REVIEW

Mechanisms of Neutrophil-Mediated Killing of Endothelial Cells

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INTRODUCTION

The killing of endothelial cells either by activated neutrophils or by addition of H_2O_2 has been extensively studied. Several different, important themes related to the mechanism of cell killing have recently emerged. 1) Depending on their origin, the susceptibility of endothelial cells to oxidant-mediated killing by neutrophils varies. 2) In some respects, the killing of endothelial cells by activated neutrophils involves a participation of endothelial cells in their own destruction. 3) The killing of endothelial cells by products of neutrophils does not occur by a single, commonly accepted biochemical event. 4) Endothelial cell killing appears to be mediated by synergistic interaction of oxygen products and proteases from leukocytes. 5) Finally, endothelial cells can be modified by contact with cytokines such that their susceptibility to killing by neutrophils is greatly enhanced.

VARIATION IN SUSCEPTIBILITY OF ENDOTHELIAL CELLS TO NEUTROPHIL-MEDIATED DAMAGE

There are several bodies of evidence suggesting that endothelial cell damage by phorbol ester-activated neutrophils varies with regard to the mechanism of injury. Human umbilical vein endothelial cells and rat pulmonary artery endothelial cells have been reported to be susceptible to damage by neutrophils in a manner that is related to the generation of toxic oxygen products from neutrophils [1–4]. The damage can be largely abrogated by catalase and by the iron chelator deferroxamine but not by superoxide dismutase (SOD), suggesting that an iron-dependent product of H_2O_2, perhaps the hydroxyl radical (HO·), is the damaging oxygen product. In addition, if neutrophils from patients with chronic granulomatous disease (CGD) are employed, little or no endothelial cell injury occurs, consistent with the conclusion that injury is due to a toxic oxygen product of the respiratory burst. In contrast, microvascular endothelial cells obtained from human omentum are injured by contact with phorbol ester-activated neutrophils obtained from normal human volunteers or from patients with CGD, and catalase is nonprotective [5,6]. The protective effects of chlormethyl ketone inhibitors, which inactivate the catalytic site in elastase, have been taken to suggest that this type of endothelial cell injury is related to the effect of a granule-released product, such as elastase. Collectively, these data indicate that, within the same species, endothelial cells may be injured by neutrophils via two independent mechanisms, one being oxygen radical-mediated and the other being independent of this pathway. In addition to these pathways of cytotoxic injury, endothelial cell monolayers may be disrupted by neutrophil products (presumably proteolytic enzymes) without being killed, the outcome of which may be loss in vivo of the endothelial cell covering of vessels and compromise of the permeability barrier [7,8].

Additional evidence suggesting that endothelial cells from different vascular beds vary significantly with respect to the mechanism of neutrophil-mediated injury comes from some recent in vivo observations in rats. Immune complex deposition (involving rabbit polyclonal IgG antibody to bovine serum albumin) has been induced in the lungs and dermis by the local injection (into airways or dermis) of antibody and the systemic injection of antigen. Immune complex deposition occurs in alveolar walls of lung and in venules and capillaries of the dermis, leading to complement-dependent and neutrophil-dependent vascular injury, which results in endothelial cell damage, increased vascular permeability, and hemorrhage. Vascular injury in the lung is significantly diminished if catalase is intermixed with the antibody or if the animals are systemically treated with the iron chelator deferroxamine [9]. In striking contrast, no protection from injury is obtained in the dermis under the same

conditions of treatment, even when catalase is covalently linked to the IgG [9]. This striking discordance in effects of interventions (catalase and deferroxamine) strongly suggests that, whereas endothelial cells in both the lung and the dermis are damaged by products of activated neutrophils, toxic oxygen products are vital for damage of lung microvascular endothelial cells and irrelevant to damage of dermal vascular endothelial cells. Very little is known regarding the mechanism by which dermal vascular endothelial cells are damaged by products of activated neutrophils. More definitive studies will probably require access to primary cultures of microvascular endothelial cells from each of the two organs, but to date we have not been successful in culturing dermal microvascular endothelial cells in sufficient quantities to carry out the necessary studies.

PARTICIPATION OF ENDOTHELIAL CELLS IN THEIR KILLING BY NEUTROPHILS

As strange as it may seem, there is now incontrovertible evidence that, in the interaction of phorbol ester-stimulated neutrophils and endothelial cells, the latter play an active role in events leading to their own damage and/or destruction. Collectively, the participation of endothelial cells in their demise by activated neutrophils can be considered an "autotoxic" response of endothelial cells. There are two bodies of evidence that support this conclusion. The first relates to the killing of rat pulmonary artery endothelial cells by human neutrophils. It has been shown that this killing requires oxygen products of the neutrophil [1–4]. On the basis of the protective effects of catalase, deferroxamine, and HO· scavengers such as dimethyl thiourea and dimethyl sulfoxide, it seems likely that HO· may be the key reactive species of oxygen responsible for the killing process. Inasmuch as it has been demonstrated that phorbol-activated human neutrophils generate O_2^- and H_2O_2 but not HO· unless iron is added to the extracellular fluid [10], the observation related to the iron requirement for neutrophil killing of endothelial cells implies that iron is available either from the extracellular fluid or from the endothelial cell itself. Accordingly, rat pulmonary artery endothelial cells have been pretreated with deferroxamine and washed and then neutrophils have been added. Their ability to kill the endothelial cells was assessed after addition of phorbol ester. The results were striking: Pretreatment of endothelial cells with deferroxamine led to almost 70% reduction in their killing by activated neutrophils [11]. It could be demonstrated by high-performance liquid chromatography that extracts of the deferroxamine-treated cells contained deferroxamine that was bound to iron (Fe^{3+}). The location within the endothelial cell of the iron-deferroxamine complex (which might

provide vital information related to where in the endothelial cell engagement of iron in a manner that leads to damage occurs) is not known; this finding demonstrates that the deferroxamine has intercepted iron within the endothelial cell and that this iron is related to the cytotoxic events. On the basis of this information, it can be concluded that neutrophil-mediated killing of endothelial cells requires the engagement of iron from the target cell. The presumption is that iron is released and converted by O_2^- to its transition state (Fe^{2+}), which permits it to react with H_2O_2 to generate HO·.

A second line of evidence for autotoxic responses of endothelial cells comes from recent observations that rat pulmonary artery endothelial cells contain xanthine dehydrogenase (xd) and xanthine oxidase (xo) in a ratio of approximately 2:1. If endothelial cells are pretreated with inhibitors of xo, namely, allopurinol and oxypurinol, and then washed, the ability of phorbol ester-activated neutrophils to kill these cells is greatly attenuated, in proportion to the concentration of inhibitor employed [12]. In parallel experiments, pretreatment of neutrophils does not attenuate their ability to kill endothelial cells, indicating that the effect of allopurinol and oxypurinol is related to their interaction with the endothelial cell. Although allopurinol might be functioning as a scavenger of HO·, it seems unlikely that this explains the protective effects of the inhibitor, since the scavenging ability of allopurinol requires millimolar concentrations. A more plausible explanation is that these compounds inhibit xd and/or xo in the treated endothelial cells, a finding that has been confirmed by direct measurements of xd and xo in the treated endothelial cells. These observations imply that availability of xd and/or xo in endothelial cells is linked to destruction of these cells by activated neutrophils. Perhaps relevant to these findings is the fact that, when phorbol ester-stimulated neutrophils interact with endothelial cells, there is a fairly rapid conversion of xd to xo (with an inversion of xd/xo the ratio from 2:1 to 1:2), with the conversion being irreversible (not reversible by the addition of reducing agents). The addition of catalase, superoxide dismutase (SOD), or deferroxamine fails to prevent conversion of xd to xo in the endothelial cells following activation of neutrophils. Also, CGD cells are fully effective in causing conversion of xd to xo, providing compelling evidence that the conversion process is not related to an oxygen products of the neutrophil [12]. Although it can be speculated that the neutrophil-derived "converting factor" may be a granule secretion product, this is not proven at present.

On the basis of these data, it seems possible that neutrophil interaction with endothelial cells results in additional xo activity, which will provide for more generation of O_2^- within the endothelial cell, adding to the oxygen radical burden of the cell. A more comprehen-

sive analysis of how this fits into the scheme of events leading to injury or death of endothelial cells is presented below.

BIOCHEMICAL EVENTS RELATED TO INJURY OF ENDOTHELIAL CELLS

There is no consensus regarding the biochemical events that can be linked to damage or killing of endothelial cells by toxic oxygen products from activated neutrophils or by the addition of reagent H_2O_2. As referred to above, rat pulmonary artery endothelial cells are killed over a period of several hours following exposure to phorbol ester-activated human neutrophils, using effector/target cell ratios as small as 2:1 [4,11,12]. Since neutrophils from patients with CGD fail to induce killing, the process of cell injury is linked to oxygen products of the respiratory burst. Furthermore, catalase but not SOD is protective, as are the HO· scavengers such as DMSO and DMTU. Although it is possible that the cell killing process could be related to the formation of iron radicals (ferryl and/or perferryl ions), the role of HO· also seems likely. The killing of endothelial cells by H_2O_2 follows a similar pattern of protective interventions, indicating that extrinsic O_2^- is not essential for the cytolytic pathway. The evidence (referred to above) that inhibitors of endothelial cell xo (and xd) will block a significant amount of neutrophil-mediated killing suggests that intracellular formation of O_2^- within the endothelial cell is somehow linked to the cell killing process. We speculate that intracellular formation of O_2^- (whether by xo, cyclooxygenase, mitochondrial enzymes, or some other source) brings about release of Fe^{3+} from its storage form in ferritin and formation of Fe^{2+}, which can then interact with H_2O_2 (which has diffused into the endothelial cell after its formation on the cell membrane of the neutrophil), following which H_2O_2 is reduced to HO· and Fe^{2+} is recycled to Fe^{3+}. The influx of H_2O_2 into the endothelial cell would also be expected to result in a fall in intracellular levels of ATP, because of both impaired synthesis and accelerated breakdown [13–21]. Even after the fall in ATP, a rise in intracellular calcium due to influx from the extracellular compartment would be expected [22–28].

It is not at all clear what biochemical events can be directly linked to cell injury. Although it is known that H_2O_2 and other oxygen products (including hypochlorous acid (HOCl), a halide metabolite resulting from the action of myeloperoxidase on H_2O_2) can react with surface molecules on cell membranes [29], to what extent these effects are relevant to cytotoxicity is not known. It is known that H_2O_2 can inactivate glyceraldehyde-3-phosphate dehydrogenase [21] and also bring about activation of ADP-ribose polymerase [14], the result of

which can cause a fall in intracellular levels of NAD. Whether these findings can be generalized to include mechanisms of cytotoxicity in endothelial cells is not known. We have not been able to demonstrate that the inhibitor of ADP-ribose polymerase, namely, 3-aminobenzidine, will protect endothelial cells from H_2O_2-mediated damage (Varani, J., and Ward, P.A., unpublished data). Furthermore, the observation that red blood cell killing by oxygen products of neutrophils involves a role for O_2^- [30] and the fact that red blood cells lack ADP-ribose polymerase suggests the likelihood of multiple explanations for biochemical events related to neutrophil or H_2O_2-mediated killing of endothelial cells.

ENDOTHELIAL KILLING: SYNERGY BETWEEN PROTEASES AND H_2O_2

Recent evidence indicates that endothelial cells (from the rat pulmonary artery) are killed following addition of H_2O_2 in a time-dependent process. The endpoint, which is release of ^{51}Cr, is ordinarily established at 4 hr for convenience, even though the release of ^{51}Cr continues for a total period of 8 hr, following which there is plateau in the release of ^{51}Cr. During the first 4 hr, the addition of protease inhibitors such as soybean trypsin inhibitor (SBTI) does not reduce the release of ^{51}Cr. However, when the endpoint of 18 hr is used, the continued presence of catalase (180 units/ml) alone produced 10% ± 1% protection, the continued presence of SBTI produced 35% ± 4% protection whereas the combination of catalase and SBTI yielded 78% ± 5% protection, implying that over this extended period there may be synergistic effects of H_2O_2 and proteases, the result of which is intensified cell injury [31]. The experiments were then extended to employ addition of porcine pancreatic elastase (2.3 units/ml), which at 18 hr caused 13% ± 1% release of ^{51}Cr, H_2O_2 (100 nmol), which in the same period of time caused 33% ± 1% release of ^{51}Cr, or the combination of elastase and H_2O_2, the result of which was 75% ± 2% release of ^{51}Cr. Similar results have been obtained with the use of trypsin and chymotrypsin. These data show beyond reasonable question that, under the experimental conditions employed, proteases and H_2O_2 act in synergistic manner to bring about cytotoxic events (defined by ^{51}Cr release) in the endothelial cell. There is at present no explanation for how this occurs, why a prolonged duration of contact of these substances with the endothelial cell is required, and whether the ^{51}Cr release at 4 hr vs. 18 hr involves different mechanisms of cytotoxicity. Obviously, much additional work is required.

EFFECTS OF CYTOKINES ON NEUTROPHIL MEDIATED KILLING OF ENDOTHELIAL CELLS

Cytokines have a plethora of biological effects on a variety of different cells. With regard to phagocytic cells, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) can "prime" these cells for exaggerated oxygen radical responses [32–34]. At much higher concentrations, direct stimulation of neutrophils and macrophages by cytokines occurs. The ability of these cytokines to alter endothelial cells in a manner that leads to increased adhesive interactions with neutrophils is a well-known phenomenon [35–37]. This is a slowly developing process of up-regulation in the endothelial cell, requires several hours, and depends on protein synthesis, probably involving increased expression on the endothelial cell of at least two different adhesion-promoting molecules. The complementary adhesion-reactive molecule on the neutrophil involves the CR3 (complement receptor) moiety and probably, to a lesser extent, at least one other adhesion-related molecule. Not only does prior exposure of endothelial cells to TNFα or IL-1β lead to augmented adhesive interactions with neutrophils, this also results in a greatly enhanced (two- to threefold) cytotoxic outcome in the presence of phorbol ester activated neutrophils [38]. Whether the increased killing of endothelial cells is a reflection solely of enhanced adhesive interactions with neutrophils or whether other mechanisms are also responsible for the enhanced cytotoxicity is not currently known. It should be noted that the ability of TNF to cause rapid conversion of xd to xo in endothelial cells may be another event that puts these cells at added risk of injury [39]. By whatever mechanism the enhanced cytotoxicity occurs, these observations may be relevant to clinical studies featuring the infusion of TNF into humans, in which case evidence for serious acute injury of the pulmonary microvasculature has been obtained.

Another finding of relevance to these issues comes from recent observations that, whereas exposure of human umbilical vein endothelial cells to TNF does not itself result in evidence of cell injury when the cells are examined immediately after exposure, if endothelial cells exposed for 18 hr to as little as 2 ng TNFα/ml are then washed and placed in a simple culture medium (Hanks balanced salt solution), there is progressive evidence of cell injury over the next 4 hr, as reflected by ^{51}Cr release [40]. The cytotoxic effects are abrogated if the TNFα used for the 18 hr treatment regimen is either heat-inactivated or blocked by the addition of specific antibody. IL-1β has similar effects, whereas M-CSF is without any effect on the endothelial cells. Furthermore, endothelial cells show a degree of specificity for the effects of TNFα inasmuch as neither human dermal fibroblasts nor squamous epithelial cells from skin develop evidence of cytotoxic effects after exposure to TNFα. Although the manner by which TNFα causes susceptibility of endothelial cells to toxic effects under these special conditions is unknown, the fact the deferroxamine is protective suggests that an iron-dependent pathway of toxicity is involved. Not surprising, allopurinol is not protective, in accordance with the fact that human umbilical vein endothelial cells are largely devoid of detectable xd and xo. However, the ability of several cyclooxygenase inhibitors (ibuprofen, indomethacin, piroxicam) to protect against the toxicity following exposure of endothelial cells to TNFα may imply that generation of O_2^- from cyclooxygenase is relevant to the mechanism of injury. These data indicate that exposure of endothelial cells to TNFα and IL-1β alters these cells in a manner that makes them significantly susceptible to injury, by either direct or indirect (neutrophil-mediated) pathways.

CONCLUSIONS

As is evident from this information, endothelial cells have very complex biological responses that have important implications for the outcome of the inflammatory response. The recognition that the susceptibility of endothelial cells to injury may vary considerably, depending on the species of origin or the source of the microvascular bed from which the cells originate, has important implications suggesting that there is a significant diversity in behavior of endothelial cells. The increasing recognition that the events related to how endothelial cells are injured by products of activated neutrophils are complex and involve factors originating both in the effector cells (neutrophils) as well as in the target (endothelial) cells adds a new dimension and suggests that a variety of different therapeutic interventions may be employed for the protection of endothelial cells during the inflammatory response. Finally, the increasing knowledge that endothelial cells can respond directly to inflammatory mediators suggests that a more detailed analysis of signal-transduction events in these cells should provide some important clues to their biology.

ACKNOWLEDGMENTS

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