Activation of Human Neutrophil by Cytokine-Activated Endothelial Cells

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Abstract—Cytokine activation of vascular endothelial cells renders the hyperadhesiveness for neutrophils. During the processes of inflammation and atherosclerosis, the production of reactive oxygen species by neutrophils contributes to endothelial cell (EC) damage and injury. However, the precise mechanisms for neutrophil activation by ECs remain unknown. Thus, we investigated what kinds of pathophysiological factors synthesized by inflammatory cytokine-activated ECs potentiated the activity of neutrophil functions. The magnitude of \( \text{O}_2^\cdot \) release from neutrophils, which is one of pivotal neutrophil functions, was measured as an indicator potentiated by activated ECs. Neutrophils release massive amounts of \( \text{O}_2^\cdot \) on coculture with activated ECs. Anti–granulocyte-macrophage colony-stimulating factor (GM-CSF) antibody (Ab) or specific platelet-activating factor (PAF)-receptor antagonist suppressed the \( \text{O}_2^\cdot \) release from neutrophils on coculture with the activated ECs by 50% to 70%. The supernatants from activated ECs also induced \( \text{O}_2^\cdot \) release by neutrophils. This stimulatory effect of activated EC supernatants on \( \text{O}_2^\cdot \) release by neutrophils was abolished by anti–GM-CSF Ab or by PAF-receptor antagonist. As we previously reported, we demonstrated the expression of GM-CSF mRNA by Northern blotting and protein synthesis of GM-CSF by ELISA on tumor necrosis factor as well as interleukin-1–activated ECs. Although phosphorylation of mitogen-activated protein kinases was observed in ECs stimulated by tumor necrosis factor and interleukin-1, treatment of ECs with PD98059 (MEK1 inhibitor) and SB203580 (p38 mitogen–activated protein kinase inhibitor) in the presence of the cytokine failed to attenuate the stimulatory effect of activated ECs on neutrophil activation. We found that activated ECs regulated neutrophil function on coculture. We show here for the first time, to our knowledge, that the collaboration between GM-CSF and PAF synthesized by activated ECs markedly potentiated neutrophil activation. (Circ Res. 2001;88:422-429.)

Key Words: endothelial cell ■ neutrophil ■ granulocyte-macrophage colony-stimulating factor ■ platelet-activating factor ■ intercellular adhesion molecule-1

Vascular endothelial cells (ECs) are the gatekeepers of the tissues from the perspective of the bloodstream, and alterations of the endothelium are important determinants in regulating traffic of circulating cells and molecules into various tissues. At the sites of inflammation and atherosclerosis, proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), and other mediators play crucial roles in cell-cell interactions. The cytokine network also plays a role in mediating the host inflammatory response. This response may occur via the increased expression of leukocyte adherence molecules on the surface of ECs as well as through elaboration of chemotactant substances. Activated ECs may acquire the capacity to perform new functions, and this process is the result of quantitative changes in certain gene products. The actions of IL-1 and TNF on ECs that promote leukocyte adhesion and activation are likely to be of importance in the development of inflammatory responses in vitro and in vivo. IL-1 or TNF-activated ECs also generate activators of neutrophils, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, platelet-activating factor (PAF), and IL-8. TNF and IL-1 could induce strong activation of mitogen-activated protein kinase (MAPK) 3 subtypes of human ECs. The MAPKs extracellular signal–regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) seem to be central elements of 3 homologous pathways used by mammalian cells to transduce the messages generated by stressing agents as well as growth factors. We have demonstrated that PD98059, which blocks the phosphorylation of ERK1/2 via inhibition of MEK1/2, and SB203580, which blocks the enzyme activity of p38 MAPK, markedly reduced the magnitude of GM-CSF and TNF-induced \( \text{O}_2^\cdot \) release by neutrophils. Thus, phosphorylation...
ulation of MAPK may be required for neutrophil activation in response to TNF and GM-CSF.

GM-CSF, a member of the hematopoietic growth factor family, is produced and released by monocytes, macrophages, T cells, fibroblasts, vascular smooth muscle cells, and ECs in response to IL-1 \textsuperscript{22} and TNF. \textsuperscript{12} In addition to its growth-promoting effects, GM-CSF stimulates a range of functional activities of mature neutrophils, \textsuperscript{14,15} monocytes, and eosinophils, \textsuperscript{16} including regulation of leukocyte adhesion, augmentation of surface antigen expression, O\textsubscript{2}\textsuperscript{•−} release, and enhancement or induction of cytokine production. \textsuperscript{17} GM-CSF produced by ECs plays an important role in the regulation of blood-vessel function.

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is also a highly potent phospholipid mediator of inflammation and cell-cell interaction. \textsuperscript{18,19} Newly synthesized PAF on the activated EC surface may act at the beginning of an inflammatory cascade as a mediator that amplifies and propagates the reaction. \textsuperscript{20,21}

Thus, leukocyte activation is a key feature of the progression associated with atherosclerosis as well as inflammation. However, little is known about the role of interaction between ECs and neutrophils in the development of inflammation and atherosclerosis. The magnitude of O\textsubscript{2}\textsuperscript{•−} release from neutrophils, as an indicator of the respiratory burst, was considered to be a marker potentiated by ECs. In the present study, we investigated what kinds of mediators synthesized by ECs caused the neutrophil activation.

### Materials and Methods

#### Reagents and Cells

PAF (C16: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), cytochrome c type III, superoxide dismutase, 3,7-dimethyl-1-propargylxanthine (DMPX), and adenosine deaminase (ADA) were obtained from Sigma Chemical. Highly purified recombinant human GM-CSF, TNF-\alpha, and IL-1\beta produced by Escherichia coli were provided by Schering-Plough, Dainippon Pharmaceutical, and Osaka Pharmaceutical, respectively. Polyclonal sheep anti-human GM-CSF antibody (Ab) was provided by Schering-Plough, Dainippon Pharmaceutical, and Otsuka Pharmaceutical, respectively. Polyclonal goat anti-human GM-CSF antibody (Ab) was provided by Schering-Plough. PD98059 and rabbit polyclonal antibodies against Thr\textsuperscript{202}/Tyr\textsuperscript{204}-phosphorylated ERK1/2, Thr\textsuperscript{180}/Tyr\textsuperscript{182}-phosphorylated p38, and Thr\textsuperscript{147}/Tyr\textsuperscript{185}-phosphorylated JNK1/2 were obtained from New England Biolabs. SB203580 was provided by SmithKline Beecham Pharmaceuticals. Specific PAF receptor antagonists, YM264 and WEB2170, were gifts from Yamanouchi Research Institute (Osaka, Japan) and Boeringer Ingelheim (Ingelheim, German), respectively. Human ECs from umbilical cord vein, prepared and characterized as previously described, \textsuperscript{22} were grown on 2.5% gelatin-precoated 60-mm tissue culture dishes (Nunc). The immunofluorescent staining for factor VIII was confirmed. Neutrophils were prepared as described. \textsuperscript{9} The final cell concentration was 6\times10\textsuperscript{5} cells/mL.

#### Activated EC-Neutrophil Interaction and O\textsubscript{2}\textsuperscript{•−} Release Assay

Confluent EC monolayers at passages 2 through 5 were removed from the dishes, and 1.5\times10\textsuperscript{5} ECs were seeded onto gelatin-precoated 24-well plates. TNF and IL-1 were added to the wells at the indicated concentrations, and the plates were incubated for 4 hours at 37°C. The wells were gently washed 3 times with warmed HBSS. When TNF and IL-1–treated EC monolayers were fixed with 1% paraformaldehyde, the fixed ECs were thoroughly washed 3 times. Reaction buffers (0.2 mL) were added to each well. Then neutrophils (6\times10\textsuperscript{5} cells) were placed in contact with EC monolayers (0.4 mL/well). When supernatants from activated ECs or cocultures were required, the buffers (0.2 mL/well) were added alone. If the supernatants, cytokine, or mediator-induced O\textsubscript{2}\textsuperscript{•−} release by neutrophils were measured, neutrophils (3\times10\textsuperscript{6} cells) were put on FCS-precoated 48-well plates. The plates were incubated for 3 hours at 37°C. The wells were aspirated, and the supernatants were centrifuged to remove cells. As previously described, \textsuperscript{9,23} the O\textsubscript{2}\textsuperscript{•−} release was determined as superoxide dismutase–inhibitable reduction of ferricytochrome c. Results from duplicate wells were averaged.

#### Western Blotting

As previously described, \textsuperscript{9} human umbilical vein ECs (HUVECs) were stimulated by cytokines for the indicated time. The blots were incubated with appropriate Ab. The membrane was incubated with anti-rabbit IgG Ab conjugated with horseradish peroxidase, and the Ab complexes were visualized using the ECL detection system as directed by the manufacturer.

#### Statistical Analysis

Results are presented as mean±SEM. Differences between groups were analyzed with unpaired Student’s \textit{t} test. \textit{P}<0.05 was considered significant.

#### Results

##### Cytokine-Activated EC-Neutrophil Interaction

Our results showed that activated ECs potentiate massive amounts of O\textsubscript{2}\textsuperscript{•−} release from neutrophils (Figure 1A). This indicates that activated ECs augmented the activity of neutrophil function. The activity of ECs treated with the combination of IL-1 (8.19±1.17 nmol/6\times10\textsuperscript{5}cells per 3 hours) and TNF (10.82±1.49 nmol/6\times10\textsuperscript{5}cells per 3 hours) was subadditive (13.86±3.64 nmol/6\times10\textsuperscript{5}cells per 3 hours). Our results demonstrated that the amount of O\textsubscript{2}\textsuperscript{•−} release from neutrophils on the coculture was dependent on the concentration of IL-1 or TNF used to activate ECs (Figure 1B). IL-1–treated ECs evoked neutrophil activation at low concentration and rapidly reached the plateau, whereas TNF-treated ECs slowly caused neutrophil activation. The O\textsubscript{2}\textsuperscript{•−} release gradually increased with time (data not shown). In our assay, the amount of O\textsubscript{2}\textsuperscript{•−} release in neutrophils stimulated by TNF-\alpha (100 U/mL=39.2 ng/mL) or GM-CSF (5 ng/mL), which is the optimal concentration, was 5.86±0.35 or 4.19±0.26 nmol/3\times10\textsuperscript{5}cells per 3 hours, respectively. \textsuperscript{9} ECs did not release O\textsubscript{2}\textsuperscript{•−} in response to these cytokines.

##### Supernatants From Activated ECs or Coculture Induced O\textsubscript{2}\textsuperscript{•−} Release by Neutrophils

The supernatants from activated ECs alone could induce O\textsubscript{2}\textsuperscript{•−} release by neutrophils (IL-1, 2.47±0.35; TNF, 3.21±0.43; and IL-1+TNF, 4.77±0.92 nmol/3\times10\textsuperscript{5}cells per 3 hours) as potent as the supernatants from coculture (IL-1, 2.25±0.31; TNF, 3.42±0.54; and IL-1+TNF, 4.92±0.98 nmol/3\times10\textsuperscript{5}cells per 3 hours) (Figure 2). The supernatants on coculture with neutrophils did not synergistically augment the
Phosphorylation of MAPK 3 Subtypes in IL-1–Activated or TNF-Activated ECs

The results presented in Figure 3 show that all 3 MAPKs (ERK1/2, p38, and JNK1/2) in ECs were phosphorylated in response to IL-1 (1.25 ng/mL) or TNF (39.22 ng/mL) for 4 hours, washed, and then cocultured with neutrophils (6×10⁵ cells) for 3 hours. Human neutrophils spontaneously released O₂⁻ in the presence of IL-1β-activated or TNF-α-activated ECs (A). CTL (control) indicates untreated ECs. Furthermore, the amount of O₂⁻ release from neutrophils on the cocultures with ECs pretreated with the indicated dose of IL-1β or TNF-α at 4 hours was examined (B). Results represent ≥5 separate experiments, each done in duplicate. *P<0.05 and **P<0.01 compared with CTL.

Neutrophils Adherent to Cytokine-Activated ECs

After 5 minutes of coculture, nonadherent neutrophils were removed. Neutrophils bound to ECs were washed 3 times with warmed HBSS and fixed with 1% paraformaldehyde. The number of neutrophils adherent to ECs was counted using a phase-contrast microscope (Nikon Diaphot). The number of neutrophils was markedly increased in adherence to activated ECs (IL-1, 34.3±5.8; TNF, 52.2±8.6; and IL-1+TNF, 82.6±10.4 cells/field), as shown in Figure 4A. Furthermore, we analyzed ICAM-1 expression on the surface of IL-1–activated or TNF-activated ECs by FACS, and ICAM-1 expression on IL-1–activated or TNF-activated ECs was upregulated in a dose- and time-dependent manner in agreement with previous studies (Figures 4B and 4C).²⁴

We next investigated the involvement of ICAM-1–mediated signaling events during neutrophil adherence in the increase of O₂⁻ release from neutrophils. ECs were fixed to prevent the formation of de novo–generated EC mediators in accordance with previous studies.¹⁶,²⁵,²⁶ In agreement with

Figure 1. Cytokine-activated ECs induce O₂⁻ release from neutrophils. ECs (1.5×10⁵ cells) were untreated or pretreated with IL-1β (1.25 ng/mL) or TNF-α (39.22 ng/mL) for 4 hours, washed, and then cocultured with neutrophils (6×10⁵ cells) for 3 hours. Human neutrophils spontaneously released O₂⁻ in the presence of IL-1β-activated or TNF-α-activated ECs (A). CTL (control) indicates untreated ECs. Furthermore, the amount of O₂⁻ release from neutrophils on the cocultures with ECs pretreated with the indicated dose of IL-1β or TNF-α at 4 hours was examined (B). Results represent ≥5 separate experiments, each done in duplicate. *P<0.05 and **P<0.01 compared with CTL.

Figure 2. Effect of supernatants from activated ECs and coculture between activated ECs and neutrophils on neutrophil activation. ECs were untreated or pretreated with IL-1β (1.25 ng/mL) or TNF-α (39.22 ng/mL) for 4 hours, washed, and then incubated with or without neutrophils (6×10⁵ cells) for 3 hours at 37°C. The conditioned medium was aspirated, and the supernatants were centrifuged to remove cells. After 3 hours of incubation with supernatants (50%), the O₂⁻ release by neutrophils (5×10⁵ cells) was measured as described in Materials and Methods. Data are representative of 5 independent experiments, each done in duplicate. *P<0.01 compared with CTL.

Figure 3. Activation of MAPK 3 subtypes in response to TNF and IL-1. HUVECs were exposed for 10 minutes to TNF (39.22 ng/mL) or IL-1 (1.25 ng/mL). Phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 was analyzed by immunoblotting using Ab against phosphorylated form of each protein (A through C). The cell lysates equivalent to 2×10⁶ cells were loaded onto each lane. Immunoblots of total ERK1/2, p38 MAPK, and JNK1/2 were performed to confirm equal protein loading. Data are representative of 3 separate experiments. IB indicates immunoblotting.
previous studies, the ability of viable neutrophils to undergo adhesion in response to cytokines was not impaired in the presence of fixed ECs (data not shown). Cytokine-treated fixed ECs had higher levels of adhesion molecules, including ICAM-1, on their surfaces than control-fixed ECs (Figures 4B and 4C). The magnitude of O$_2^-$ release from neutrophils on incubation with the cytokine-treated fixed ECs in the presence of activated EC supernatants was significantly greater than that on incubation with nontreated fixed ECs (Figure 5A). Cytokine-treated fixed ECs also augmented the recombinant human (rh) GM-CSF (50 or 500 pg/mL)–induced O$_2^-$ release by neutrophils but not other doses of rh GM-CSF (5 pg/mL and 5 ng/mL) (Figure 5B). However, this enhancement of adhesion to activated ECs in O$_2^-$ release by neutrophils was small. On the other hand, eosinophil adhesion to VCAM-1 markedly enhanced O$_2^-$ release. 16,26 The amount of O$_2^-$ release from neutrophils in contact with TNF-activated fixed ECs gradually increased in a manner dependent on the dose and time used to activate ECs (data not shown).

The results shown in Figures 5A and 5B indicate that the amount of O$_2^-$ release from neutrophils induced by activated-EC supernatants was identical with that induced by nonglycosylated GM-CSF (50 pg/mL) from E. coli. Because the stimulatory effect of rh GM-CSF from E. coli was greater than that of GM-CSF from mammalian cells on a molar basis,14 nonglycosylated GM-CSF (50 pg/mL) from E. coli is equivalent to glycosylated GM-CSF (500 pg/mL) from HUVECs. Taken together, these results suggest that cytokine-activated ECs might release an equivalent of $\approx$500 pg/mL GM-CSF present in supernatants.

Effects of Various Inhibitors on EC-Neutrophil Interaction and Supernatant-Induced O$_2^-$ Release Assays
Neutralizing Ab against GM-CSF (55 µg/mL) and a specific PAF receptor antagonist (YM264 2.5×10$^{-5}$ mol/L) inhibited the amount of O$_2^-$ release from neutrophils on coculture with activated ECs by 60% to 70% and, to a lesser degree, by 50% to 60%, respectively (Figure 6A). The inhibitory effects of PAF receptor antagonists (YM264 and WEB2170) on the O$_2^-$ release were dependent on the concentration, and YM264 was more potent than WEB2170, in agreement with a previous study (data not shown). 27 Anti–GM-CSF Ab and YM264 almost completely abolished the O$_2^-$ release from neutrophils induced by activated EC supernatants (Figure 6B). It is suggested that supernatants from cytokine-activated ECs contain soluble factors that are predominantly GM-CSF and PAF. Neither YM264 nor WEB2170 had any effect on control or TNF-primed N-formyl-methionyl-leucyl-phenylalanine–induced O$_2^-$ release by neutrophils.

Because IL-1 and TNF activated MAPK, as shown in Figure 3, we investigated the involvement of MAPK cascade in ECs in neutrophil activation. We have demonstrated that PD98059 and SB203580 reduced the magnitude of GM-CSF–induced and TNF-induced O$_2^-$ release by neutrophils. 9 When PD98059 and SB203580 were added to EC monolayers 15 minutes before the treatment with IL-1 or TNF, treated for 4 hours, and then washed, the amounts of O$_2^-$ release on the coculture between PD98059- and SB203580-treated activated ECs and neutrophils for 3 hours were not altered in comparison with the coculture between activated ECs and neutrophils (data not shown). Although both PD98059 and SB203580 suppressed the O$_2^-$ release by neutrophils on the
coculture, in agreement with our previous study (Figure 6A), the pretreatment of activated ECs with both PD98059 and SB203580 had no significant effect on the activity of neutrophil function. The results indicate that MAPK cascade might have an association with neutrophil activation but not with EC activation in response to inflammatory cytokines.

It is well established that ECs exposed to hypoxic conditions have an altered phenotype, as demonstrated by increase in adenosine production. Adenosine inhibits priming of neutrophils and the respiratory burst of cytokine-triggered adherent neutrophils via A2 receptor. To test whether adenosine derived from ECs attenuates neutrophil activation, we examined the effect of 2 adenosine inhibitors, DMPX, which is an A2-specific receptor antagonist, and ADA, which can deaminate adenosine to inosine, on the O2 release from neutrophils on coculture. As shown in Figures 6A and 6B, the inhibitory effects of adenosine were partially but not significantly blocked by both DMPX (2.5 \times 10^{-5} \text{ mol/L}) and ADA (1.25 \text{ ng/mL}). These data indicate that adenosine did not mediate the activity of neutrophil function by cytokine-treated ECs compared with ischemia/reperfusion-treated ECs.

Blocking monoclonal Ab against ICAM-1 (84H10, Immunotech) did not significantly inhibit the O2 release from neutrophils on the coculture (data not shown).

**GM-CSF Production of Activated ECs**

HUVECs spontaneously released low levels of GM-CSF (16.9 \pm 2.7 \text{ pg/mL}) under control conditions. The concentrations of GM-CSF produced by IL-1\(\beta\) (1.25 \text{ ng/mL}) and TNF-\(\alpha\) (39.2 \text{ ng/mL})-activated ECs were 317.6 \pm 22.8 and 421.5 \pm 39.4 \text{ pg/mL}, respectively. When ECs were treated with the combination of IL-1 and TNF, the additive response...
in the production of GM-CSF from activated ECs was observed (758.3±81.4 pg/mL). The levels of GM-CSF production by activated ECs in the presence of SB203580 (1×10⁻⁶ mol/L) were higher than those in the absence of SB203580; however, there were no significant differences of GM-CSF production by IL-1–activated and TNF-activated ECs in the presence or absence of SB203580 or PD98059. Similar to ICAM-1 expression on the surface of ECs, the levels of GM-CSF production by activated ECs were dependent on cytokine concentration used to activate ECs (data not shown). The coculture between IL-1β or TNF-α–activated ECs and neutrophils had no significant increase in GM-CSF production (data not shown).

**Effect of PAF on GM-CSF–Induced O₂⁻ Release From Neutrophils**

When varying concentrations of exogenous PAF were added in the presence of rh GM-CSF (50 pg/mL), PAF affected nonglycosylated rh GM-CSF (50 pg/mL)–induced O₂⁻ release by neutrophils, which is equivalent to the concentration of glycosylated GM-CSF from activated ECs. The maximum enhancement of rh GM-CSF (50 pg/mL)–induced O₂⁻ release was elicited by 20 to 50 nmol/L PAF, which was the physiological concentration of PAF synthesized by activated ECs (Figure 8A).30 Interestingly, the enhancement of GM-CSF–induced O₂⁻ release by exogenous PAF (20 nmol/L) was not obtained at higher level of rh GM-CSF (500 to 5000 pg/mL) compared with the physiological level of GM-CSF produced by activated ECs (Figure 8B).

**Discussion**

IL-1 and TNF have multiple actions on ECs that seem to promote both inflammatory and coagulation responses. Although many TNF activities are indistinguishable from those of IL-1, TNF and IL-1 activities are often additive and always seem to be independent (Figures 1A, 2, 3A, and 7).1 The cytokines may promote injury and angiogenesis through indirect actions dependent on leukocytes. Through its effect on major histocompatibility complex (MHC) antigen expression, TNF may promote lymphocyte-dependent inflammation (an action not shared by IL-1). Inflammatory mediators are not always secreted; in some cases, they may be expressed on the cell surface of the stimulated cells. However, whether secreted or surface-expressed, the mediators share a common mode of action: they bind to specific protein receptors on the surface of their target cell.

TNF and IL-1 could evoke strong activation of MAPK 3 subtypes in HUVECs. ERK is involved in the regulation of transcriptional and translational activities and is essential for control of cell growth and differentiation. p38 MAPK and JNK/SAPK are recognized as stress-sensitive kinases. Our studies indicated a parallel pathway in ECs leading to activation of the p38 MAPK and JNK1/2 in a fashion similar to ERK1/2 activation in response to TNF and IL-1 (Figure 3).7,8 Two MAPK inhibitors, both PD98059 and SB203580, had no significant influence on GM-CSF production of the activated ECs (Figure 7), whereas PD98059 and SB203580 markedly inhibited the O₂⁻ release from neutrophils.9 Al-
though both EC and neutrophil activation have common cascade via ERK and p38 MAPK in response to inflammatory cytokines, the degree of contribution to EC activation via MAPK signaling might be distinct from that to neutrophil activation, or JNK/SAPK might have an important pathway in EC activation in response to TNF and IL-1.

ECs produce PAF in response to TNF and IL-1, whereas thrombin, H2O2, histamine, leukotrienes, and phorbol 12-myristate 13-acetate also trigger PAF production. Endothelial PAF remains mainly cell-associated and can be recognized by neutrophils that come into contact with the ECs. EC-associated PAF is known to participate in regulating neutrophil attachment to activated ECs. PAF cooperates with P-selectin and E-selectin in mediating increased neutrophil-EC adherence by triggering an increase in affinity of CD11b/18 (Mac-1). ECs express ICAM-1 (CD54), a counter-ligand for CD11/18 integrin that promotes adhesion and transendothelial migration of neutrophils. IL-8, also a potent priming agent for O2− release and phospholipase A2 activation, is known to be produced by TNF or IL-1–treated ECs; however, Hill et al. have shown that neutralizing Ab against IL-8 was unable to prevent priming of neutrophil function.

In our previous study, we demonstrated that GM-CSF mRNA and protein were synergistically expressed on coculture between activated ECs and monocytes, and TNF or IL-1–activated ECs synthesized GM-CSF with maximal production at 4 hours. GM-CSF stimulates a range of functional activities of neutrophils, including regulation of adhesion, augmentation of surface antigen expression, O2− release, and enhancement or induction of cytokine production. Thus, it is possible that GM-CSF may contribute to the pathophysiological events involved in atherosclerosis and inflammation. The ability of neutrophils to release large amounts of O2− in response to GM-CSF is presumably controlled by the known receptors for the cytokine. GM-CSF also stimulated not only O2− release but also PAF synthesis from neutrophils with time and dose relationships.

Neutrophils are the first inflammatory cells to appear at the site of vessel damage, exacerbating and propagating the inflammatory response. It has also been reported that neutrophils generate reactive oxygen species, which may cause lipid oxidation and lead to serious derangements of cell metabolism, including DNA destruction and modification of proteins, thereby contributing to the pathogenesis of atherosclerosis. Neutrophils also possess a variety of granule proteases suggested to contribute to inflammatory tissue injury. The increased adhesion and migration of neutrophils through the endothelium are important events in tissue injury.

The response of O2− release by neutrophils is suppressed by EC-derived adenosine, which possesses anti-inflammatory potential and is most likely mediated through the A3 receptor. Therefore, ECs regulated neutrophil activation in the process of inflammation. However, it is unlikely that adenosine from cytokine-activated ECs is associated with neutrophil activation potentiated by activated ECs.

We demonstrated that collaboration between GM-CSF and PAF at physiological concentration is essential to enhance neutrophil activation. The results from both a little enhancement of the O2− release by neutrophil adherence to activated ECs and no significant reduction of the O2− on coculture in the presence of anti-ICAM-1 Ab were obtained. Taken together, soluble factors produced by activated ECs, rather than Mac-1–ICAM-1–mediated signaling events during neutrophil adherence, induced neutrophil activation. The selective synthesis of PAF and GM-CSF by activated ECs may play a role in regulating and amplifying the priming and activation of neutrophils on the EC-neutrophil interaction. ICAM-1–β2 integrin–dependent signaling pathways in neutrophil activation had a minor effect on neutrophil O2− release on the coculture in our assay. Thus, we speculated that adhesion of neutrophils to activated ECs is unlikely to be the main mode of cytokine-activated EC potentiation of neutrophil.

Moreover, we found that the most enhanced effects of both exogenous PAF (20 nmol/L) and adhesion to cytokine-treated fixed ECs on O2− release by neutrophils were evoked by 50 pg/mL of nonglycosylated GM-CSF (Figures 5B and 8B). This concentration of GM-CSF was identical to that produced by activated ECs. On the basis of these findings, we speculate that, first, neutrophil recruitment via mediators and cytokines produced by activated ECs, such as PAF and IL-8, is an initial step; second, activated EC-associated PAF primes neutrophils adherent to ECs via the Mac-1–ICAM-1 pathway; and, third, potentially more GM-CSF synthesized by activated ECs augmented neutrophil activation during the transendothelial migration.

In conclusion, neutrophils primed by activated EC-associated PAF may be strongly potentiated by activated EC-synthesized GM-CSF during the transmigration in vivo. GM-CSF and PAF might be major contributors to neutrophil activation, whereas the Mac-1–ICAM-1 pathway might be a minor contributor. We have demonstrated for the first time, to our knowledge, that in collaboration with activated EC-derived PAF, GM-CSF synthesized by activated EC induced large amounts of O2− release from neutrophils and, furthermore, that cooperation of both GM-CSF and PAF at physiological concentrations was required for augmented neutrophil activation.

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