Oxygen Radicals Induce Human Endothelial Cells to Express GMP-140 and Bind Neutrophils

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Abstract. The initial step in extravasation of neutrophils (polymorphonuclear leukocytes [PMNs]) to the extravascular space is adherence to the endothelium. We examined the effect of oxidants on this process by treating human endothelial cells with H2O2, t-butylhydroperoxide, or menadione. This resulted in a surface adhesive for PMN between 1 and 4 h after exposure. The oxidants needed to be present only for a brief period at the initiation of the assay. Adhesion was an endothelial cell-dependent process that did not require an active response from the PMN. The adhesive molecule was not platelet-activating factor, which mediates PMN adherence when endothelial cells are briefly exposed to higher concentrations of H2O2 (Lewis, M. S., R. E. Whatley, P. Cain, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1988. J. Clin. Invest. 82:2045-2055), nor was it ELAM-1, an adhesive glycoprotein induced by cytokines. Oxidant-induced adhesion did not require protein synthesis, was inhibited by antioxidants, and, when peroxides were the oxidants, was inhibited by intracellular iron chelators.

Granule membrane protein-140 (GMP-140) is a membrane-associated glycoprotein that can be translocated from its intracellular storage pool to the surface of endothelial cells where it acts as a ligand for PMN adhesion (Geng, J.-G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. Nature (Lond). 343:757-760). We found that endothelial cells exposed to oxidants expressed GMP-140 on their surface, and that an mAb against GMP-140 or solubilized GMP-140 completely blocked PMN adherence to oxidant-treated endothelial cells. Thus, exposure of endothelial cells to oxygen radicals induces the prolonged expression of GMP-140 on the cell surface, which results in enhanced PMN adherence.

Leukocytes circulate in the blood, yet their function, mediation of inflammatory reactions and attenuation of infection, is manifested in the extravascular space. This results from the directed migration of polymorphonuclear leukocytes (PMNs) and monocytes into infected or perturbed areas by an exquisitely regulated communication between the circulating leukocytes and the endothelial cells that form the interface between blood and the extravascular space. Activated endothelial cells express several molecules that induce unactivated PMN to bind to them. Stimulation of endothelial cells for several hours with tumor necrosis factor-α (TNF-α), interleukin-1, or endotoxin (3) causes endothelial cells to bind PMN. This process is dependent on new protein synthesis, and, depending on the time of stimulation, is mediated by the surface expression of endothelial leukocyte adhesion molecule-1 (ELAM-1) (3), a member of the recently identified selectin (7,20) family. There are also mechanisms that act more rapidly to induce endothelial cell-dependent PMN adhesion. One is unique in that the mediator, platelet-activating factor (PAF), is a phospholipid. Endothelial cells do not normally contain PAF, but synthesize appreciable amounts of it within minutes of appropriate stimulation (23, 24, 40). While all of this PAF remains as- sociated with the endothelial cell (30), a portion of it is expressed on the endothelial cell surface where it is recognized by the PMN receptor for PAF (42). This endothelial cell-dependent adhesion process requires activation of the PMN by endothelial cell-associated PAF and the functional upregulation of the PMN CD11/CD18 adhesive glycoprotein2 (42).
The third known mechanism of endothelial cell–dependent PMN adhesion is demonstrated by endothelial cells stimulated with phorbol esters (7), or rapidly acting agonists like thrombin and histamine through the expression of a second member of the selectin family of adhesive receptors for leukocytes. These rapidly acting agonists transiently induce translocation of the adhesive glycoprotein GMP-140 (20) (also known as PADMGE protein; 5, 3) from specialized intracellular granules, the Weibel-Palade bodies, to the surface of the stimulated cells (5, 9, 21). Both PAF and GMP-140 are expressed by endothelial cells stimulated with rapidly acting agonists, where they act in concert to mediate maximal PMN adhesion.²

PMN adhesion and recruitment appear to play a critical role in the oxidant-induced tissue injury that accompanies a variety of pathologic processes (15, 19, 32). In many instances, PMNs are attracted to the affected area by the initial insult and then exacerbate the tissue damage by releasing a variety of injurious agents, including H₂O₂. The mechanisms by which PMNs infiltrate areas subjected to oxidant damage are not completely defined. However, this process is a major pathologic one as about half of the damage observed upon reperfusion of ischemic tissue is prevented by either removing circulating PMN or inhibiting their function, by treatment with a variety of antioxidants, or by chelation of transition metals such as ionic iron (8). We observed (14) that H₂O₂ itself is an agonist for PAF synthesis by endothelial cells, and that it also induced PMN adhesion to these cells. These responses occurred within minutes of exposure to H₂O₂, providing that the concentration was at least 1 mM, and PMN adhesion was completely dependent on the PAF-mediated mechanism.

Recently Nathan (27, 28) observed that activated leukocytes, if they have spread onto a favorable surface, generate large amounts of oxygen radicals for prolonged periods of time. This raised the possibility that endothelial cells may be exposed to relatively high concentrations of oxidants for several hours. Indeed, activation of 10⁵ PMN in the presence of endothelial cells generated H₂O₂ equivalent to 0.3 mM (34), while other studies (reviewed in 14) have shown that endothelial cells are exposed to oxidants for prolonged periods in hyperoxic pulmonary injury and other pathologic states. Furthermore, PMN accumulate at the intimal surface in these conditions (1, 6). We report that endothelial cells exposed for 1–4 h to submillimolar concentrations of H₂O₂, or other oxidants, bound significant numbers of PMN. Prolonged surface expression of GMP-140 was solely responsible for this process, and therefore was unlike PMN adhesion in response to receptor-mediated agonists where GMP-140 expression is only transient, and is only partially responsible for PMN adhesion. This is a new mechanism for PMN adhesion to endothelium in which a pathologic agonist induces unregulated expression of a proadhesive molecule.

Materials and Methods

Materials

HBSS and M199 were from Whittaker M. A. Bioproducts (Walkersville, MD), and human serum albumin (25%) was from Miles Laboratories, Inc. (Elkhart, IN). Purified human thrombin was the kind gift of Dr. John Fen- ton (Albany, NY), and recombinant human TNF-α was provided by Genen- tech, Inc. (San Francisco, CA). GMP-140 was solubilized and purified as described (7). PAF was from Avanti Polar Lipids, Inc. (Birmingham, AL). [³H]Acetate was from New England Nuclear (Boston, MA), and [¹¹¹In]oxine was purchased from the Radiopharmacy Service at the University of Utah. L659,989 was the kind gift of John Chabala of Merck, Sharp and Dohme Research (Rahway, NJ) and WEB 2086 was kindly provided by Peggy Gunning of Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). mAbs H18/7 and 60.3 were generously donated by Michael Bevi- acqua (Boston, MA), and Patricl Beatty and John Harlan (Seattle, WA), respectively. 2-7- Bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescin, acetoxymethyl ester, and propidium iodide were from Molecular Probes (Eugene, OR). Ascorbate was from Eastman Kodak Co. (Rochester, NY). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation and Culture

Human umbilical vein endothelial cells were cultured in 24-mm multiwell plates (Costar Data Packaging Corp., Cambridge, MA) as described (40). Only monolayers of primary cultures that were tightly confluent were used for these studies. Human intestinal smooth muscle cells (CRL 1692) and HL60 cells were obtained from the American Type Culture Collection. HL-60 cells were cultured as described (42). PMN were isolated from fresh human blood and labeled with [¹¹¹In]oxine as described (40).

Cell Adhesion and Labeling Techniques

Endothelial cells were treated with HBSS containing 0.5% human serum albumin (HBSS/A) and various agonists for the stated period of time, washed twice with HBSS and the adherence of [¹¹¹In]-PMN or [¹¹¹In]-HL-60 cells to monolayers of endothelial cells was performed as described (40, 42). Synthesis of PAF was determined by following the incorporation of [³H]acetate into a phospholipid that comigrated with PAF on thin-layer chromatography plates as described (23). This product previously has been shown to be authentic PAF (30). The effects of PAF receptor antagonists were examined following a 5-min preexposure of PMN to the antagonists as described (42). Endothelial cells were labeled with 2-7-bis-(2- carboxyethyl)-5-(and-6) carboxyfluorescin, acetoxymethyl ester (CFPM) by incubating the monolayers with 2.8 μM CFPM for 5 min at 37°. The monolayers were then washed with HBSS and incubated in this buffer for an additional 2 min to allow hydrolysis of the ester bond. The monolayers were then washed twice with HBSS before agonist addition. Propidium iodide and trypsin blue exclusion were determined by exposing control or oxidant-treated monolayers to 30 μM propidium iodide or 0.4% trypsin blue for 5 min. The monolayers were washed twice with HBSS and then visualized by fluorescence microscopy using a Nikon DM580/61-A filter cube when propidium iodide was the dye. Endothelial cell monolayers were fixed with glutaraldehyde by exposing washed monolayers to 2.5% glutaralde- hyde for 30 min. Microwave fixation (2) of monolayers was performed for 1 min in a Litton microwave set at maximum irradiation. PMNs were fixed by collecting freshly isolated PMN (5.5 x 10⁶/ml in HBSS/A) by centrifugation. They were then resuspended at the original concentration in a minimum of two times with equivalent results.

Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).
period of time before this medium was removed and the monolayer fixed with 1% formaldehyde (9). The fixed monolayers were washed thrice with HBSS, and then the monolayer was incubated with 1/1,000 dilutions (into PBS containing 1% BSA) of supernatants of endothelial cells treated with HBSS/A with or without 250 μM t-BuOOH for the stated time, or with various concentrations of human vWF (the kind gift of Gerald Reth, University of Washington, Seattle, WA). After 90 min at 37°, the wells were washed six times with PBS/T, and then incubated for 10 h in 1N NaOH, before the reaction was stopped with 8N H2SO4. Bound antibody was quantitated by ELISA by coating microtiter dishes with 10 μg/ml rabbit antihuman vWF (Behring Diagnostics, La Jolla, CA) at 4° overnight, followed by a 1/1,000 dilution of goat antihuman IgG (Atlantic Antibodies, Scarborough, ME). These wells were then washed six times with PBS/T, incubated for 90 min at 37° with a 1/10,000 dilution of rabbit anti-goat IgG conjugated to HRP (type VI, Sigma Chemical Co.). The wells were washed six times with PBS/T and incubated for 20 min at 37° with o-phenylenediamine (0.1 ml at 0.4 mg/ml) before the reaction was stopped with 8N H2SO4. Bound antibody was quantitated by determining the optical density at 492 nm.

Results

Peroxides Induce Human Endothelial Cells to Bind PMN

We previously observed (14) that exposure of endothelial cells to millimolar concentrations of H2O2 rapidly stimulates the synthesis of PAF, and induces the adherence of PMN. Adhesion of PMN under these circumstances is mediated by PAF synthesized and expressed by the endothelial cells (40). The data in Fig. 1 showed that much lower concentrations of H2O2 also induced the adherence of PMN to monolayers of endothelial cells if the incubation was prolonged. The optimal concentration of H2O2 lay between 150 and 350 μM when the incubation time was 2 h (Fig. 1a). Alteration of the endothelial cell surface in response to H2O2 developed rapidly with maximal PMN adherence occurring after 1–3 h of continuous incubation with H2O2 (Fig. 1b).

We next investigated the effect of a lipid-soluble peroxide, t-BuOOH, on human endothelial cells. We found that it too stimulated these cells to bind PMN with a concentration relationship like that of H2O2 (Fig. 1a). The magnitude of the endothelial cell–dependent adhesion in response to this peroxide was equivalent to that induced by TNF-α, which, in general, was greater than that in response to agents such as thrombin (41). The time relationship of PMN adhesion also was similar to that of H2O2 (Fig. 1b), except that, although not shown in this experiment, there was no adherence to endothelial cells treated with t-BuOOH for 30 min or less. The generation of an adhesive surface on the endothelial cells in response to t-BuOOH was considerably faster than that induced by TNF-α (Fig. 1), but was much slower than that induced by thrombin (40), histamine4 or high concentrations of H2O2 (14).

The endothelial cell monolayer did not need to be exposed to H2O2 or t-BuOOH continuously in order to induce PMN adhesion. When endothelial cells were pulsed for 15 or 30 min with t-BuOOH and then incubated in the absence of exogenous peroxide for 105 or 90 min, for a total of 2 h of incubation, the amount of adherence was 36 and 116%, respectively, of that in response to a continuous 2-h exposure to t-BuOOH (Fig. 2). The same protocol using H2O2 as the oxidant showed that it, too, did not have to be present.
The nature of the protocol that we employed, where the PMNs were not exposed to peroxide, suggested that oxidant-induced PMN adhesion described in Figs. 1 and 2 was an endothelial cell–dependent process. To confirm this and to determine if an active PMN response was required for adhesion, we used several strategies. PMN-dependent adhesion requires that the adhesive glycoprotein complex CD11/CD18 be activated (39). We found that mAb 60.3, which blocks the function of this class of integrins (39), failed to prevent the PMN adherence described in Figs. 1 and 2. We also found that there was little diminution of adherence in response to oxidant treatment when the PMN were first fixed by treatment with formaldehyde (Fig. 3). That this effectively prevented PMN-dependent processes was shown by the lack of adherence in response to direct PMN agonists, 10⁻⁷ M phorbol myristate acetate or 10 μM A23187 (Fig. 3 legend). In addition, we found that direct treatment of PMN with peroxides under the conditions of these experiments did not induce them to become adhesive (not shown). Therefore, PMN adherence to oxidant-treated endothelial cells was an endothelial cell–dependent process that required no active response from the adherent PMN.

We asked whether the generation of an adhesive surface after treatment with the peroxides was a response specific to endothelial cells. We treated cultured human smooth muscle cells with either 250 μM H₂O₂ or t-BuOOH for 2 and 4 h. These cells normally do not bind, nor can they be induced to bind, PMN. This treatment did not alter the number of PMNs that adhered to these cells (5 vs 3% adherence to un-treated smooth muscles cells: the same experiment adherence to endothelial cells was 40%). We conclude that the generation of an adhesive surface was not a general response of cells to oxidant stress.

Induction of PMN binding required that the oxidant-treated endothelial cells be viable. We fixed the endothelial cell monolayer with glutaraldehyde or by microwave irradiation (2), and then exposed them to either H₂O₂ or t-BuOOH. There was no increased adhesion of PMN to endothelial cells that had been fixed before oxidant exposure (not shown). In contrast, endothelial cells that were first treated with TNF-α, H₂O₂ or t-BuOOH and then fixed by either method did demonstrate increased PMN adhesion (not shown).

We used phase-contrast microscopy to examine endothelial cells that had been treated with H₂O₂ or t-BuOOH, and the number of PMN that adhered to these cells was determined as described under Fig. 1. The PMNs were either freshly isolated, or had been fixed with formaldehyde (2). We also used several strategies to confirm that the adhesion of PMN to oxidant-treated endothelial cells was not a general response. PMN adherence to oxidant-treated endothelial cells was an endothelial cell–dependent process that required no active response from the adherent PMN.
found that the morphology of these monolayers differed from control monolayers and from each other (Fig. 4). A typical H$_2$O$_2$-treated monolayer appeared pyknotic and desiccated. Additionally, the cells often had retracted so that the monolayer developed large gaps. This, however, did not account for the increased PMN adherence as visual inspection showed that the majority of the PMN was adherent to endothelial cells, and not the exposed substrate. Furthermore, endothelial cells exposed to 2.5 µg/ml cytochalasin B displayed extreme cellular retraction, but no increase in PMN adherence (not shown). In contrast to the appearance of H$_2$O$_2$-treated monolayers, the nuclei of t-BuOOH-treated endothelial cells were distorted and swollen (Fig. 4). Additionally, within several hours the cells developed blebs that subsequently pinched off from the cell surface. The marked differences in the appearance of monolayers treated with H$_2$O$_2$ or t-BuOOH suggested that the mechanisms of action of the two peroxides also might be different.
The effect that oxidant exposure had on the viability of the endothelial cell monolayer was assessed by examining the retention of carboxyfluorescein, a negatively charged, water-soluble fluorescent dye, that had been introduced into the cells before their exposure to peroxide. Fig. 4 shows that endothelial cells were highly fluorescent after labeling, and that they retained the carboxyfluorescein dye after 2 h of exposure to either H₂O₂ or t-BuOOH. We also found (Fig. 4) that the cells excluded propidium iodide, which stains the nucleus of dead cells, and trypan blue (not shown) after 2 h of treatment with the oxidants. Half of the cells in monolayers exposed to H₂O₂ for 4 h still retained carboxyfluorescein and excluded propidium iodide, but few of the cells treated with t-BuOOH maintained a functional permeability barrier. Lysis of the monolayer by a brief exposure to digitonin or Triton X-100 immediately decreased cellular fluorescence to background levels, showing that the dye was not retained by the oxidant-treated cells by interaction with cellular components. Viability, as measured by dye retention, correlated with the ability of monolayers to recover their normal morphology after exposure to the peroxides: monolayers that were treated for 4 h with H₂O₂, washed, and returned to growth medium for 16 h appeared healthy, while those exposed to t-BuOOH for the same period did not recover their usual morphology. These results show that expression of the pro-adhesive surface was a function of viable cells, but that lethally injured cells still remained adhesive for PMN.

**Generation of the Endothelial Cell Pro-adhesive Surface Is a Response to Oxygen Radicals**

The appearance of the adhesive endothelial cell surface was the result of oxidative reactions carried out in or near the endothelial cells. First, PMN adherence was dependent on the peroxide function of t-BuOOH, as treatment of the endothelial cell monolayers with equivalent concentrations of t-butanol did not promote PMN adherence (not shown). Second, PMN adherence was also induced by intracellularly generated oxygen radicals. Endothelial cell monolayers treated with menadione, a quinone that undergoes reductive cycling with the production of O₂⁻ and H₂O₂ (29, 31), also became adhesive for PMN (Fig. 3). Adherence in response to menadione usually was less than that induced by maximally effective concentrations of t-BuOOH (n = 8).

The third line of evidence that the peroxide induction of endothelial cell–dependent PMN adhesion required intracellular metabolism was that this process required intracellular iron. Transition metals, such as iron, have peroxidase activity and catalyze radical formation during peroxide breakdown (25). We found that adherence in response to either H₂O₂ or t-BuOOH was inhibited by coincubation with the Fe⁺³ chelator desferal (Table I). The essential Fe⁺³ was localized within the endothelial cells because conalbumin, a chicken transferrin not transported by human cells, was unable to inhibit the adhesion induced by either H₂O₂ or t-BuOOH (not shown). In contrast to the results obtained with t-BuOOH or H₂O₂, PMN adhesion induced by endothelial cell metabolism of menadione was not blocked by desferal (28 ± 2% adherence with 20 mM desferal, 26 ± 2% without desferal; unstimulated adherence was 5 ± 1%).

The impression that there were differences in the mechanism of action of these oxidants was strengthened when the effect of the hydrophobic transition metal chelator, α,α'-dipyridyl, on endothelial cell–dependent adherence was examined. α,α'-Dipyridyl blocked almost all of the adhesion due to t-BuOOH, while it inhibited only 44% of the adhesion induced by H₂O₂. These chelators did not affect TNF-α–induced adherence. These results suggest that the exogenous peroxides had to be metabolized to more potent oxidizing radicals in an intracellular, iron-dependent reaction, while direct intracellular generation of oxygen free radicals did not require free iron to exert their effects.

Finally, we used free radical scavengers to directly test whether induction of the pro-adhesive state by peroxides was due to radical formation. Butylated hydroxytoluene, a lipid-soluble antioxidant, effectively inhibited adherence induced by either H₂O₂ or t-BuOOH (Table I). Nordihydroguaiaretic acid, an inhibitor of lipoxygenase-catalyzed reactions and a nonspecific inhibitor of radical reactions, also inhibited PMN adhesion, but not as effectively as butylated hydroxytoluene. Ascorbate, a water-soluble antioxidant, inhibited 90% of the adherence induced by either H₂O₂ and t-BuOOH. Thus, termination of oxidative radical chain reactions by lipid- or water-soluble antioxidants prevented the appearance of the adhesive endothelial cell surface that resulted from peroxide exposure.

**PAF Does Not Mediate Adherence after Prolonged Oxidant Exposure**

Our initial experiments to identify the molecular mechanism of adherence in response to prolonged oxidant exposure examined the potential role of endothelial cell PAF in this event. We first examined the time course of PAF synthesis after oxidant exposure by pulse labeling the cultures with [³H]PAF at either 100 or 240 min of stimulation with H₂O₂. We found (Fig. 5 a) that 250 μM H₂O₂ induced PAF synthesis immediately after exposure to H₂O₂ and during the subsequent half-hour of exposure. This synthesis had fallen to undetectable levels after 60 min of exposure. In a separate experiment (not shown), there was no detectable synthesis of [³H]PAF at either 100 or 240 min of stimulation with H₂O₂. The effect of t-BuOOH on the endothelial cells differed from that of H₂O₂ in that there was no detectable synthesis of
Figure 5. (A) Rate of synthesis of [3H-acetyl]PAF by endothelial cells at various times after stimulation with H2O2, t-BuOOH, or thrombin; (B) effect as a function of time of a PAF receptor antagonist on PMN adherence. Endothelial cells were washed with HBSS/A and at time = 0 the buffer was switched to 37°C HBSS/A with or without 250 μM t-BuOOH, 250 μM H2O2 or 2 U/ml human thrombin. 10 min before the stated time, 25 μCi of [3H]acetate was added to the incubation medium, and the incubation with the radiolabel was allowed to continue for 10 min at 37°C. Incorporation of [3H]acetate was stopped by removing the incubation medium (<1% of the [3H]PAF is released into the overlaying medium) and adding 1 ml methanol to the monolayer. [3H]PAF was quantitated using total radioactivity in the lipid extract and the ratio of [3H]PAF to recovered radioactivity from the chromatography plate. In B, endothelial cell monolayers were washed and treated with HBSS/A with or without 350 μM H2O2 for the stated period of time. This was then removed, the monolayers washed, and PMN that had been preincubated for 5 min with 100 μM L659,989, or an equivalent amount of DMSO vehicle, were layered over the monolayer. This allowed the concentration of L659,989 to be maintained at 100 μM throughout the coincubation. Nonadherent PMNs were removed and the tightly adherent PMNs were quantitated as in Fig. 1. When the effect of a second PAF receptor antagonist, WEB 2086, was examined (not shown) its concentration was 10 μM, and the concentration of H2O2 or t-BuOOH was 250 μM. The incubation time was 2 h. In parallel experiments, both L659,989 and WEB 2086 inhibited PAF-induced PMN adherence to a gelatin matrix in a fashion that depended on the concentration of PAF. Data are expressed as a mean and range of duplicate values.

Figure 6. Adherence of PMN or undifferentiated HL-60 cells to endothelial cells stimulated with menadione, t-BuOOH, or H2O2. Endothelial cells grown to confluence were treated with HBSS/A, or 300 μM t-BuOOH, 1 mM H2O2, 300 μM menadione, or 1,000 U TNF-α in HBSS/A. After 4 h of incubation at 37°C, the incubation medium was removed, and the monolayer washed twice before the ability of the incubated endothelial cell monolayers to bind 111In-PMN or 111In-HL-60 cells was determined as described under Fig. 1 using a 5-min incubation period. The values presented are the percent of cells that bound compared to the total added to each well, and are given as a mean and range of duplicate values.

Figure 7. Effect of actinomycin D or emetine on PMN adherence to endothelial cells treated with t-BuOOH, H2O2, or TNF-α. Endothelial cells were treated for 15 min at 37°C with or without 5 μg/ml of actinomycin D or 5 μM emetine in HBSS/A. The cells were washed, and then treated with HBSS/A buffer, or 250 μM t-BuOOH, 250 μM H2O2, or 1,000 U TNF-α in HBSS/A for 2 h at 37°C in the continued presence of the protein synthesis inhibitors. The incubation buffer was removed, the monolayers were washed twice, and PMN adherence was measured as described under Fig. 1. Data are expressed as the percent of adherent cells compared to total cells added to each well, and are presented as a mean and range of duplicate values.
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[1H]PAF at any of the times examined. We next examined
the ability of endothelial cells to retain for prolonged times
the PAF synthesized immediately upon exposure to H₂O₂.
We continuously exposed endothelial cells to H₂O₂ and
[1H]acetate (four times the amount of [1H]acetate used in
the pulse labeling experiment) and then extracted and purified cellular PAF. There was no detectable [1H]PAF re-
mainin in the exposed cells after 90 or 120 min of stimula-
tion (not shown).

We employed L659,989, a competitive antagonist of the
PAF-receptor (10), to directly assess the role of endothelial
cell-associated PAF as a signal for the adherence of PMN.
As we previously observed, exposure of endothelial cells to
H₂O₂ for short periods of time induced PMN adherence
(14) (Fig. 5 b). L659,989 inhibited 64% of the PMN adher-
ance induced by exposure of endothelial cell monolayers to
H₂O₂ for 30 min. However, after 1 h of exposure, L659,989
had no measurable effect on H₂O₂-induced PMN adher-
ence. We confirmed this result with a second, structurally
unrelated receptor antagonist, WEB 2086, which also had no
effect on t-BuOOH- or TNF-α-induced adherence (not shown).
These results show that while H₂O₂ induces en-
thelial cells to bind PMN by a PAF-dependent mechanism
soon after exposure to it, this mechanism does not account
for PMN adherence after exposure to H₂O₂ for 60 min or
longer, and plays no role in t-BuOOH-induced adhesion at
any time.

ELAM-1 Does Not Mediate Adherence after
Oxidant Exposure

ELAM-1 directly mediates neutrophil, monocyte, and HL-
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cells as a probe for the expression of ELAM-1. The data in

PMN adherence was performed
in parallel as described under
Fig. 1. There was no detect-
able increase in S12 binding
to monolayers incubated with
HBSS/A alone, nor was there
an increase in binding of the
isotype-matched irrelevant an-
tibody T10 to t-BuOOH-treated monolayers. Data are expressed as the amount of [125I]-labeled second antibody associated with the
monolayer, and are presented as a mean and range of duplicate values.

Fig. 6 show that although the oxidants induced PMN adher-
ence in this experiment, they uniformly failed to stimulate
the adherence of undifferentiated HL-60 cells. We also ex-
amined the effect of protein synthesis inhibitors on the devel-
opment of the pro-adhesive surface. This approach relied on
the observation that endothelial cells do not normally ex-
press either ELAM-1 or its messenger RNA (3, 4). Treat-
ment of endothelial cell monolayers with actinomycin D, an
inhibitor of transcription, failed to inhibit adherence in
response to 250 μM H₂O₂ or t-BuOOH even though it
blocked 70% of the adherence induced by TNF-α (Fig. 7).
A similar effect was obtained when emetine was used to in-
hbit translation. Finally, as a direct and independent assess-
ment of the role of ELAM-1, we employed H18/7, an mAb
against ELAM-1 that blocks about one-half of the adherence
induced by TNF-α (3). In this one experiment, H18/7
blocked 50% of the adherence induced by TNF-α, as expected,
but failed to inhibit adherence in response to t-BuOOH (not
shown). These results exclude ELAM-1, or any newly syn-
thetized protein, as the mechanism for the adherence of
PMN to oxidant-treated endothelial cells.

**GMP-140 Mediates Adhesion after Prolonged Exposure
of Endothelial Cells to Peroxides**

GMP-140 is a recently characterized glycoprotein that has a
predicted structure similar to that of ELAM-1 (11). It is a
membrane-bound component of specialized granules in en-
thelial cells, the Weibel-Palade bodies, that are rapidly
translocated to the cell surface in response to agonist stimu-
lation (5, 9). GMP-140 mediates PMN adhesion to appropri-
ately stimulated endothelial cells, GMP-140-coated surfaces,
and COS cells transfected with its cDNA (7). Therefore, we
asked whether treatment of endothelial cell monolayers with
peroxide induced the translocation of GMP-140 to the cell
surface. We found (Fig. 8) increased surface expression of
GMP-140 as determined by increased binding of S12, an
mAb against GMP-140, on endothelial cells treated with...
We also found that PMN adherence to peroxide-treated endothelial cells could be inhibited by pretreating the PMN with solubilized, purified GMP-140 (Fig. 10). Excess GMP-140 completely suppressed adhesion to both H_2O_2- and t-BuOOH-treated endothelial cell monolayers, and, since the PMNs were not aggregated, this effect was not due to an artifactual decrease in available PMN. Control incubations showed that the interaction between PMN and oxidant-treated endothelial cells was not affected by an irrelevant protein, fibrinogen, and that excess GMP-140 did not inhibit PMN adherence to endothelial cells expressing ELAM-1 in response to TNF-α stimulation. Our experiments demonstrated that, in response to oxidant treatment, human endothelial cells express GMP-140 for prolonged periods of time, and that it is the sole mechanism for the increased adhesion of PMN to oxidant-perturbed endothelial cell monolayers.

**Discussion**

We demonstrated that primary cultures of endothelial cells became adhesive for PMN after treatment either with a water-soluble or a lipid-soluble peroxide, or after intracellular generation of oxygen radicals. The increase in adhesion was equivalent to that induced by cytokine stimulation, up to 10-fold over basal values, but was not due to the same adhesion mechanism. Formation of the adhesive surface could be initiated by a short exposure of the endothelial cells to the oxidants, and clearly preceded irreversible oxidative damage to the cells. Adherence of PMN to oxidant-treated endothelial cells was due to an alteration of the endothelial cells themselves, rather than the activation of the adhesive response of PMN. Although H_2O_2 can directly stimulate
the adhesive response of the U937 monocytic cell line (33), and our preliminary data showed a similar direct activation of PMN adhesion by high concentrations of peroxides (not shown), there were insufficient residual peroxides remaining in the system to induce this response. This was verified by demonstrating that PMN did not adhere to a human smooth muscle cell line, and that the mAb 60.3 did not block the adherence of PMN to oxidant-treated endothelial cells. Additional evidence that this was an endothelial cell–dependent process was the ability of formaldehyde-fixed PMN to adhere to oxidant-treated endothelial cells. This observation also demonstrated that the adhesive mechanism required no active response from the PMN.

The pro-adhesive molecule expressed by the oxidatively stressed endothelial cells was not the phospholipid PAF, although this lipid does mediate PMN adherence to endothelial cells treated with H2O2 for shorter periods of time (14) (Fig. 6). The surface expression of this potent phospholipid mediates PMN–endothelial cell adherence under other circumstances (14, 23, 24, 40, 42), but our data showed that there was little or no PAF present at the times examined here. Furthermore, we were unable to detect significant synthesis and accumulation of PAF in response to t-BuOOH treatment, even though it induced as much adherence as H2O2. As expected from these observations, PAF receptor antagonists failed to antagonize PMN adherence in response to prolonged peroxide treatment.

We found that PMN adherence to oxidant-treated endothelial cells did not require de novo protein synthesis. This suggested a role for a preformed mediator, and eliminated a role for ELAM-1 in this process. The lack of an effect by the mAb H18/7, directed against ELAM-1, supported this conclusion. Further evidence that ELAM-1 was not involved was the complete lack of adherence of the myelocytic cell line HL-60 to the oxidant-treated cells. The failure of HL-60 cells to bind was not expected as HL-60 cells, and monocytes, also bind to GMP-140 (7, 13). Indeed, treatment of human umbilical vein endothelial cells with a different lipid-soluble peroxide, cumene hydroperoxide, induces monocyte adherence to the exposed monolayer (26). One potential explanation is that the recloned line of HL-60 cells that we employed recognized ELAM-1 (since they bound to TNF-α–treated cells), but not GMP-140. If true, this would suggest that HL-60 cells possess different receptors for these selections that can be independently expressed.

The sole molecular mechanism responsible for the adherence of PMN to endothelial cell monolayers treated with low concentrations of oxidants for prolonged periods of time was the expression of GMP-140 on the cell surface. This was demonstrated by the complete inhibition of adherence by an mAb that blocks GMP-140–mediated PMN adherence, and by competitively blocking GMP-140 receptors on PMN with solubilized GMP-140. There was a marked difference between the expression of GMP-140 after oxidant treatment and its expression after stimulation of the endothelial cells with the rapidly acting agonists histamine or thrombin (9).

We found that GMP-140 was expressed on the peroxide-treated endothelial cells over a period of several hours, which is in sharp contrast to its transient expression after stimulation with the rapidly acting agonists where its presence on the cell surface is measured in just minutes (9). We have not identified the reason for this difference, but it is possible that the mechanisms responsible for its reinternalization (9) were not induced by the oxidizing agents, or that this mechanism was impeded by the oxidants. Also, peroxided-treated endothelial cells bound more PMN than did thrombin-treated monolayers, even though thrombin is a potent agonist for GMP-140 expression (9). Again, this may relate to the inability of peroxide-treated cells to reinternalize surface GMP-140, resulting in a higher steady-state abundance of GMP-140. Alternatively, it may reflect the lack of synthesis of PMN inhibitory compounds that decrease PMN adhesion to activated endothelial cells (38). The overall effect, however, is to produce an endothelial surface with very high adhesivity for prolonged times that is completely due to expression of GMP-140. This is in contrast to rapidly acting agonists where adhesion results from both GMP-140 and PAF expression (18), and in contrast to adhesion induced by short exposures to higher concentrations of H2O2, which is solely due to the expression of PAF (14).

The mechanism by which oxidants induced the translocation of GMP-140 from the Weibel-Palade bodies to the cell surface is not known, but free radicals were essential components. Free radicals have previously been shown to trigger exocytosis of histamine-containing granules from mast cells (18). In our experiments, there were differences between radical generation by H2O2 and t-BuOOH: t-BuOOH–mediated adherence was more sensitive to the actions of a hydrophobic iron chelator and lipid-soluble antioxidants. This suggests that the effects of both peroxides were mediated by the iron-catalyzed propagation of free radical oxidation reactions, but that lipid-soluble radicals (perhaps t-BuOO radicals) were essential intermediates in t-BuOOH-mediated GMP-140 surface expression. The lack of effect of conalbumin, a transferrin that is not translocated by human cells, suggests that the iron-catalyzed reactions occurred within the intracellular compartment. This is consistent with our observation that menadione, an intracellular source of O2·− and H2O2 (29, 31), also resulted in the formation of a pro-adhesive endothelial cell surface. However, the effect of menadione, unlike peroxide-stimulated GMP-140 expression, did not depend on free iron, a result also observed during the study of menadione-induced toxicity (17). Since the formation of radicals by alternative methods circumvents the requirement for iron, the essential role for ionic iron shows that H2O2 and t-BuOOH must be metabolized to free radicals in order to induce GMP-140 expression.

Extracellular generation of peroxides and oxidizing radicals might also induce GMP-140 translocation, and a potential source of these could be the radicals produced by activated neutrophils. Such radicals damage endothelial cells (12, 16, 35, 36), and do so in an iron-dependent fashion (12, 35). Thus extravascular PMN, when stimulated by appropriate agonists to produce oxidizing radicals, would be able to recruit other, circulating PMN by alteration of the intravascular surface. Endothelial cells have been widely employed as a target for oxidizing reagents because of the relevance of this model to a large number of diverse and important disease processes including adult respiratory distress syndrome, reperfusion injury, and atherosclerosis (15, 19, 32, 37). Recruitment of PMN to the sites of oxidative damage may serve to enhance the fundamental pathologic events and thereby exacerbate the disease process. Induction of Weibel-Palade body translocation to the cell surface and the attendant
expression of GMP-140 on the surface of these endothelial cells may be one mechanism involved in the etiology of these diseases.

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