Tissue Injury in Inflammation

Oxidants, Proteinases, and Cationic Proteins

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In circumstances of acute injury to tissues of the body, it is currently popular to incriminate byproducts of oxygen metabolism as the ultimate injurious agents. A disparate array of conditions is encompassed in this concept, including injury to the lungs by external agents that induce oxidant formation, such as paraquot (1), bleomycin (2), and perhaps even endotoxin (reference 3, and Brigham, K. L. et al., personal communication), or that contains oxidants e.g., cigarette smoke (4); myocardial damage after infarction (5, 6); and inflammatory tissue injury at sites of accumulation of phagocytic cells (7–11), e.g., like that in the respiratory distress syndromes (7, 8). In the case of the lung and heart, oxygen metabolites are particularly attractive as intracellular toxic agents because of the high oxygen tensions to which the cells of these tissues are exposed. At these and other sites, cells of the inflammatory system (especially neutrophils, mononuclear phagocytes, and eosinophils) are invoked as additional sources of such metabolites due to their ability to undergo the well described respiratory burst that accompanies phagocytosis and other membrane stimulatory events (see below). However, the case for oxygen radicals may not be as clear or as complete in vivo as the existing reports suggest. It is the purpose of this discussion to attempt, at this admittedly intermediate stage of knowledge, to put the issues of oxidant tissue damage into perspective.

Earlier concepts of the mechanisms of inflammatory injury, derived from Metchnikov (12, 13), had focused on release of what are now known to be lysosomal constituents from inflammatory cells after they had reached the tissue and had subsequently lysed (see discussion in reference 14). Studies in the 1960s showing the potent injurious effects of injecting neutrophil contents (e.g., references 15 and 16) exemplify this approach. A decade later, it had become apparent that inflammatory cells were actively capable of secreting these constituents (14, 17–19) and that the suicide sac concept of lysosome-induced injury was overstated. While initially received with skepticism, it is now reasonable to suggest that granulocytes often remain intact in inflammatory lesions, exhibit an active secretory process, and are removed without lysis by macrophages (20), (also suggested by Metchinokov [12]). Over the same recent period, the concept that macrophages are secretory cells has become generally accepted (21, 22). In keeping with these developments, inflammatory injury was seen as resulting from lysosomal discharge, primarily of proteases, but also of low molecular weight cationic proteins and other, largely proteinaceous, mediators or injurious agents (16–23). For example, destruction of glomerular basement membrane in experimental nephrotoxic nephritis induced by antibodies directed against the glomerular basement membrane was shown to involve neutrophils and to be accompanied by release, in vivo, of neutrophil proteases (24). A mechanism of secretion of such materials subsequent to leukocyte adhesion to surfaces was proposed (25–27), which one of us colloquially termed frustrated phagocytosis. These types of studies, and particularly the concurrent investigation of protease involvement in tissue remodeling and arthritis (28), stimulated a large field of investigation of leukocyte proteases (see e.g., reference 29). Nevertheless, as favored agents of tissue injury they were soon eclipsed by the developing interest in oxygen metabolites.

We live in an oxidant-rich, oxygen-dependent environment bequeathed us by the biochemical miracle of photosynthesis. However, excess molecular oxygen and its metabolic byproducts can be highly toxic. These toxic products are formed normally in aerobic cells through a variety of metabolic reactions that are essential for the existence of the cell, including mitochondrial electron transport, reactions of mixed-function oxidases of the endoplasmic reticulum, cytoplasmic reactions of enzymes such as xanthine oxidase, and the like. Cellular protective mechanisms against these reactive products include the superoxide dismutases, catalase, and the glutathione peroxidase–reductase cycle. A variety of pathologic conditions has been proposed to result from accentuation of the intracellular metabolic processes that lead to formation of toxic oxygen metabolites, e.g., radiation damage and myocardial injury after infarction (5, 7, 8, 30). We have confined the discussion here to consideration of damage induced by oxidants released at a focus of inflammation; the common major source of these oxidants is phagocytic cells.

Phagocytosis-associated respiratory burst

That phagocytic cells produce potentially toxic metabolites of oxygen was first reported in 1961, using a system in which hydrogen peroxide (H_2O_2) was detected in the medium after exposure of neutrophils to particulate stimuli (31). It had been reported previously that neurotrophils exposed to opsonized microorganisms consumed oxygen from the surrounding buffer in a nonmitochondrial (cyanide-insensitive) event (32, 33). Thus, it was proposed that the oxygen consumed in this phagocytosis-dependent respiratory burst was converted to an agent capable of killing ingested microorganisms. That this impressive event was, in fact, pertinent to phagocytic killing of microorganisms was demonstrated convincingly by the experiment of nature, chronic granulomatous disease, in which absence of the respi-

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ratory burst was associated with markedly deficient killing of many species of bacteria and with life-threatening infections by those species (34–36).

The list of oxygen metabolites generated in the phagocytosis-dependent respiratory burst subsequently expanded to include hypohalides, especially hypochlorite (OCl^-), formed through activity of the azurophilic granule enzyme myeloperoxidase (37); superoxide anion (O_2^-) (38), now recognized to be the initial conversion product of the consumed oxygen; and hydroxyl radical ($\cdot OH$) (39, 40), a highly potent oxidant formed by the interaction of O_2^- and H_2O_2 in the presence of iron or copper (Haber-Weiss reactions) or between H_2O_2 and iron (Fenton reaction) (for review see reference 41). More recently identified are chloramines, formed by the reaction of hypochlorite with ammonia or amines (42). Other microbicidal products of the reduction of oxygen may be formed, but these have not yet been well substantiated.

The evolving knowledge of phagocytosis-associated oxidative metabolism was placed in the perspective of microbicidal activity, the unexamined assumption being that the products of the respiratory burst were released primarily into the phagocytic vacuole.

In the mid 1970's it became clear that a substantial fraction of the oxygen metabolites stimulated by phagocytosis were released to the outside of neutrophils (43). An in vitro model of tissue-bound immune complexes had played a central role in forming the concept that cellular damage at sites of inflammation might be due to release by neutrophils of toxic granule constituents to the outside of the cell (26). Using this same model, it could be demonstrated that both neutrophils (44) and monocytes (45) can release large concentrations of toxic oxygen species to the outside without cell lysis. Thus, the experimental basis for hypothesizing that granule enzymes and cationic proteins played a role in tissue injury was extended to invoke the possibility that toxic oxygen metabolites might also be involved (44). More direct demonstration of the participation of oxygen byproducts in tissue damage has ensued; but as we shall describe, the field has come full cycle, and the involvement of proteases and cationic proteins in inflammatory tissue injury is once again under intense study. Thus, it seems important to attempt to integrate current knowledge of the various proposed mediators of inflammatory tissue injury.

Injury

First, it would seem appropriate to define injury or damage; the phenomenon under scrutiny. These are terms that have been used generally and loosely and seldom with explanation or definition. Webster defines injury as "loss, pain, distress or impairment" and points out that the word injury is the least specific of a variety of synonyms. In the context of tissue effects, injury has to be considered from the standpoint of both functional and structural changes. For example, when pulmonary microvascular endothelial cells express ultrastructural blebs or projections after fixation and processing, is this, as is often claimed, endothelial injury? Unfortunately, we are only beginning to develop means to assess the function of such endothelial cells in vivo (45–60). Moreover, investigations to determine the degree of endothelial injury that results in release of endothelial constituents into the circulation, or defective uptake and metabolism of labeled molecules, are fraught with problems relating to alterations in vascular surface area (e.g., reference 48). Thus, in vivo we can often only guess at the presence of functional abnormalities at the cellular level.

It is easy to fall back on the obvious requirement that injury must represent structural alteration of a given degree, e.g., lysis of cells, denudation of epithelium or endothelium, blockage of a vessel or duct, or breakage or erosion of connective tissue elements. However, injury or damage in the clinical setting is much more subtle than this, and may often be manifested in functional changes without obvious structural counterparts. To the other extreme, functional damage can only be defined in the context of what is normal, and that subject itself is open to multiple and often subjective interpretations.

Suffice it to say that we would make a plea for more careful operational definition when using the terms injury and damage in studies of inflammatory effects on cells and tissues.

Biochemical effects of exogenously produced oxidants

Although we know that oxygen metabolites are toxic for cells in culture, leading to a variety of dysfunctions, such as inability to replicate, and to overt lysis, remarkably little is understood of the mechanisms of injury at the biochemical level. However, new interest in these mechanisms of toxicity, along with new methodology for their study, is developing. This would seem to be a field of investigation of great potential importance. We are gradually progressing beyond the point of talking about membrane perturbation or even lipid peroxidation (51, 52). By contrast, the specific lipids that are oxidized are being identified (52), and even more importantly, their effects on the physiology and biochemistry of the cell are being analyzed. Many questions arise. Are the oxidant molecules, or the products of their interaction with membrane lipids, responsible for changes such as altered ion fluxes and inappropriate phospholipase or protein kinase activation (53, 54)? Are these molecules responsible for alteration or fragmentation of nuclear DNA (55, 56); and does this proceed by logical, biochemical pathways to an extensive depletion of cellular ATP and consequent exhaustion of cellular energy (57)? Alternatively, are proteins (including structural molecules of the tissues) the targets of the oxidant effects (58, 59)? Much is already known of the effects of oxidation of methionyl residues in proteins (and peptides) in which this amino acid plays a critical role. For example, with $alpha_1$ antiproteinase (60), inactivation may allow uncontrolled and damaging effects of neutrophil elastase; with C5a (61), oxidation may serve as a beneficial inactivation process to limit the extent of neutrophil emigration into tissue. But what of the effects of methionine oxidation in proteins of the cell? Which proteins are most susceptible (see for example reference 62)? Are there other especially susceptible groups in proteins or other molecules (59)?

An interesting dilemma in studies of oxygen radical production by phagocytic cells is how such reactive species gain access to the extracellular milieu without expending themselves on susceptible chemical groups on the way out of the cell. To explain this, anion channels (63) and extracellular generation of the reactive species (64) by a transmembrane enzyme (65) have been invoked. For the purpose of this discussion, an equally relevant question is whether the extracellular oxidant radicals act only on the molecules of the external membrane of target cells. If they do, how are the effects transmitted to intracellular structures known to be damaged, e.g., the nucleus (55, 56)? The importance of external actions of oxygen metabolites is also emphasized by the potent protective enzymes and scavengers present in the cytoplasm of most mammalian cells (66–68).

It may seem of little interest to study how cells die. Nevertheless, one may argue that such detailed understanding of these intracellular processes are required to develop methods for prevention of oxidant effects.

Release of oxygen metabolites by phagocytes in tissues

When oxygen metabolites are released into the phagolysosome of neutrophils or macrophages, their effects are presumably confined to this site, where they are not likely to be harmful to the host. In regard to injury, however, we need to examine the conditions during phagocytosis in which oxidants are released to the outside. The aforementioned inefficiency of the phagocytic process, whereby cells that react with stimulatory surfaces too large to engulf discharge constituents at that site, may play an important role in tissue injury. Conditions that promote the binding of phagocytes and their stimulants to parenchymal cells and connective tissue elements (69) would therefore be expected to enhance injury resulting from this mechanism.

Because chemotactic factors might be expected to bind to tissue elements (70), would we not expect injury during emigration? Interestingly, in concentrations that are probably physiologic, chemotactic factors are very poor stimulants of oxygen radical formation (71). In fact, the receptors on neutrophils to initiate chemotaxis and the oxidative burst may be different, or at least in different affinity states (72, 73). This would explain the observations that neutrophils can accumulate in the lung without injury (measured morphologically and by lack of enhanced vascular permeability [74]), and that neutrophils can migrate through epithelial monolayers without altering transmonolayer electrical resistance (75, 76). By contrast, there appear to be circumstances in which additional priming factors render phagocytes capable of vigorously releasing oxygen radicals upon stimulation with chemoattractants (71). Bacterial lipopolysaccharides (endotoxins) are particularly effective in this regard, and this observation has stimulated an even greater interest in the participation of endotoxins in many forms of phagocyte-mediated tissue injury.

Demonstration of oxidant effects in vivo

A major problem in determining the involvement of oxidants in tissue injury is the difficulty of formally demonstrating such effects in vivo. In the test tube and culture dish it is easy to show the toxic effects. In the intact organ most investigators have relied on scavengers of oxygen metabolites to implicate these in the injurious processes (reviewed in references 9, 10, and 66). The inevitable complexity of the system being studied, questions of scavenger specificity, the ephemeral nature of the metabolites, the presence of multiple natural scavenger molecules in blood, and issues of local concentrations of scavengers at necessary sites, all lead to difficulties of interpretation in many of these studies, particularly if they are negative. Moreover, the oxidants may act indirectly to promote injury, for example by initiating generation of chemotactic factors (77).

More recently, important attempts are being pursued in a number of laboratories to demonstrate that oxygen byproducts are generated in tissues by detecting the products of their action (50, 78–82). That they have affected specific target cells can be determined, e.g., by measuring glutathione levels in such cells (83, 84). These experiments are difficult to carry out and interpret, but they have the potential of greatly clarifying the role of oxygen metabolites in injury to tissues. In particular, they should allow the determination of especially susceptible cell types, which, in turn, might permit determination of the molecular basis of the susceptibility, e.g., arrangement or composition of surface molecules, or lack of effective protective processes.

Proteinases and cationic proteins

Inflammatory reactions are highly complex, interacting and redundant processes. This redundancy applies as well to the injurious aspects of inflammation as to its protective role, as indicated by the marked inflammation, with injury, that occurs at sites of infection in patients with chronic granulomatous disease (36). Hence, it comes as no surprise that oxygen metabolites represent only one way, out of many, to injure cells and tissues. Proteinases and cationic proteins seem particularly important, and consideration herein of their participation in inflammatory tissue injury emphasizes the multifaceted character of the process and the cooperative actions between different injurious mechanisms.

The ability of phagocyte-derived proteinases to digest key structural elements of connective tissues has received much attention, for example in the joint and lung (28, 29, 85, 86). Less well studied, but currently receiving increasing scrutiny, are the toxic effects of phagocyte proteinases, especially elastase, on cells. Thus, endothelial monolayers in vitro have been shown to be susceptible to leukocyte elastases, which cause detachment (87), altered barrier properties (88), or frank lysis (69); and elastase infusion into isolated lungs causes increased vascular permeability (89). The further demonstration that primed and stimulated neutrophils can lyse cultured endothelial cells by mechanisms that are blocked by inhibitors of elastase and mimicked by the isolated enzyme (69), certainly lends credence to a potential role such a mechanism in cellular injury. However, this finding has yet to be confirmed or demonstrated in vivo. The problems with studying the action of proteases in vivo are considerable and are similar to those outlined for oxygen metabolites. Thus, neutrophil elastase has been detected in the lavage fluid from patients with the adult respiratory distress syndrome (ARDS)[1] (90, 91). Although much of the enzyme was already complexed with $alpha_1$ anti-proteinase, an injurious effect of the elastase before such inactivation was suggested (91). However, one could argue that the presence of this enzyme reflected no more than the known presence of large numbers of neutrophils in such lavages (92), and that if the enzyme were liberated after the altered vascular permeability event, and, thus after the $alpha_1$ anti-proteinase had accumulated, then it might have been inactivated before it had a chance to exert a deleterious effect. In support of this argument, deliberate instillation of leukocyte elastase into lungs causes emphysematous changes, which are not observed in patients recovering from ARDS.

The emigration of inflammatory cells may also be associated with injurious action of leukocyte-derived proteinases. Evidence is accumulating (93, 94) that neutrophils require proteolytic action to migrate through connective tissue barriers, e.g., in their passage out of the blood vessel. That this activity can be exhibited even in the presence of plasma antiproteases (69, 93) could indicate that neutrophils can express the enzyme activity locally at the site of cell–substrate contact (26, 95, 96).

Experiments performed more than twenty years ago implicated neutrophil-derived cationic proteins (peptides) in vascular

1. *Abbreviation used in this paper:* ARDS, acute respiratory distress syndrome.

permeability reactions in experimental animals (97–99) (as well as in the bactericidal properties of phagocytes [see reference 100]). It must be reemphasised that in our concept of injury, microvascular permeability may occur without the advent of cellular injury, even though overt endothelial damage will clearly also result in permeability. Nevertheless, the contribution of cationic peptides to tissue injury is of considerable interest and has received only sporadic investigation. Certainly, highly positively charged molecules such as polylysine or protamine are toxic to cells in vitro and in vivo (101–104) and induce vascular permeability (105, 106). These effects may involve alterations in surface charge and/or effects on membrane enzyme functions (107). Of more importance may be the degree and mechanisms of toxicity of naturally produced cationic molecules, including the leukocyte-derived peptides and polyamines (100). It should be noted in this regard that a number of phagocyte-derived enzymes, including neutrophil elastase, are highly positively charged and may act in part through their cationic nature, either directly, or by enhanced binding to cell surfaces. These interactions are likely to be fruitful areas of investigation.

Interaction between oxidants and other agents in tissue injury

Finally, it is worth questioning the potential interaction between oxidants and other injurious agents. Inactivation of alpha$_1$ antiproteinase by oxidants in tobacco smoke will diminish the antiproteinase screen, which has been suggested to contribute to the excess protease activity believed to be involved in the pathogenesis of emphysema (4). More prevalent, perhaps, might be local inactivation of the antiproteinase by oxidants from leukocytes (108), which might be expected to allow local uncontrolled action of enzymes released from those same cells. In conjunction with the expression of protease activity in a presumed protected site (between the phagocyte and its target), significant injury might ensue.

In another, less well defined area of interaction, oxidant effects on proteins might be expected to render them more susceptible to proteinases (109) that are more effective against denatured proteins. Thus, it would seem important to understand better the denaturing effect of oxygen metabolites. Similarly, effects of oxidants on membrane lipids may alter surface membrane properties to render proteins in them more susceptible to the effects of proteinases or cations. One of the attractions of these possible synergistic actions is their potential ability to explain the incomplete inhibitory actions of either proteinase inhibitors alone or scavengers of oxygen metabolites alone in many injurious circumstances in vitro or in vivo (e.g., reference 69).

Therapeutic implications

The concepts outlined above lead to some suggestions with regard to protection against cellular injury in vivo. However, a cautionary comment is appropriate. Massive stimulation of cellular production of oxygen metabolites, for example by ionophore or tetradecanol phorbol myristate (TPA or PMA), will certainly lead to cell injury in vitro, in isolated organs, and in vivo (reviewed in references 9, 10, and 66). However, natural stimuli are generally more subtle and, as indicated, may involve other mechanisms in addition.

The likely involvement of oxidants in inflammatory injurious processes raises questions about reducing the oxygen tension in blood or tissues as a beneficial procedure. The action of bleomycin on the lung is less in Denver at 5,000 feet than at sea level and is markedly enhanced by short (2-min) exposure to hyperoxia (110). However, production of oxygen metabolites by phagocytic cells is not so easily altered by manipulation of oxygen tensions (R. B. Johnston, unpublished observations). Such an approach may therefore be less helpful in inflammatory circumstances.

Nontoxic scavengers of oxygen metabolites, such as dimethylthiourea, dimethylsulfoxide (111, 112), or n-acetyl cysteine (3) might hold promise for protection in vivo. However, they would need to be used cautiously in view of the beneficial, protective effects of oxidants, and probably in combination with agents directed towards other toxic processes.

Additional emphasis should be given to natural scavengers. Are acute phase proteins such as ceruloplasmin (113) effective protectants in vivo? Erythrocytes have also been suggested to exhibit protective effects (114, 115). Can we manipulate these properties to our advantage?

What are the relative merits of administering extracellular scavengers for oxygen radicals, e.g., superoxide dismutase and catalase, compared with inducing persistence of these enzymes in body fluids (116) or attempting to stimulate increased intracellular levels of these and other protective enzymes (66, 117, 118)? Directed delivery of such enzymes encased in liposomes might also be considered (117). A prima faciae case was made above for the importance of the extracellular actions of toxic oxygen metabolites, and at this site the protective enzymes do not alter significantly the ability of phagocytes to kill bacteria (119). Similarly, an ability to specifically induce increases in protective enzymes in tissue cells at risk for oxidant damage would certainly be expected to protect without predisposing to infection.

An area that has not been explored systematically is suppression of the production, rather than actions, of toxic oxygen metabolites. Some anti-inflammatory drugs, e.g., corticosteroids, may work in part by this mechanism (120). Obviously there is risk for increased infections in this approach and a need to block the generation of oxygen metabolites specifically rather than by inhibiting stimulus–response coupling in general and, thereby, other cell responses. However, possible differences in the mechanisms involved in stimulation of neutrophil movement and phagocytosis, compared with the oxidative burst, suggest this to be a fruitful area for investigation.

Acting at a point after the initial synthesis of O_2^-, iron chelators are receiving considerable attention (2, 79) because they are presumed to interfere with the production of the especially reactive oxidant hydroxyl radical via iron-dependent pathways (9, 10, 121). In any event, strategies to prevent oxidant injury must take into account the protective antimicrobial function of phagocytic oxygen metabolites and maintain a careful balance between beneficial and harmful aspects of these processes.

Furthermore, these suggestions all focus on oxidant injury but, as indicated, we need also to pay attention to other possible causes of damage. For example, combinations of antiprotease agents, antioxidant therapy and scavengers of cationic materials (e.g., heparin) may well prove useful. It seems likely that because of the intersecting effects of multiple processes, no one inhibitor, scavenger, or antiinflammatory agent will represent the magic bullet.

Finally, but most importantly, at the cellular level we still know far too little about what constitutes injury, its pathogenesis and its repair. Without this information, approaches to therapy must still be considered to be largely empirical.

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