Mechanisms of Cellular Injury in Interstitial Lung Disease*

Peter M. Henson, Ph.D.

The pathogenesis of fibrosing interstitial pneumonitis is currently thought to involve an early but progressive inflammatory response and injury to parenchymal cells. This injury could derive from the inflammation, be independent of it, or even could induce an inflammatory reaction. Unfortunately, while these ideas set a useful framework within which to pursue investigation of this group of diseases, they are no more than broad brushstrokes, and certainly at this point do not constitute precise, testable hypotheses. Inflammation itself is a complex network of processes involving reciprocal interactions between circulating inflammatory cells and the blood vessels and parenchymal cells of the lung. These inflammatory cells may exhibit different forms of activation, produce different patterns of mediators, and accumulate in different proportions in different types and stages of inflammation.

The concept that the degree or permanence of cell injury, especially epithelial injury, contributes to fibrotic responses is of great current interest and derives from the work of Hakkinen et al and Terasaki et al. The latter's experiments may be used to illustrate this idea. Tracheal rings implanted subcutaneously remained intact unless they were deep epithelialized, whereupon fibrosis occurred. This fibrotic reaction was prevented by simultaneous inclusion of isolated epithelial cells. In essence these studies suggest a competition between reepithelialization and fibrosis. Too much epithelial damage, permanent injury to the type II cells (which repopulate the epithelium of the alveoli), or repeated deep epithelialization would, in this scenario, result in fibroblast replication and collagen deposition. Abnormalities of the basement membrane that might serve to prevent normal epithelial regeneration would easily fit within this hypothesis. To explore these ideas, we need to know the following: whether epithelial cells exert controlling influences on fibroblasts and by what means; whether the degree of epithelial damage indicates the extent of subsequent fibrosis; the contribution of endothelial injury to these processes; and, most important, the factors that control the rate and extent of type II cell proliferation.

It is important in such a discussion to consider the definition of "injury." The term is employed very loosely, often encompassing phenomena that in other circumstances might be considered physiologic. Usage also tends not to discriminate between changes in cells, vessels, or the whole lung. Permeability is commonly considered to be a manifestation of injury. This concept not only can be challenged in itself (for certain forms of permeability change) but often does not distinguish endothelial from epithelial alterations.

"Injury" must include both structural and functional elements, the former being easier to delineate. A strong plea can be made, therefore, to define operationally the terms injury and damage in writings on this subject. For the purposes of this discussion, cellular injury will indicate severe morphologic alteration up to and including lysis and detachment and inability to replicate.

Within the framework outlined above, mechanisms of cellular, and especially endothelial and epithelial injury would take on new relevance (Fig 1). Certainly, externally administered materials that promote pulmonary fibrotic reaction such as bleomycin and paraquat are cell toxins. Because they are thought to act via oxygen dependent processes, great emphasis has been placed on oxygen-radical mediated cellular toxicity. In experimental animals, local administration of tetradecanol phorbol myristate (TPA or PMA) produces fibrosis in the pulmonary parenchyma and pleural cavity. Since this agent is known to be an extremely potent stimulant to oxygen radical production (presumably by its activation of protein kinase C), coupled with some oxygen radical scavenger data, involvement of these oxygen-derived toxins in fibrotic processes has been inferred. Once again, this brings up the question of a role for the inflammatory process in lung cell injury because infiltrating inflammatory cells are prolific producers of oxygen radicals.

In this discussion, it is not crucial to enter into the controversy about the possible fibrogenic or antifibrogenic role of the polymorphonuclear neutrophil leukocyte in pulmonary fibrosis. More broadly, it seems reasonable to sug-

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gest that accumulation of inflammatory cells (including neutrophils) may under some circumstances (time course, environment, stimuli, etc) promote resolution of inflammation and under others might promote more injury and fibroblastic response. Given the long time frame of the human diseases, these issues will be hard to resolve. However, it does seem important to examine ways in which the inflammatory process, including that involving a number of different cell types, causes injury to lung cells.

Inflammatory cells have to bind to endothelial and epithelial cells in the course of their migration out into the air spaces of the lung (Fig 2). This occurs under the influence of specific stimuli to the migratory cells, the chemotaxins. It may therefore be questioned whether such stimulation and interaction lead to damage to the parenchymal cells over and through which the neutrophils, monocytes, etc, are migrating. The answer seems to be not necessarily. Neutrophils are able to accumulate into lavageable compartments of the lung without demonstrable alteration in permeability or overt morphologic evidence of injury (Worthen GS, Staub N, personal communications). In vitro, neutrophils can migrate through monolayers of epithelial cells without altering morphology or even sensitive electrical properties of the monolayer. These data suggest that inflammatory cell infiltration of the lung can occur without significant injury. In other words, inflammatory responses are often important and protective and are not automatically associated with malfunction and disease.

By contrast, in other circumstances, inflammatory cell accumulation certainly is related to cellular injury and pulmonary functional abnormalities. We have suggested that three factors contribute to this. First, quantitative issues (numbers of inflammatory cells) are important. Second, the time during which a stimulated inflammatory cell remains in contact with a lung cell is critical; and third, the nature of the stimulus must be considered.

In the system mentioned above in which neutrophils are induced to migrate through epithelial monolayers, no injury ensues if the neutrophils can continue to move on through the supporting filter. However, if the pores of this filter are too small to allow neutrophils to penetrate, they accumulate underneath, in direct contact with the epithelial monolayer, and cause a loss in the barrier functions of the artificial "epithelium." Similarly, if the neutrophils become strongly adherent to the luminal surface of such epithelial cells as a consequence of a phagocytic stimulus, epithelial integrity is also disrupted. Translated to an in vivo setting, circumstances in which inflammatory cells become trapped against or more persistently adherent to, endothelial or epithelial cells might be expected to result in greater degrees of "injury" (see Haslett et al, p 113S).

The nature of the stimulus gains prominence, when considered in the context of inflammatory cell "priming." Resting neutrophils, monocytes, and macrophages are in fact poorly responsive to chemoattractants and other nonparticulate stimuli with regard to production of oxygen radicals or secretion of proteases. Teleologically, this might again allow migration to occur without significant injury. When exposed to a variety of priming agents, however, much larger secretory responses are observed. Of particular importance as a priming agent is bacterial lipopolysaccharide (LPS), although other endogenous agents, including the phospholipid platelet activating factor, can also subserve this function. Conceptually, then, if leukocytes are primed by LFS, they will produce a much greater oxidative burst when exposed to inflammatory and chemotactic stimuli.

Since LPS also induces a neutrophil adhesion that is probably stronger and certainly more prolonged than that caused by the chemoattractants alone, the net result of LPS exposure is (1) prolonged adhesion to parenchymal cells, and (2) enhanced production of oxygen radicals and proteases. Together these might be expected to cause enhanced injury.

Early attempts to test this possibility have been encouraging. Bacterial endotoxin is known to produce lung injury in man and many animal systems. However, in rabbits, minute amounts of intravascular LPS (<0.05 ng/ml) act synergistically with neutrophil chemoattractants such as C5 fragments formed from C5 cleavage to induce vascular and epithelial permeability changes in the lung and morphologic evidence of endothelial damage. Neither agent alone at these concentrations produced these effects, and the increased vascular permeability was dependent on the presence of circulating neutrophils. It is important to emphasize that such studies do not exclude the possibility of an additional direct toxic effect of LPS (especially at higher concentrations). Additionally, the interactions are described here to exemplify a phenomenon. It would be expected that other agents, which induce leukocyte adhesion, priming, and activation, could act similarly. A part of particularly interesting candidates for some of those effects are the peptide interleukin 1 and the phospholipid, platelet activating factor.

These synergistic actions of neutrophils primed with, for example, LPS, and stimulated with chemoattractants have been further pursued in vitro. Cultured endothelial cell monolayers were disrupted (overt cytolsis was the end point) by incubation with these combinations. No 2 of the agents together were effective, and evidence was presented that the effects of the LPS and chemoattractant were exerted predominantly on the neutrophil. In combination with
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DOSAGE AND ADMINISTRATION

The usual dose of TORNALATE is two inhalations at an interval of at least one to three hours.

ADVERSE REACTIONS—The most common adverse reactions reported with TORNALATE are associated with the respiratory tract, including cough, dyspnea, pharyngitis, and laryngitis. Other adverse reactions that have been associated with beta-adrenergic agents include nervous system events such as nervousness, tachycardia, palpitations, tremors, and tremors.

The effects of TORNALATE may last up to eight hours or longer. It should not be used more than twice a day. In patients with chronic obstructive pulmonary disease, the use of TORNALATE is not recommended.

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earlier reports of neutrophil toxicity to endothelial cells\(^8\) or of detachment of endothelial cells induced by neutrophils,\(^9\) these data suggest that leukocytes can cause significant endothelial cell alterations in vitro, even if they are not inherently toxic under normal stimulatory conditions, and support the extensive literature on in vivo effects. It should be emphasized here that studies with mononuclear phagocytes in systems such as this are needed, as are more investigations of effects on pulmonary epithelial cells.

Earlier studies of cytotoxic actions of inflammatory cells focused on lymphocyte toxicity in the immune reaction and/or antibody-dependent cellular cytotoxic reactions. These immunologic reactions cannot be discounted in interstitial lung disease. Kravis et al\(^a\) showed immunologic reactivity to type I collagen in I.LD. The well-known association of immune complexes with these diseases and the newer data on lymphocyte accumulation and activation all lend support to the possibility of lymphocyte-dependent toxicity. However, we do not know the antigens or targets. Moreover, the mechanisms of injury by T cells or natural killer cells are still unclear or at least controversial. For example, is the immunoglobulin seen in the alveolar walls in patients with I.LD on the endothelial or the epithelial surface, and, if so, does this promote antibody-dependent toxic reaction to that cell type? It would seem fruitful to pursue investigations of lymphocyte-(or monocyte-)-dependent injury in I.LD along with the basic mechanisms by which such damage is caused.

The actual mechanisms by which the inflammatory cells damage the endothelium or epithelium are still unclear. Much emphasis has been given to oxygen radicals.\(^2\) However, other agents are receiving attention in recent studies, and the whole picture may turn out to be highly complex. Part of the problem lies in difficulties of studying these processes in vivo and the need to use inhibitors or scavengers whose specificity is often problematic. The ephemeral nature of these toxic agents and problems of local action (and the presence of potent plasma scavengers) make this even more difficult to study. Nevertheless, a wealth of indirect data support oxygen radicals as important agents of inflammatory cell toxicity. Newer investigations are probing the actual mechanisms by which such radicals injure cells\(^a\) and are beginning the important task of showing that such molecules were in fact generated in the lung in vivo.

A second series of materials that have been involved as promoting tissue injury are cell-derived proteinases. For example, these have been suggested as one mechanism of lymphocyte-dependent toxic reactions.\(^3\) In the above-mentioned experiments on neutrophil-dependent injury to cultured endothelial and epithelial cells, the toxic reactions could not be attributed to oxygen radicals but were inhibited by low molecular weight inhibitors of neutrophil elastase.\(^3\) Neither the major plasma inhibitor of elastase \(\alpha\)-antiprotease nor plasma itself prevented the toxicity. This could explain why injury can occur in vivo. It may be postulated that inflammatory cells that are closely adhered to endothelial or epithelial cells release or express proteases at these points of adhesion which exclude molecules of the size of \(\alpha\)-antiprotease (see E. Campbell, p 138). These concepts are supported by the finding that "primed" neutrophils express more extracellular elastase when stimulated,\(^4\) and that some of this may remain associated with the cytoplasmic membrane. Clearly, these proteases are also likely to contribute to disruption of elements of the pulmonary connective tissues and basement membranes.

A final group of agents that may contribute to the toxic reactions are the cationic proteins found in phagocytic inflammatory cells. Eosinophil basic proteins are known to be toxic to airways epithelial cells.\(^5\) However, neutrophil cationic proteins may also contribute to tissue alterations. They certainly can alter vascular permeability.\(^6,7\) Additionally, some of the granulocyte proteinases are themselves cationic, which may promote their binding to target cells and perhaps produce direct toxic effects. These are areas that should be further investigated.

This short list of groups of cell-derived toxic agents is not meant to be exclusive. Significantly absent are phospholipases, membrane-active lipids, and complement-like hydrophobic membrane-inserting proteins. Nevertheless, it should serve to emphasize that there is more than one way to kill a cat, or lung cell.

**Conclusions**

It is suggested that parenchymal cell injury represents a key element in the initial and progressive alteration in fibrosing interstitial lung diseases. The direct causes of such injury are not yet known, but inflammatory processes could be the initiators (as well as representing an amplifying consequence of the damage). Inflammatory cells can induce injury and lysis to endothelial and epithelial cells in vitro and in vivo. At this point most work has focused on the neutrophil, and in a different context, the lymphocyte. Areas for further investigation should therefore include study of the toxic potential of other inflammatory cells in the tissue culture dish as well as in the whole animal (and man), and, to determine in the arena of interstitial lung diseases, the involvement of immunologic toxic reactions involving either antibody, lymphocytes or both.

The fundamental mechanisms by which inflammatory cells injure their targets is a further area of developing and important interest. Emphasis should certainly be placed on oxygen radicals but not to the exclusion of alternative or additional processes. Of particular importance, for example, might be possible denaturing effects of oxygen radicals on cell surface proteins, which would render them more susceptible to proteases.

Finally, it would seem unlikely that complex, progressive, and prolonged disease processes such as those exemplified by the fibrosing interstitial pneumonitides can be explained by simple schemes or single toxic events or sequences. The studies of acute events mentioned above are important and are certainly likely to be relevant on an individual cell-to-cell basis. However, the whole disease must be appreciated in a completely different time frame, allowing checks, balances, and feedback loops of which we have had but the most superficial glimpses. Here are the future frontiers in the study and the treatment of these diseases.

**References**

Accumulation of Lung Tissue Oxidized Glutathione (GSSG) as a Marker of Oxidant Induced Lung Injury*

Carl W. White, M.D.; Robert F. Mimmack, B.S.; and John E. Repine, M.D.

Oxidants have been implicated recently in diverse models of interstitial lung disease (ILD) including injuries caused by bleomycin, asbestos, and anthracyclic drugs. These oxidants may be produced endogenously by lung cells (anthracyclic drugs) or exogenously by inflammatory cells that have been recruited and/or activated in the lung. If oxidants do have primary and/or facultative roles in causing ILD, then markers of lung tissue oxidation are needed to help determine basic mechanisms of ILD and purported interventions.

The importance of the glutathione redox cycle as a defender of lung cells against hydrogen peroxide and other oxidative injuries is increasingly well recognized. It seems likely that a metabolite of the glutathione system (oxidized glutathione, GSSG) could provide a marker of oxidant induced injury. More specifically, since optimal function of the pentose phosphate shunt and glutathione reductase systems is needed to maintain cell glutathione in the reduced state (GSH), and since very little lung glutathione is present in the oxidized (GSSG) form under normal conditions, GSSG may be a useful marker of oxidative stress in lung tissue (Fig 1). Our hypothesis was that exposure to hyperoxia would promote excessive production of oxygen metabolites and lead to accumulation of oxidized glutathione (GSSG) in the lung. To test this premise initially, we chose hyperoxia-induced lung injury, which is similar to some models of accelerated ILD in that an early phase of endogenous oxidant production occurs and is followed by a stage of chronic alveolar inflammation, which may facilitate additional lung injury.

**MATERIALS AND METHODS**

Following injection with saline or antioxidant enzymes (polyethylene glycol [PEG]-attached superoxide dismutase [SOD] and catalase [CAT]), male Sprague-Dawley rats (350–400 g) were exposed to hyperoxia (>99% O₂, 1 atm) or to normoxia. After 54 hours of exposure, rats were anesthetized (pentobarbital, 110 mg/kg). Tracheostomies and thoracotomies were performed. Following heparin (150 U) administration via the pulmonary artery, catheters were placed and lungs were rapidly perfused blood-free and freeze-clamped at liquid nitrogen temperature. Measurement of oxidized and total glutathione in lung tissues was done using recently described tissue preparation and assay methods. In additional rats' pleural effusion volumes were measured, and lungs were lavaged with saline for measurement of albumin concentrations.

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