Proteases and Lung Injury
A State-of-the-Art MiniReview

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HISTORICAL PERSPECTIVES

It is widely supposed today that the destructive changes in lung parenchyma associated with pulmonary emphysema are mediated, in large part, by unrestrained proteolytic activity in lung connective tissue. This condition is thought to arise whenever elastolytic proteases, or combinations of such enzymes acting synergistically with other proteases, are released from lung cells and are ineffectively downregulated by endogenous proteinase inhibitors in the lower respiratory tract. Margiinized neutrophils, resident alveolar macrophages, and (more recently) freshly recruited mononuclear leukocytes in transit between blood capillaries and alveolar air-spaces have all been implicated as likely sources of lung-damaging elastases. Alpha-proteinase inhibitor (a-Pi, alpha-antitrypsin), present in alveolar secretions as a transudated protein from the pulmonary circulation, and low molecular weight bronchial mucus inhibitor, a product of local secretory cells of the pulmonary epithelium, have been implicated as important regulators of neutrophil and monocyte elastases.

The hypothesis that an imbalance between the foregoing factors, favoring protease activity, is pathogenetic in emphysema rests largely on the signal observations of Laurell and Eriksson and Gross and colleagues (now reproduced in many other laboratories). Laurell and Eriksson recognized that selected cases of early-onset familial emphysema were associated with heritable a-Pi deficiency, while Gross et al showed that intrapulmonary instillation of elastolytic proteases produced anatomic derangements in experimental animals characteristic of human emphysema. (Others have recorded characteristic physiologic derangements in these models, as well.) Recent progress in this area has been exciting and has been summarized at several major symposia over the last decade. However, protease-antiprotease imbalance as the mechanism of alveolar effacement in most forms of emphysema remains to be proven.

HIGHLIGHTS OF RECENT DEVELOPMENTS

Mononuclear Phagocytes; Role in Elastase Homeostasis

Newer studies have pointed to several mechanisms by which mononuclear phagocytes can affect the homeostatic balance between elastolytic enzymes and their inhibitors in the lung tissue. First, cultured human alveolar macrophages secrete small amounts of a metallo-enzyme with weak elastin-degrading activity. Secretion of the enzyme is constitutive (not increased by several stimuli, including cigarette smoke). Human macrophage elastase is not inhibited by a-Pi; mouse macrophage elastase even degrades and inactivates this important inhibitor. Second, alveolar macrophages of cigarette smokers generate increased amounts of oxygen radicals and peroxide, substances which can inactivate a-Pi by oxidation (see following section). Third, bloodborne monocyte precursors of the macrophage appear to contain small amounts of a serine elastase with many shared properties of neutrophil elastase (the enzyme that most previous workers have implicated in destructive lung disease). This enzyme appears to be lost during maturation of the monocyte, but may be secreted by newly-recruited monocytes while en route between lung capillary and alveolar air-space. The foregoing represent potentially adverse (tissue-damaging) roles for mononuclear phagocytes in elastase homeostasis. At the same time, these cells (when differentiated into macrophages) also synthesize and secrete inhibitors of neutrophil elastase (a-Pi, a-Pi2) and they also internalize the neutrophil enzyme (as well as other neutrophil glycoproteins) through receptor-mediated endocytosis. These last activities are potentially beneficial (tissue-protective) with respect to elastase homeostasis. Clearly, more work is required before the true role of mononuclear phagocytes in the pathogenesis of emphysema can be appreciated.

Oxidative Inactivation of Elastase-inhibitors

While genetic a-Pi-deficiency may account for some forms of emphysema, the bulk of patients with the disease are genetically normal for this major elastase inhibitor. A potential link between their disease and protease-antiprotease imbalance was suggested by the observation that a-Pi can be inactivated by cigarette smoke in vitro, implying that smokers may develop an acquired deficiency in a-Pi, locally, at sites of accumulation of tobacco smoke residues in the lung. The mechanism appeared to be based on oxidation of the inhibitor. These findings stimulated many subsequent studies, with sometimes conflicting results, but over the last 5 years, a number of potentially important findings have emerged. Aalpha-Pi contains methionine in its elastase-combining site; oxidation of the active-site methionine by chemical or by cell-derived oxidants (including those from pulmonary macrophages) decreases the affinity of the inhibitor for neutrophil elastase. Indeed, a new animal model of a-Pi-deficiency has become available, based on systemic treatment of dogs with the chemical oxidant, chloramine-T. The capacity of different brands of commercial cigarettes to inactivate a-Pi in vitro is directly proportional to the oxidizing activity of their aqueous smoke solutions, and addition of hydrogen peroxide (1 product of activated macrophages in smokers) to such smoke solutions greatly augments both the oxidizing and the a-Pi-inactivating capacities of smoke extracts. Bronchopulmonary lavage fluids obtained from chronic human smokers show decreased activity of a-Pi/µg inhibitor protein, provided that lavage effluents are collected a short time after cigarette smoke inhalation. In 1 such study, oxidized methionine residues were detected in a-Pi recovered from smokers’ lung washings, but not in a-Pi from control nonsmokers, while no evidence of gross molecular alterations in smokers’ a-Pi could be found (Table 1). These findings have been reviewed and discussed in greater detail elsewhere. Further studies are now required to confirm the observations reported above and to clarify their possible pathophysiologic role. Newer data bearing on this topic, some of it contradictory, will be presented at the present meeting.
Table 1—Studies on \( \alpha_{\text{Pi}} \) in Smokers' and Nonsmokers' Pooled BAL* Fluids

<table>
<thead>
<tr>
<th>Category</th>
<th>No.</th>
<th>Male</th>
<th>Female</th>
<th>Age (yr)</th>
<th>Smoking history (pk-yr)</th>
<th>( \mu g ) PE inhibited per ( \mu g ) ( \alpha_{\text{Pi}} )</th>
<th>MetSO mol/mol of inactive ( \alpha_{\text{Pi}} )</th>
<th>Ag Ab EP of ( \alpha_{\text{Pi}} )</th>
<th>SDS-PAGE of ( \alpha_{\text{Pi}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>24</td>
<td>11</td>
<td>13</td>
<td>25±6.8</td>
<td>0</td>
<td>0.59±0.08</td>
<td>0</td>
<td>Charge (mobility) and antigenic behavior of ( \alpha_{\text{Pi}} ) indistinguishable; no complexes detected in either BAL pool</td>
<td>mol wt (migration index) indistinguishable</td>
</tr>
<tr>
<td>Smokers</td>
<td>26</td>
<td>17</td>
<td>9</td>
<td>28±3.2</td>
<td>18±3.5</td>
<td>0.34±0.10†</td>
<td>3.8±0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data modified from Carp et al (PNAS 1982; 779:2041-45)

*BAL, bronchoalveolar lavage; PE, pancreatic elastase (measured enzymatically using Ac-Ala-Ala-p-nitroanilide as substrate); \( \alpha_{\text{Pi}} \), \( \alpha_{\text{Pi}} \)-proteinase inhibitor (measured by radial-immunodiffusion); MetSO, methionine sulfoxide (measured as methionine in amino acid analyzer after cyanogen-bromide cleavage of \( \alpha_{\text{Pi}} \) and acid-hydrolysis under reducing conditions). Ag Ab EP, crossed-antigen-antibody electrophoresis against anti-human \( \alpha_{\text{Pi}} \) antiserum; SDS-PAGE, polyacrylamide gel electrophoresis after boiling in the presence of 0.3% Na-dodecylsulfate and 0.1% 2-mercaptoethanol. See reference 27 for further details of all the foregoing methods.

†Significantly different from nonsmokers' value, \( p<0.05 \) (t-test)

Immunochemical Assays of Elastin Degradation

A basic tenet of the protease hypothesis of emphysema holds that lung elastin breakdown is accelerated in this disease and that this process eventually culminates in alveolar wall destruction.** This is certainly true in animal models of the disease produced by protease instillation, and emphysematous human lungs have also been found to contain reduced amounts of elastin.** Recently, progress has been made in developing sensitive immunochemical assays for measuring rates of elastin degradation in experimental animals and man. These methods should allow a direct test of the assumption that lung elastinolysis is increased in emphysema (or at least in its early stages). With refinement, such assays may also facilitate screening of individuals at risk for development of emphysema and they may even prove useful in monitoring future treatment modalities involving protease-inhibitor therapy. The presently available methods depend on raising antibodies to elastin peptides** or to cross-linking amino acids unique (in mammals) to elastin, such as desmosine.** A state-of-the-art symposium on this topic was held recently and will be published elsewhere.**

CURRENT MAJOR QUESTIONS; PREVIEW OF SESSION

Is the Mononuclear Phagocyte and/or the Neutrophil the Major Source of the Lung's Elastase "Burden"?

Although our understanding of macrophage biology has advanced considerably, the role of these cells (and of their monocyte precursors) in the pathogenesis of emphysema is still unclear. Several of the speakers in the present session are confronting these questions in one or another way and perhaps their contributions today will shed new light on this important matter. Thus, Dr. Hoidal has explored the effects of neutrophil-depletion in hamsters upon the development of experimental emphysema induced by intratracheal cadmium chloride. He will present evidence suggesting that these cells may not be obligate mediators of lung injury in his model. Does this result, by default, further implicate the mononuclear phagocyte in this disease? Dr. Campbell will describe his latest studies on sequestration of active neutrophil elastase by alveolar macrophages, on its release after hypoxic cell-injury and he will address the implications of these processes for the macrophage's role in lung injury. Dr. Sandhaus' presentation will concern the elastase of human monocytes (a neutrophil-type enzyme) and its replacement, during cell maturation, by the metallo-elastase characteristic of mature macrophages. Perhaps his findings can be of help in assessing the role of freshly recruited monocytes in the pathogenesis of destructive lung disease.

Some as yet unpublished observations from our own laboratory may be of interest in the present connection. Using a radioimmunoassay for urinary desmosine to monitor the rate of elastin-degradation in the body** we compared excretion of this elastin breakdown-product in normal individuals and in 2 patients with cyclic neutropenia. We were interested in learning whether normal elastin catabolism is influenced by cyclic depression of circulating neutrophils. Desmosine excretion was not decreased in either patient during periods when their peripheral blood neutrophil counts fell to undetectable levels. These results suggest that (in PIM subjects) the basal turnover of elastin in normal, uninflamed connective tissue is probably mediated by enzymes derived from sources other than the neutrophil. However, neutrophils may still play an important role in accelerating elastin-turnover, following their emigration into inflamed or injured tissues and may even affect basal elastin turnover in PITZ subjects.

Is Oxidation of \( \alpha_{\text{Pi}} \) a Physiologically Significant Consequence of Chronic Smoking in Man? If So, Do Endogenous Anti-oxidants Modulate Lung Injury in Smokers?

Conflicting results have emerged recently regarding the pathophysiologic significance of oxidative damage to \( \alpha_{\text{Pi}} \) in smokers. On the one hand, several laboratories have reported decreased functional activity of \( \alpha_{\text{Pi}} \) in lung washings obtained from smokers** or have found direct evidence of oxidized \( \alpha_{\text{Pi}} \) in such fluids** or indirect evidence of oxidized \( \alpha_{\text{Pi}} \) in smokers' serums.** On the other hand, other workers have failed to confirm a loss of \( \alpha_{\text{Pi}} \) activity due to smoking in man (Stone et al, this meeting; Bieth et al, personal communication). We will hear Dr. Stone's results in the present
session. It may be essential to control rigorously the time interval between last cigarette smoked and lung washing, since we found earlier that α1-Pi activity or pancreatic elastase is diminished during the first 2 hr after acute cigarette smoke inhalation in rats, but returns to control values by 4 to 6 hours (unpublished observations). In the aforementioned positive studies,⁵⁶,⁵⁷ volunteers were allowed to smoke up to the time of lung washing; by contrast Bieth et al⁵⁸ used volunteers who had not smoked for 24 hr prior to lavage. Interestingly, neither Cadek et al⁵⁹ nor Carp et al⁶⁰ found any inactive α1-Pi in nonsmokers' lung washes, whereas both Stone and Bieth report that approximately one-half of the α1-Pi present in their nonsmoking controls is inactive. This additional discrepancy requires explanation. (It may be worth noting that inactive α1-Pi was not found in lung washings of control subjects during a recent study of ARDS patients.⁶¹ This work will also be reported in the present session [Cochrane].)

Should the "oxidant-hypothesis" be confirmed by future work, it will be important to determine if oxidation of other susceptible amino acids in the inhibitor takes place in smokers, in addition to the methionine oxidation already observed.⁶² Some circumstantial evidence which favors this possibility will be presented below (see footnote ‡ in Table 2). Equally important will be a study of the role of anti-oxidant systems in the lung and in serum, especially their possible modulation of protease-antiprotease homeostasis in smokers. Several laboratories have already begun such investigations and, later in this Conference, Dr. Taylor will discuss variations in ceruloplasmin and other antioxidants among healthy smokers and COPD patients.⁶³

Another potentially important antioxidant described recently,⁶⁴ which has aroused the attention of emphysema investigators because of its ability to reduce oxidized methionine in proteins (including chemically-oxidized α1-Pi⁶⁵) and to reactivate the latter, has been studied recently by Dr. Carp in my laboratory. This enzyme, methionine sulfoxide peptide reductase,⁶⁶ has several interesting biologic properties, a few of which are summarized in Table 2. However, to date, we have been unable to implicate the reductase in resistance to emphysema among smokers (Table 2). Our studies will be reported in greater detail elsewhere.⁶⁷

Are There Effects of Cigarette Smoking on Elastin Synthesis Which Might Contribute to Emphysema Development?

Chronic cigarette smoke inhalation exacerbates emphysematous lesions induced by endotracheal elastase in hamsters (Dr. Niewoehner will present this finding in this morning's session). One contributory factor which might help explain the foregoing observation could be interference with lung elastin-repair mechanisms in smoke-exposed animals. Dr. F. Laurent, working in my laboratory, and our collaborator, Dr. Kagan at Boston University, have obtained data (which I will present after Dr. Niewoehner's report) showing that water-soluble components of the gas phase of filtered cigarette smoke inhibit formation of covalent desmosine cross-links during conversion of tropoelastin to elastin in vitro. These same smoke components also suppress lysyl oxidase-catalyzed oxidation of lysine ε-amino groups in elastin (the chemical step preceding formation of all elastin cross-links, including desmosine) in a dose-dependent fashion. However, gas phase smoke does not block the oxidation of diananimopentane by lysyl oxidase. Thus, gas phase cigarette smoke possesses substrate-directed (rather than enzyme-directed) inhibitory components capable of interfering with elastin cross-linking in vitro. Similar effects occurring in smokers' lungs could impede elastin repair and contribute to the development of pulmonary emphysema, since repair of elastin (following protease attack) stabilizes lung connective tissue and limits the degree of anatomic deformity in this disease.⁶⁸ Although our data do not permit conclusions regarding cigarette smoke-induced inhibition of elastin cross-link formation in vivo, Osman et al⁶⁹ recently showed that chronic exposure of hamsters to whole, unfiltered

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**Table 2—Some Biological Properties of Methionine-sulfoxide Peptide Reductase in Mammalian Species (modified from Carp et al⁶⁶)**

<table>
<thead>
<tr>
<th>Property</th>
<th>Test Material</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution in cells and tissues</td>
<td>whole lung homogenate (rat, man)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>type II cell lysate (rabbit, rat)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>neutrophil lysate (rat, man)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>alveolar macrophage lysate (rat, man)</td>
<td>no</td>
</tr>
<tr>
<td>Capacity* to reactivate α1-Pi</td>
<td>α1-Pi oxidized with chloramine-T⁺</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>α1-Pi oxidized with myeloperoxidase + H₂O₂ + Cl⁻</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>α1-Pi exposed to cigarette smoke⁻⁺</td>
<td>no‡</td>
</tr>
<tr>
<td>Does the enzyme show induction</td>
<td>whole lung homogenate</td>
<td>no</td>
</tr>
<tr>
<td>after adaptation to oxidant-injury?</td>
<td>type II cell lysate</td>
<td>no</td>
</tr>
<tr>
<td>(2 ppm O₃ × 4 hr, rat)</td>
<td>neutrophil lysate</td>
<td>no</td>
</tr>
<tr>
<td>Does methionine sulfoxide reduc-</td>
<td>PMN lysates from nonsmokers (8)</td>
<td>119 ± 56‡</td>
</tr>
<tr>
<td>tase activity of PMN lysates vary</td>
<td>PMN lysates from healthy smokers (6)</td>
<td>87 ± 36‡</td>
</tr>
<tr>
<td>directly with resistance to COPD?</td>
<td>PMN lysates from former smokers with COPD (4)</td>
<td>135 ± 44‡</td>
</tr>
<tr>
<td>(man)</td>
<td>PMN lysates from nonsmoking PM subjects with early-onset emphysema (3)</td>
<td>124 ± 24‡</td>
</tr>
<tr>
<td></td>
<td>PMN lysates from smokers with bullous emphysema (2)</td>
<td>133 ± 10‡</td>
</tr>
</tbody>
</table>

*Sources of enzyme tested for reactivating activity were: human neutrophil lysate (for α1-Pi treated with chloramine-T or myeloperoxidase or cigarette smoke), human lung homogenate (for α1-Pi treated with chloramine-T); rabbit type II cell lysate (for α1-Pi treated with chloramine-T).
†Reactivation of chloramine-T-treated α1-Pi by neutrophil enzyme was inhibited by excess methionine sulfoxide.
‡Inactivation of α1-Pi in vitro by cigarette smoke is prevented by antioxidants such as thymol⁷⁰ or catalase⁷¹. Therefore, failure of methionine sulfoxide peptide reductase to reactivate smoke-treated α1-Pi might be explained if α1-Pi exposed to cigarette smoke undergoes oxidation of other amino acids (tyrosine? lysine?) in addition to methionine.

PICOMOLES N-acetyl methionine sulfoxide reduced/hr/mg PMN lysate protein (± 1 SDM)

†Not statistically significantly different from one another or from nonsmokers' values (p<0.1)

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Lung Defense, Injury and Repair

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cigarette smoke significantly suppressed incorporation of 3H-lysine into newly synthesized lung elastin, during the repair phase following elastase-mediated lung injury.

Some Suggestions for Future Study

It clearly will be important to unravel the role of the mononuclear phagocyte in lung elastase homeostasis (especially the part played by monocytes and immature macrophages). In this connection, newer techniques permitting resolution of alveolar macrophage subpopulations may be of value. It will likewise be important to clarify the pathophyslogic significance (if any) of elastase-oxidation in smokers' lungs. Also, the newly discovered effect of smoke on elastin synthesis requires confirmation, and immunochemical tests for elastin degradation fragments need to be refined to improve sensitivity and prove reproducibility. Perhaps one good way to approach these questions would be to evaluate the parameters presently suspected of being important in the pathogenesis of emphysema, in individuals with radically different susceptibilities to the disease. Thus, one might compare older individuals relatively free of lung disease despite extensive smoking histories with younger PiMM individuals who have early-onset emphysema despite not smoking heavily. These subjects should be compared with respect to the following: (a) monocyte and neutrophil elastase content; (b) elastase secretion by cultured alveolar macrophage; (c) antioxidant enzymes and cofactors in plasma, lung lavage fluids and alveolar macrophages; (d) O2 and H2O2 generation by resting and stimulated alveolar macrophages; and, (e) elastin-synthesis rates in fibroblast cultures (derived from skin or lung biopsies) with and without the addition of aqueous cigarette smoke solutions.

ACKNOWLEDGMENT: I wish to thank Dr. S. Y. Yu (VA Hospital, St. Louis) for kindly supplying 115I-labeled desmosine used in some of the studies described in this article.

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The Role of Tissue Repair and Leukocytes in the Pathogenesis of Emphysema*

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An obstacle to understanding the role of inflammatory cells in the pathogenesis of emphysema has been the lack of a practical and economic animal model. The classic models have employed intratracheal instillation of exogenous proteases to evaluate the pathogenesis of emphysema. In these models, host phagocytes do not contribute to the production of emphysema and, in fact, phagocyte depletion has exacerbated the lesions. An ideal model would employ cigarette smoke. However, with the possible exception of dogs, attempts to produce emphysema in animals exposed to cigarette smoke have met with limited success.

Exposure to cadmium salts has been reported to cause emphysema-like lesions in animals although the results have been conflicting. CdCl₂ aerosol produced centrilobular lesions. An initial inflammatory lesion occurred in the region of the terminal bronchioles followed by repair with ultimate distortion and dilatation of airspaces surrounding respiratory bronchioles. Although pulmonary function and connective tissue measurements were not done, the resultant lesion resembled human centrilobular emphysema. When CdCl₂ solutions were instilled endotracheally, a similar series of events ensued, and resulted in a lesion similar to that of human paracase atrial emphysema. It has been suggested, therefore, that CdCl₂ models may more closely mimic the pathogenesis of the disease in smokers than the models based on the administration of exogenous elastase.

The current experiments were designed to clarify the relationship between lung phagocytes and the development of emphysema. In the initial experiments, CdCl₂ was instilled intratracheally into hamsters. Hamsters exposed to CdCl₂ developed an acute predominantly neutrophil (PMN) response in the lungs with an increased phagocyte-derived oxidant and elastase load. However, despite the pronounced oxidant and elastase load, the dominant lesion was fibrosis. Furthermore, subsequent measurements on excised lungs demonstrated increased elastic recoil and reduced lung volumes consistent with a restrictive pattern of lung dysfunction.

An explanation for the absence of emphysema may be that while connective tissue destruction was occurring, the structural defects were limited by the ability of the host to synthesize new connective tissue fibers. Prominent fibrosis present in all of the models of CdCl₂ exposure supported this possibility. It was recently reported that the feeding of lathyrogens, β-amino-propionitrile (BAPN) or penicillamine, resulted in a marked worsening of elastase-induced emphysema when compared with the effect elastase in animals fed a normal diet. These lathyrogens inhibit the cross-linking of collagen and elastin and thus impede the formation of new connective tissue.

Experiments were therefore designed to evaluate the importance of the repair process. Hamsters were fed either a regular diet or the same diet supplemented with BAPN. As in the earlier experiments, hamsters fed the regular diet who received CdCl₂ developed an acute inflammatory reaction which evolved into lung fibrosis and a restrictive pattern of lung dysfunction. In striking contrast, animals on BAPN-supplemented diet who received CdCl₂ developed morphologic and functional changes of bullous emphysema. No abnormalities were observed in animals which received BAPN but no CdCl₂.

The role of PMN in the production of emphysema was evaluated by selective mediator depletion. Specific anti-neutrophil antibody (ANS) was administered starting 2 days before instillation of CdCl₂. ANS produced a profound neutropenia and prevented PMN recruitment into the lungs. This effect was sustained by repeated administration of ANS for 1 week following CdCl₂ administration. In the absence of PMN, the elastase load recovered in lung lavage of CdCl₂-BAPN treated animals was profoundly diminished. Despite this effect, the same degree of emphysema developed in

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