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PAF, CYTOKINES, TOXIC OXYGEN PRODUCTS AND CELL INJURY

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Introduction

It is becoming increasing clear that injury to cells or tissues related to the inflammatory reaction can be ascribed to a complex array of mediators generated and released from activated phagocytes such as neutrophils, monocytes and macrophages. Furthermore, there is now accumulating evidence for cell-cell interactions which involve phagocytic cells and result in amplified damage at the cellular or tissue level. We will provide insights into mechanisms of injury to tissues or cells at two levels: *in vivo* lungs or dermal inflammatory reactions initiated by the local deposition of immune complexes and *in vitro* killing of endothelial cells by activated neutrophils.

Role of Platelet Activating Factor (PAF) Receptors in Immune Complex Induce Vasculitis

PAF is a lipid mediator consisting of a glycerol backbone with substitutions at position 1 containing an alkyl group, position 2 an acetyl group and position 3 a phosphorylcholine group. PAF is synthesized in a variety of cells including endothelial cells, neutrophils, monocytes and macrophages, but to date the only cells known to secrete PAF are phagocytic cells (Benveniste *et al.*, 1983, reviewed). When deposition of IgG-containing immune complexes occurs in the dermis or lungs of rats, the acute microvascular injury that develops is complement and neutrophil dependent (Cochrane *et al.*, 1966; Johnson *et al.*, 1974). Vascular damage associated with these reactions is associated with extensive evidence of endothelial cell damage as reflected in the development of haemorrhage and transvascular leakage of water and proteins. Precisely how vascular endothelial cell damage occurs is not fully known. Local injection of superoxide dismutase is protective (Johnson *et al.*, 1981), being associated with a diminished accumulation of neutrophils, suggesting that O_2^- participated in events leading to the recruitment of neutrophils (Warren *et al.*, 1990).

The fact that treatment with the inhibitor of xanthine oxidase, allopurinol, reproduces the effects of SOD in the dermal model of vascular injury suggests that O_2^- may be derived from products of

tissue xanthine oxidase (Warren *et al.*, 1990). Curiously, in contrast to immune complex injury of the alveolar compartment of lung, immune complex vasculitis of the dermis is not susceptible to the protective effects either of catalase or of the iron chelator, desferrioxamine. It is currently assumed that injury of dermal vascular endothelial cells by immune complex stimulated neutrophils is due to products other than oxygen derivatives, perhaps related to granule-released proteases. This possibility is supported by the fact that damage of human microvascular endothelial cells by activated neutrophils is resistant to the protective effect of SOD or catalase but apparently susceptible to agents which inhibit leukocytic proteases (Smedly *et al.*,1986).

Recent evidence suggests that injury associated with immune complex induced vasculitis of skin involves PAF (Hellewell *et al.*, 1986). More specifically, the addition of the PAF receptor antagonist, L-652,731, to the antibody used to form deposits of the immune complexes results in greatly attenuated injury as measured by leakage into the tissue of colloidal protein. Proof that access of PAF receptors on neutrophils is vital for the full development of injury was obtained by the use of neutrophil-depleted rats in which the dermal skin sites were reconstituted with homologous neutrophils (Warren *et al.*, 1989a). If neutrophils were used to reconstitute the skin sites pretreated with PAF receptor antagonist (L-652,731), the subsequent injury was attenuated by approximately 50%. In contrast, the use of the cis-isomer of this antagonist (L-652,763), which has much less blocking activity (100-1000 fold) for the PAF receptor, failed to provide any protection from immune complex-induced injury. These data imply that engagement of PAF receptors on neutrophils is required for maximal vascular injury following tissue deposition of immune complexes. One mechanism by which this might be explained would be "priming" of neutrophils by PAF, resulting in enhanced production of O_2^- and H_2O_2 by immune complex-stimulated phagocytic cells. The source of PAF in these reactions may well be the neutrophils themselves as well as tissue macrophages.

Participation of TNF in Immune Complex Induced Injury

While it has been shown that alveolar macrophages can be stimulated *in vitro* to generate TNFα (TNF) IL-1ß (IL-1) (Le *et al.*, 1987, reviewed) the extent to which these cytokines participate in inflammatory reactions following deposition of immune complexes in tissue is not clear. Recently we have utilised a lung model of immune complex induced injury in order to address this question (Warren *et al.*, 1989b). Deposits of immune complexes have been formed in pulmonary alveolar walls. Between 2 and 4 hrs during development of these reactions, substantial amounts of TNF and IL-1 can be detected in bronchoalveolar lavage fluids from these animals. The issue, of course, is whether the presence of either cytokine is of biological significance. In order to assess this question, rats were treated with antibody to murine TNFα, an antibody preparation which is specific, cross-reacts with rat TNF and blocks its biological reactivity. The addition of antibody to TNF (intratracheal) resulted in neutralization of intrapulmonary TNF and a marked reduction in intensity of lung injury as measured by leakage of colloid (irrelevant IgG) from the vasculature into the lung parenchyma. Further evidence of diminished lung damage was demonstrated by the use of morphometric approaches. The reason behind the protective effects of anti-TNF was unexpectedly related to a greatly diminished influx of neutrophils into the lung parenchyma. By the use of morphometric approaches and by extraction from lung tissue of myeloperoxidase, it was apparent that neutrophil accumulation in the lung was decreased by more than 60%, accounting for the diminished lung injury.

The manner by which antibody induced attenuation in the recruitment of neutrophils into lung sites containing the immune complexes is unclear. What is surprising is that C5a generated by the immune complexes is not able to completely mediate the recruitment of neutrophils in the absence of TNF. It may well be that TNF is generated from alveolar macrophages following their activation by immune complexes, as shown recently by immunohistochemical approaches (Warren *et al.*, 1989b). After TNF is released it may induce the nearby vascular endothelial cells to express cell

surface adhesive molecules (e.g. ELAM's) which will permit adhesive attachment of blood neutrophils. Whatever the explanation, these data demonstrate a key interaction between macrophages and neutrophils, the products (TNF) of the former being required for recruitment of the latter, with the end result being release from neutrophils of toxic oxygen products and proteases with subsequent damage of vascular endothelial cells, alveolar lining cells and basement membrane.

Mechanisms of Endothelial Cell Injury

In order to more fully understand the manner by which neutrophils can injure endothelial cells, a number of *in vitro* studies have been undertaken using cultured endothelial cells and phorbol ester activated neutrophils. It has been known for some time that activated neutrophils can cause injury in endothelial cells maintained in culture (Sacks *et al.*, 1978), although in some laboratories human umbilical vein endothelial cells appear to be resistant to the damaging effects of neutrophils (Smedly *et al.*, 1986). It is probable that the explanation for these discrepancies is due to the technical details of the experimental protocols as well as the possibility that in the process of cell culture the cell content of antioxidant factors (e.g. glutathione, catalase, SOD, glutathione peroxidase/reductase) has been modified.

Role of Toxic Oxygen Products from Neutrophils

Our own studies, which have utilized rat pulmonary artery endothelial cells and phorbol ester activated human neutrophils, demonstrate that in a time-dependent and cell-dependent (ratio of neutrophils to endothelial cells) manner, endothelial cell injury and death occurs (Varani *et al.*, 1985). That this outcome which can be ascribed to toxic oxygen products from the neutrophils, has been shown by the protective effects of catalase (with SOD having little protective activity) and by the inability of neutrophils (from patients with chronic granulomatous disease of childhood) to bring about injury to the endothelial cells. It should be mentioned that elastase released from the neutrophils can cause detachment from the surface to which the endothelial cells have become attached (Herlan *et al.*, 1981). This may also contribute to the loss of integrity of vascular lining cells *in vivo* following release from neutrophils of proteases. Further details on the mechanism of neutrophil-induced killing of endothelial cells *in vitro* include the protective effects of the iron chelator, desferrioxamine, as well as agents such as dimethylsulfoxide and dimethylthiourea, both of which are highly active in scavenging the hydroxyl radical, $OH^{\bullet}$ (Varani *et al.*, 1985). Recently accumulated evidence also indicated that the sources for iron in this reaction is the endothelial cell, not the neutrophil (Gannon *et al.*, 1987). Thus, the endothelial cell seems to cooperate with the neutrophil in events that lead to death of the former.

Killing of Endothelial Cells by Neutrophils: Synergy of H_2O_2 and Proteases

In most *in vitro* studies of killing of endothelial cells by phorbol ester-activated neutrophil, the endpoint for ^{51}Cr release is 4 hrs, during which time the addition of antiproteases (e.g. soybean trypsin inhibitor, SBTI) does not protect the cells from injury. However, if the time is extended to 18 hrs, the addition of catalase (1800 units/ml) or SBTI (100 µg/ml) each yielded 30-35% protection while the combination resulted in 78% protection, indicating a clear, synergistic effect (Varani *et al.*, 1989). This phenomenon could be documented by direct experimentation: for example, addition of 2.3 units human leukocytic elastase or 100 nmoles H_2O_2 resulted at 18 hr in 13% and 33% ^{51}Cr release, respectively, while the combination caused 75% ^{51}Cr release. Furthermore, the addition of H_2O_2 for the first 2 hrs followed by washing of the endothelial cells and addition of protease reproduced the outcome found with the simultaneous presence of H_2O_2 and protease for 18 hrs. Although these experiments do not define the mechanism for effects of protease and H_2O_2 in the killing of endothelial cells and one may argue about the prolonged time course, the results unequivocally indicate that endothelial cells can be damaged or killed by a

synergistic interaction involving leukocytic elastase and H_2O_2.

Role of Endothelial Cell Xanthine in Killing by Activated Neutrophils

Rat pulmonary artery endothelial cells contain xanthine dehydrogenase (xd) and xanthine oxidase (xo) in a ratio of approximately 2:1 (Phan *et al.*, 1989). Recent evidence has suggested that pretreatment of the cells with either allopurinol and oxypurinol results in a marked reduction in both xd and xo activities. Interestingly, such manipulations also lead to a greatly reduced killing of the endothelial cells by phorbol ester activated human neutrophils suggesting that products of either xd or xo may play a role in endothelial cell killing by neutrophils. Interaction of neutrophils (stimulated with phorbol ester) and endothelial cells leads to a rather dramatic, irreversible conversion of xd to xo, causing a reversal in the ratio of activity (xd/xo) to approximately 1:2. The manner by which this conversion process occurs in unknown, but the conversion of xd to xo appears not to be related to products of the respiratory burst of the neutrophil since neutrophils from patients with chronic granulomatous disease of childhood have the full capacity to cause conversion of xd to xo. Thus, it appears that neutrophil interaction with intact endothelial cells can lead to an increase in the level of intracellular xo. It is speculated that, following diffusion with endothelial cells of H_2O_2 derived from activated neutrophils, rapid breakdown of ATP will commence, resulting in the appearance of xanthine and hypoxanthine, both of which can serve as substrates for xo (Spragg *et al.*, 1985). The production of O_2^- within the endothelial cell would then cause reduction of Fe^{3+} (to Fe^{2+}) and its release from ferritin, following which interaction of Fe^{2+} with H_2O_2 would generate $OH^{\bullet}$, the putative toxic oxygen species involved in the ultimate series of events leading to damage or death of endothelial cells. It is now probable that endothelial cells contribute to their own destruction by activated neutrophils by providing two important factors: iron and O_2^- for xo. This curious anomaly of biology may be linked to some evidence that the presence of xo in rat pulmonary artery endothelial cells may be a vestige of an oxygen-dependent microbicidal pathway in endothelial cells.

Direct Effects of Cytokines and other Peptides in Endothelial Cells

It is now well-established that cytokines such as TNF and IL-1 can interact with endothelial cells to bring about an upregulation of ELAM's on the surface of the endothelial cells, greatly increasing the adhesive interaction with neutrophils (Prober *et al.*, 1986; Beveilacqua *et al.*, 1987) This process of upregulation is time-and dose-dependent and requires active protein synthesis. The functional counterpart of this increased adhesive interaction between endothelial cells and neutrophils has been shown to be related to a marked accentuation in the killing of the treated endothelial cells by phorbol ester stimulated neutrophils. Antibodies to one family of adhesive molecules located on the neutrophil, the CD11b/CD18 molecules (CR3 receptor) attenuated the killing of the endothelial cells, indicating that these molecules on the neutrophil will collaborate in events that exist between endothelial cells and neutrophils and which will lead to endothelial cell killing (Bevilacqua *et al.*, 1985).

Another interesting and recently discovered finding is that interactions between rat pulmonary artery endothelial cells and one of three inflammatory peptides (TNF, C5a and N'formyl-Met-Leu-Phe) leads to a conversion of xd and xo that is rapid (complete within 5 min.) and does not require protein synthesis (Friedl *et al.*, 1989). With any of the three peptides, the ED50 is in the very low dose range (1-20 nM), entirely consistent with concentrations of these peptides that can be presumed to be present *in vivo* during inflammatory reactions. It is curious that other inflammatory peptides (IL-1, bradykinin, C3a) as well as phorbol ester itself lack the ability to cause conversion of xd to xo. This finding raises the possibility that, by causing additional xo activity, the direct interaction of TNF, N'formyl-Met-Leu-Phe or C5a with endothelial cells puts these cells at added risk for damage by activated neutrophils. Thus, at least in the case of TNF, there may be two completely unrelated mechanisms by which endothelial cells become more susceptible to injury by

activated neutrophils: increased adherence interactions with neutrophils and conversion of xd to xo.

Not to be forgotten in consideration of endothelial cells is the fact, referred to above, that, while endothelial cells may be sensitive to damage by phorbol activated neutrophils, in not every case can the pathways leading to cell damage be linked to neutrophil-derived oxygen products. As mentioned, microvascular endothelial cells obtained from human placental tissue are not protected by catalase from killing induced by phorbol ester stimulated neutrophils, whereas endothelial cells from human umbilical veins are almost totally protected by the presence of catalase. In rats, again using immune complex as the trigger of neutrophil recruitment and subsequent neutrophil-dependent microvascular damage either in the dermis or the lung, the lung microvascular bed is almost totally protected from injury either by the presence of catalase or desferrioxamine whereas no such protection is seen in the dermis, even when skin and lung inflammatory reactions have been induced in the same animals (Warren *et al.*, 1990). Not even covalent linkage of catalase to the IgG antibody affords protection in the dermis while substantial protection was found in the lung. These findings lead to the nearly indisputable conclusion that microvascular endothelial cells in the dermal and in the pulmonary interstitial compartment are resistant or sensitive, respectively, to the damaging effects of toxic oxygen products from neutrophils. What is now key is to obtain primary cultures of endothelial cells from these tissues and to assess whether it is content of antioxidant factors or other explanations (such as presence or absence of xo) that account for these differences. These data suggest that in the human it will be important to compare different vascular beds for sensitivity to damage by toxic oxygen products. If this is confirmed, then it implies another level of complexity in understanding how the inflammatory process brings about injury to cells and to tissues.

References

Benveniste, J., Arnous, B., (1983). Platelet activating factor and structurally related ether-lipids. Elsevier Scientific Publishing Co., Amsterdam. The Netherlands.

Bevilacqua, M.P., J.S. Prober, M.E. Wheeler, R.S. Cotran, and M.A. Gimbrone, Jr. (1985). Interleukin 1 acts on cultured human vascular endothelial cells to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. J. Clin. Invest. 76, 2003-2011.

Bevilacqua, M.P., J.S. Prober, D.C. Mendrick, R.S. Cotran, and M.A. Gimbrone, Jr. (1987). Identification of an inducible leukocyte adhesion molecule, ELAM-1. Proc. Natl. Acad. Sci. 84, 9238-9242.

Cochran, C.G., and B.S. Aiken (1966). Polymorphonuclear leukocytes in immunologic reactions. The destruction of vascular basement membrane *in vivo* and *in vitro*. J. Exp. Med. 124 733-742.

Friedl, H.P., G.O. Till, U.S. Ryan and P.A. Ward (1989). Mediator-induced activation of xanthine oxidase in endothelial cells. FASEB J. 3, 2512-2518.

Gannon, D.E., J. Varani, S.H. Phan, U.S. Ryan, and P.A. Ward (1987). Source of iron in neutrophil-mediated killing of endothelial cells. Lab. Invest. 57: 37-44.

Harlan, J.M., P.D. Killen, L.A. Harker, G.E. Striker, and D.G. Wright (1981). Neutrophil-mediated endothelial injury *in vitro*: mechansims of cell detachment. J. Clin. Invest. 68, 1394-1401.

Hellewell, P.G., and T.J. Williams (1986). A specific antagonist of platelet-activating factor suppresses oedema formation in an Arthus reaction but not oederma induced by leukocyte chemo attractants in rabbit skin. J. Immunol. 137, 302-307.

Johnson, K.J., and P.A. Ward (1974) Acute immunologic pulmonary alveolitis. J. Clin. Invest. 54, 349-356.

Johnson, K.J., J.C. Fantone, J. Kaplan, and P.A. Ward (1981). *In vivo* damage of rat lungs by oxygen metabolites. J. Clin. Invest. 67, 983-991.

Le, J., and J. Vilcek (1987). Tumour necrosis factor and interleukin 1: Cytokines with multiple overlapping biological activities. Lab. Invest. 56, 234-247.

Phan, S.H., D.E. Gannon, J. Varani, U.S. Ryan, and P.A. Ward, (1989). Xanthine oxidase activity in rat pulmonary artery endothelial cells and its alteration by activated neutrophils. Am. J. Pathol. 134, 1201-1211.

Prober, J.S., M.P. Bevilacqua, D.C. Mendrick, L.A. Lapierre, W. Fiers, and M.A. Gimbrone, Jr. (1986). Two distinct monokines, interleukin 1 and tumour necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J. Immunol. 136, 1680-1687.

Sacks, T., C.F. Moldow P.R. Craddock, T.K. Bowers, H.S. Jacob (1978). Oxygen radical mediated endothelial cell damage by complement-stimulated granulocytes: an in vitro model of immune vascular damage. J. Clin. Invest. 61, 1161-1167.

Smedly, L.A., M.G. Tonnesen, R.G. Sanhaus, C. Haslett, L.A. Guthrie, R.B. Jonston, P.S. Heson, and G.S. Worthen (1986). Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. J. Clin. Invest. 77, 1233-1242.

Spragg, R.G., D.B. Hinshaw, P.A. Hyslop, I.U. Shraufstatter, and C.G. Cochrane (1985). Alterations in adenosine triphosphate and energy charge in cultured endothelial and P388D1 cells after oxidant injury. J. Clin. Invest. 76, 1471-1476.

Varani, J., S.E.G. Fligiel, G.O. Till, R.G. Kunkel, U.S. Ryan and P.A. Ward (1985). Pulmonary endothelial cell killing by human neutrophils. Possible involvement of hydroxyl radical. Lab. Invest. 53, 665-674.

Varani, J., I. Ginsburg, L. Schugar, D.F. Gibbs, J.Bromberg, K.J. Johnson, U.S. Ryan, and P.A. Ward (1989). Endothelial cells killing by neutrophils: Synergistic interaction of oxygen products and protease. Am. J. Pathol 135, 435-438.

Warren, J.S., D.M. Mandel, K.J. Johnson, and P.A. Ward (1989a). Evidence for the role of platelet-activating factor in immune complex vasculitis in the rat. J. Clin. Invest. 83, 669-678.

Warren, J.S., K.R. Yabroff, D.G. Remick, S.L. Kunkel, S.W. Chensue, R.G. Kunkel, K.J. Johnson, and P.A. Ward (1989b). Tumor necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. J. Clin. Invest. 84, 1873-1882.

Warren, J.S., K.R. Yabroff, D.M. Mandel, K.J. Johnson and P.A. Ward (1990, in press). Role of O_2^- in neutrophil recruitment into the sites of dermal and pulmonary vascultis. Free Rad. Biol. Med.