBSS, and resuspended in PBS.

**In vitro angiogenesis assay.** For the angiogenesis assay, freshly isolated HUVEC were used immediately after isolation without further culture. HUVEC (1 × 10⁶/ml) were incubated with 25 μg of fibronectin-coated (10 μg/ml, 30 min) Biosilon microbeads (diameter 150 μm; Nunc, Wiesbaden, Germany) for 4 h at 37°C in medium 199 with HEPES completed with the supplement pack for endothelial growth medium under gentle rotation. Subsequently, HUVEC were grown to confluence on the microbeads by incubation under static cell culture conditions. The in vitro angiogenesis assay was performed in 24-well plates (Nunc) in duplicate. HUVEC-coated microbeads were embedded into three-dimensional fibrin matrices (400 μl of medium 199 supplemented with 2% FCS, 1 mg/ml fibronogen, and 54 U thrombin per well). Angiogenesis was induced by addition of the supernatant obtained from fMLP-activated PMN (100 μl) or the proangiogenic factors bFGF, VEGF, or IL-8. After 3 days of incubation at 37°C and 5% CO₂, the capillary-like structures were counted with a Nikon microscope (DM510; Düsseldorf, Germany) and a ×10 objective. A sprout was defined as a capillary-like structure when it had a length at least twice the diameter of one microbead (300 μm). Sprouts that connected two microbeads were counted twice. For staining of the nuclei, samples were treated for 5 min at room temperature with 5 μg/ml acridine orange and analyzed by fluorescence microscopy (Nikon DM510) with an epifluorescence adapter (Nikon B-2A).

**RT-PCR.** Total RNA was isolated by the guanidine isothiocyanate method (10) with Tri Reagent (Sigma). RNA (2 μg) was transcribed into cDNA with oligo(dT)₁₅ primers (Promega, Mannheim, Germany) and 200 U of reverse transcriptase Moloney murine leukemia virus (Promega). PCR amplification was carried out with specific primer sets (Metabion, Munich, Germany) for IL-8 (upstream primer: 5’-GGA-CAA-GAG-CCA-GGA-AGA-ACC-C, downstream primer: 5’-CTT-CAA-AAA-CTC-CTC-CAC-ACC-C; 335-bp product) and VEGF (upstream primer: 5’-AGA-AGG-AGG-AGG-GCA-GAA-TC, downstream primer: 5’-TTG-GTG-ATT-TGT-CGG-C-C; 250-bp product). For positive control, a specific primer set (Metabion) for β-actin (upstream primer: 5’-TGT-CCA-CCT-TCC-AGC-AGA-TGT-G, downstream primer: 5’-AGT-CCT-CGG-CCA-CAT-TGT-GAA-C; 300-bp product) was used. PCR (27 cycles for IL-8, 31 cycles for VEGF, and 23 cycles for β-actin; 60 s at 94°C, 60 s at 59°C, 60 s at 72°C) was performed with 1.25 U Taq DNA polymerase (Promega). PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide under ultraviolet light.

**Chemosatx assay.** The chemotaxis assay was performed in duplicate under sterile conditions with 48-well Transwell microtaxis chambers with 8-μm-pore polyvinylpyrrolidone-free filters (Neuroprobe, Gaithersburg, MD) coated with fibronectin (50 μg/ml) and gelatin (1 mg/ml). HUVEC (2 × 10⁴/50 μl) in medium 199 supplemented with 1% FCS were loaded into the upper chamber. The stimulus was applied to the lower chamber. After incubation for 4 h at 37°C and 5% CO₂, HUVEC were harvested by trypsinization, washed twice with HBSS, and resuspended in PBS.

**ELISA for IL-8 and VEGF.** The concentrations of IL-8 and VEGF in the supernatant of activated human PMN were measured in duplicates with ELISA kits (KHO0111 for human VEGF and KHC0081 for human IL-8) according to the supplier’s instructions (Biosource Europe).

Fig. 1. Activated human polymorphonuclear neutrophils (PMN) induce the sprouting of capillary-like structures: analysis of the proangiogenic effect induced by the supernatant of PMN (5 × 10⁶/ml) stimulated for 1 h at 37°C with 100 nM N-formyl-methionyl-leucyl-phenylalanine (MLP). Top: light microscopy of capillary-like structures at day 3 after addition of the supernatant of PMN. Bottom: nuclei of HUVEC were stained by acridine orange (arrows) and analyzed by light (left) and fluorescence (right) microscopy.
CO₂, the filters were removed and the cells were fixed with 100% methanol. Migrated HUVEC were stained with Haemacolor staining solution according to the supplier’s instructions (Merck) and quantified by counting the cells under the microscope.

**Antibodies.** The anti-human VEGF MAb (clone JH121) was obtained from Oncogene Research Products (Boston, MA). The antihuman IL-8 MAb (clone B-K8) was obtained from Biosource Europe.

**Statistical analysis.** Data represent means ± SD where applicable. Statistical significance was determined with Student’s t-test using Sigma Plot 8 software (SPSS, Chicago, IL). P values of <0.05 were taken to indicate statistical significance.

**RESULTS**

**Proangiogenic effect of human PMN.** To study the question of whether activated human PMN have the ability to directly induce angiogenesis, the supernatants of fMLP-activated neutrophils were collected at different time points and tested for their ability to stimulate the sprouting of capillary-like structures with an in vitro angiogenesis assay. At day 3 after the onset of the experiment, we observed a substantial sprouting of capillary-like structures in the presence of the supernatant obtained from neutrophils activated with 100 nM fMLP for 1 h at 37°C (Fig. 1). These structures showed a multicellular organization as detected by staining the nuclei with acridine orange. To quantify the proangiogenic effect, the number of capillary-like structures per microcarrier was counted at day 3 after the onset of the experiment (Fig. 2). We found that the supernatant obtained within 1 h after PMN stimulation induced an ∼7.3-fold increase of angiogenesis from 0.06 sprouts/microcarrier in the unstimulated control to 0.44 sprouts/microcarrier. This effect was comparable in size to the response observed in the presence of 100 ng/ml bFGF, which induced the formation of 0.43 sprouts/microcarrier. A significant increase of angiogenesis was also induced by the supernatants obtained within 4 and 8 h after PMN stimulation (0.23 and 0.24 sprouts/micro-

![Fig. 2. Activated human PMN release a proangiogenic entity on stimulation: quantitative analysis of the proangiogenic effect induced by the supernatant of PMN (5 × 10⁷/ml) stimulated for indicated times at 37°C with 100 nM fMLP. For positive control, sprouting of capillary-like structures was stimulated by basic FGF (bFGF; 100 ng/ml). Direct application of fMLP had no effect on angiogenesis (data not shown). Data are means ± SD numbers of capillary-like structures per microbead; n = 10. *P < 0.05 vs. control (PBS); n.s., not significant.](image-url)

**Fig. 3. Mechanism underlying the release of VEGF and IL-8. A:** human PMN were treated with 10 μM cycloheximide (Cyc), 10 μM actinomycin D (Act D), or vehicle (DMSO) for 30 min at 37°C before stimulation for 1 h at 37°C with 100 nM fMLP. The concentrations of IL-8 and VEGF were determined by ELISA. Values are means ± SD; n = 4. *P < 0.05. B: semiquantitative RT-PCR for IL-8 and VEGF. PMN were left untreated (0 h, 1 h) or were stimulated for 1 h at 37°C with 100 nM fMLP. For negative control, RT-PCR was carried out in the absence of mRNA. Data represent the mean optical density (OD) ratio of the PCR products obtained (OD_IL-8/OD_β-actin or OD_VEGF/OD_β-actin); n = 4. M, DNA marker, *P < 0.05 vs. unstimulated control at 0 h; *P < 0.05 vs. unstimulated control at 1 h.
In contrast, the supernatant collected within 5 and 30 min after neutrophil stimulation showed no significant effect on angiogenesis compared with the unstimulated control (0.07 and 0.11 sprouts/microcarrier, respectively). This was also true for the supernatant collected after 24 h of PMN stimulation (0.11 sprouts/microcarrier). Together, these findings suggest that activated human PMN release a proangiogenic entity after 1, 4, and 8 h of stimulation that is able to induce sprouting of capillary-like structures.

Molecular nature of proangiogenic entity released by activated human PMN. Human PMN are known to release IL-8 as well as VEGF, both of which are able to promote angiogenesis. Therefore, we measured the concentration of IL-8 and VEGF in the supernatants of human PMN that were used to induce angiogenesis. By means of ELISA technique, we found that PMN released 78 pg/ml IL-8 and 39 pg/ml VEGF within 1 h after the onset of stimulation (Fig. 3A). Thus human PMN secreted substantial amounts of IL-8 and VEGF as early as 1 h after the onset of stimulation.

To study the mechanisms underlying the secretion of IL-8 and VEGF, PMN were treated with actinomycin D or cycloheximide to inhibit mRNA or protein synthesis, respectively. We found that both inhibitors almost completely abrogated the release of IL-8 by human PMN stimulated for 1 h with 100 nM fMLP. This suggests that the IL-8 release at this time point required de novo mRNA and protein synthesis. In contrast, inhibition of mRNA or protein synthesis had no effect on the secretion of VEGF after 1 h of stimulation by fMLP, demonstrating that the VEGF secretion did not require de novo synthesis. Thus VEGF release at this time point was obviously due to a mobilization of preformed stores. These results were further confirmed by measuring the mRNA levels of IL-8 and VEGF with semiquantitative RT-PCR (Fig. 3B). Compared with unstimulated controls (0 h), we observed a marked up-regulation of IL-8 mRNA in human PMN stimulated for 1 h with 100 nM fMLP. In contrast, stimulation of PMN for 1 h with 100 nM fMLP did not affect the expression of VEGF mRNA compared with the unstimulated controls. This con-

![Fig. 4.](image-url) The proangiogenic activity of the supernatant derived from human PMN is neutralized by antibodies against VEGF and IL-8. The supernatant of PMN (5 x 10⁶/ml) stimulated for 1 h at 37°C with 100 nM fMLP was treated with MAb against VEGF and IL-8 or left untreated. For control, sprouting of capillary-like structures was stimulated by VEGF (100 ng/ml) in the presence of the anti-IL-8 MAb (data not shown). Data are means ± SD numbers of capillary-like structures per microbead in % of values seen on application of the PMN supernatant alone (without MAb, 100%); n = 10. *P < 0.05.

![Fig. 5.](image-url) Induction of endothelial IL-8 mRNA expression: semiquantitative RT-PCR for IL-8. A: HUVEC were activated for indicated times with 10 ng/ml VEGF or 5 ng/ml IL-8. Data are mean OD ratios of the PCR products obtained (OD_{IL-8} / OD_{β-actin}); n = 4. *P < 0.05 vs. unstimulated control (0 h). B: HUVEC were activated for indicated times by the supernatant of PMN (5 x 10⁶/ml) stimulated for 1 h at 37°C with 100 nM fMLP or treated with PBS only (control). Data are mean OD ratios of the PCR products obtained (OD_{IL-8} / OD_{β-actin}); n = 4. *P < 0.05 vs. unstimulated control at 0 h; #P < 0.05 vs. unstimulated control at 6 h.
firmed that the release of IL-8 after 1 h of PMN stimulation depended on gene activation, whereas VEGF was secreted from preformed stores.

To study the biological relevance of VEGF and IL-8 secretion for the observed induction of angiogenesis, we neutralized VEGF and IL-8 in the supernatant of PMN by the addition of specific monoclonal antibodies in excess (Fig. 4). We found that the proangiogenic activity of the supernatant obtained 1 h after PMN stimulation was almost completely absent when VEGF, IL-8, or both were neutralized with specific antibodies. This demonstrates that the proangiogenic effect exerted by activated PMN critically depended on both cytokines. For positive control, angiogenesis was induced by VEGF (100 ng/ml), which significantly induced angiogenesis compared with untreated controls. The effect was completely absent when VEGF was neutralized by an antibody against VEGF, demonstrating that the cytokine was successfully neutralized by the protocol used. Thus the neutralization experiments demonstrated an important role of IL-8 and VEGF in the induction of angiogenesis in our model. However, the supernatant of PMN contained only 78 pg/ml IL-8 and 39 pg/ml VEGF after 1 h of stimulation, as detected by ELISA technique (compare Fig. 3). Because the supernatant was used at a dilution of 1:5 to induce angiogenesis, this corresponds to an effective dose of ∼16 pg/ml IL-8 and ∼8 pg/ml VEGF. The application of both cytokines at these concentrations alone or in combination was not able to induce sprouting of capillary-like structures (data not shown), demonstrating that VEGF and IL-8 were required but not sufficient to induce angiogenesis.

However, the neutralization experiments demonstrated an important role of both factors in the induction of angiogenesis. Therefore, we addressed the question of whether these factors may represent a part of a paracrine feedforward mechanism involving endothelial IL-8. First of all, we studied the responsiveness of HUVEC to IL-8 and VEGF with a microchamber chemotaxis assay and found that both factors were able to mediate chemotactic migration of HUVEC (data not shown). Subsequently, HUVEC were stimulated with IL-8 (5 ng/ml) or VEGF (10 ng/ml) to test whether these factors may induce endothelial IL-8 production (Fig. 5A). After stimulation of HUVEC for 0, 2, 4, and 6 h and 1 day, induction of endothelial IL-8 mRNA was measured by semiquantitative RT-PCR. Compared with the unstimulated controls (0 h), treatment of HUVEC with IL-8 resulted in a transient upregulation of endothelial IL-8 mRNA after 2, 4, and 6 h of stimulation. Similar results were obtained on stimulation of HUVEC with VEGF. Thus both factors were able to induce the upregulation of endothelial IL-8 mRNA. Next, the effect of the supernatant obtained from activated PMN was used to stimulate HUVEC (Fig. 5B). Similar to the effect of VEGF or IL-8 alone, the supernatant obtained 1 h after PMN stimulation induced a transient upregulation of endothelial IL-8 mRNA at 6 h after the onset of the experiment. This suggests that activated human PMN trigger the induction of endothelial IL-8, which may represent a paracrine feedforward signal amplifying the proangiogenic effect of PMN.

Fig. 6. A role for a paracrine feedforward mechanism involving endothelial IL-8. A: semiquantitative RT-PCR for endothelial IL-8. HUVEC were treated with PBS alone (control) or were activated for 6 h with 10 ng/ml VEGF without addition of an antibody, in the presence of the isotype-matched control antibody, or in the presence of the neutralizing anti-IL-8 MAb. Top: agarose gel of the PCR products. Bottom: data are mean ± SD OD ratios of the PCR products obtained (OD_{IL-8}/OD_{β-actin}); n = 4. *P < 0.05. Similar results were obtained on stimulation of HUVEC with 100 ng/ml VEGF (data not shown). B: sprouting of capillary-like structures was stimulated by VEGF (100 ng/ml) without addition of an antibody, in the presence of the isotype-matched control antibody, or in the presence of the neutralizing anti-IL-8 MAb. For negative control, samples were treated with PBS alone (control). Data are mean ± SD numbers of capillary-like structures per microbead in % of the values seen on application of VEGF (without MAb, 100%); n = 4. *P < 0.05.
To further prove the hypothesis that endothelial IL-8 upregulation depends on a paracrine feedforward mechanism, HUVEC were stimulated with VEGF (10 ng/ml) in the presence of a neutralizing antibody against IL-8 (Fig. 6A). We found that VEGF-induced upregulation of endothelial IL-8 mRNA expression was almost completely inhibited in the presence of the antibody against IL-8, demonstrating that the VEGF-mediated response depended on a paracrine feedforward mechanism involving endothelial IL-8. For control, the upregulation of endothelial IL-8 mRNA expression was measured in the presence of an isotype-matched control antibody, which had no effect. To study the biological relevance of the induction of endothelial IL-8 gene expression, we measured the VEGF-induced angiogenic response in the presence of the neutralizing anti-IL-8 antibody with an in vitro angiogenesis assay (Fig. 6B). We observed that the VEGF-mediated proangiogenic effect was markedly impaired on neutralization of IL-8 by the specific antibody. In contrast, the isotype-matched control antibody had no effect on the VEGF-induced response. These findings demonstrate that VEGF-induced angiogenesis critically depends on the release of endothelial IL-8. During the acute inflammatory response, human PMN extravasate from the blood into the tissue to exert their defense functions. As illustrated in Fig. 7, our data suggest that bacteria-derived tripeptides such as fMLP stimulate the extravasated PMN to release IL-8 and VEGF, which activate endothelial cells and induce angiogenesis by a paracrine feedforward mechanism involving endothelial IL-8. These findings imply that the inflammatory reaction must be placed within a greater context of tissue homeostasis, because it not only covers host defense mechanisms but also promotes angiogenesis, which may be critical in reestablishing tissue integrity by facilitating wound healing and repair.

**DISCUSSION**

Neovascularization by sprouting angiogenesis plays an important role in inflammation and facilitates tissue remodeling and repair. In this study, we present evidence that human PMN have the ability to directly induce the sprouting of capillary-like structures in vitro. Moreover, we found that the ability of human PMN to promote angiogenesis depended on the factors VEGF and IL-8. The release of VEGF by human PMN was found to be independent of de novo mRNA or protein synthesis, suggesting that VEGF is released from a preexisting intracellular pool—a finding that is in accordance with a previous study (12). In contrast, the release of IL-8 was found to depend on de novo synthesis. Thus the proangiogenic capacity of human PMN consists of at least two components: de novo synthesis of IL-8 and release of VEGF from preformed stores.

The neutralization experiments revealed that VEGF and IL-8 were both required to induce angiogenesis. In vivo studies in the murine system showed that PMN are the main source of macrophage inflammatory protein-2 (MIP-2), the homolog of human IL-8, within the first hours after the onset of inflammation (3). In the present study, IL-8 and VEGF were detected in a range of picograms per milliliter in the supernatant of PMN that were activated for 1 h—a finding that is in accordance with the literature (9, 12). However, these concentrations are rather low to induce angiogenesis (15, 18). This prompted us to study the question of whether PMN may induce an endothelial feedforward mechanism that may amplify the proangiogenic response. In accordance with the literature, we found that VEGF (16) and moreover IL-8 induce the upregulation of endothelial IL-8 mRNA. Accordingly, the supernatant of activated PMN also was found to trigger the upregulation of endothelial IL-8 mRNA, suggesting that the ability of PMN to promote angiogenesis is at least in part due to the fact that PMN activate a paracrine feedforward mechanism involving endothelial IL-8. This was confirmed by the finding that the VEGF-induced upregulation of endothelial IL-8 expression was almost completely abrogated in the presence of a neutralizing anti-IL-8 antibody. The inhibition of VEGF-mediated angiogenesis on neutralization of IL-8 demonstrated the biological significance of this mechanism. The importance of VEGF and MIP-2, the murine analog of human IL-8, in neutrophil-mediated angiogenesis in vivo was recently demonstrated in an animal study using mice (20).

**Fig. 7.** Model for the induction of PMN-mediated angiogenesis. During the acute inflammatory response, human PMN extravasate from the blood into the tissue. Bacteria-derived tripeptides such as fMLP stimulate the extravasated PMN to release IL-8 and VEGF, which activate the endothelial cells and promote angiogenesis by a paracrine feedforward mechanism involving the upregulation of endothelial IL-8.
However, application of recombinant factors at a dose corresponding to the concentration in the PMN supernatant failed to induce sprouting of capillary-like structures in the present study, demonstrating that both factors are required but not sufficient to induce angiogenesis. This strongly suggests that PMN secrete additional factors that are required to obtain the full proangiogenic response. TNF-α may be one of these factors that play a role in PMN-mediated angiogenesis. It is released by activated PMN (9), and it has been shown to induce IL-8, VEGF, and bFGF expression in microvascular endothelial cells (22). Moreover, neutralizing antibodies against IL-8 and VEGF blocked TNF-α-induced neovascularization (22). Another possible candidate is hepatocyte growth factor, which is secreted by PMN on fMLP stimulation and is also known to induce angiogenesis (13). Thus further investigations are required to identify the exact molecular nature of all PMN-derived factors that are released on activation and promote angiogenesis. However, the ability of human PMN to directly induce sprouting of capillary-like structures in vitro suggests a role for this cell type in orchestrating the initiation of inflammation-mediated neovascularization during wound repair and tissue remodeling. Moreover, our findings may show that not one but a variety of different factors is involved in the generation of functional vessels in vivo.

At present, the mechanisms that provide sustained and functional angiogenesis in the adult are only incompletely understood. However, various proangiogenic approaches are in clinical trials (8). To date, it seems to be a rather optimistic view that the in vivo application of one single factor can induce the formation of functional vessels and an intact microcirculation in vivo. Because tumor vessels are highly disorganized and show an abnormal microcirculation (8), it is also questionable whether identification of the factors that induce tumor angiogenesis provides insight into the mechanisms required for the generation of functional microvascular networks in physiological settings. Thus the understanding of the mechanisms underlying angiogenesis in the context of wound healing and tissue repair, an example for the generation of functional vessels and lymphatics in the adult, not only may provide a deeper understanding of this immediate physiological process but also may provide new concepts for therapeutic intervention.

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