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Collagen Biosynthesis

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Collagen is the major structural protein of the lung. At least five genetically distinct collagen types have been identified in lung tissue. However, the precise role of collagen in nonrespiratory lung function is not well understood, in part because of the difficulties inherent in studying lung collagen, regardless of the type of assay used. A major problem is the insolubility of lung collagen; generally less than 20% of total lung collagen can be solubilized as intact chains, even with harsh extraction procedures. Since such collagen may not be representative of total lung collagen, errors in quantitating collagen types, for example, may arise from using such material. Measurement of total lung collagen content may also pose problems, unless appropriate parameters of normalization are chosen. Biopsy dry weight, protein content, and DNA content, for example, may all change in certain disease states. Despite these difficulties, a number of changes in lung collagen have been documented in experimental pulmonary fibrosis, including increased collagen content, increased collagen synthesis rates, and changes in collagen type ratios. Many questions remain. For example, why do diverse toxic substances appear to cause essentially the same fibrotic response, even though initial sites of damage may vary? Conversely, why do similar toxic substances, such as ozone and NO_2, cause diverse responses (fibrosis and emphysema, respectively)? Much work remains to be done to elucidate the mechanisms underlying the lung's choice of response.

Introduction

Collagen biosynthesis in the lung is a remarkably complex process that subserves several functions. In the normal lung of a healthy individual, several genetically distinct collagen types (Table 1) are the primary component of the acellular structural matrix. In the lungs of individuals exposed to toxicants, interstitial and basement membrane collagens may be degraded; other collagens may be synthesized as "scars" to fill the areas formerly occupied by cells and matrix components. "Scar" collagen may be qualitatively (structurally) different from "normal" collagen; it may be laid down focally in different regions of the lung than collagen normally occupies, and the total amount of collagen in the lung may also be altered after toxicant exposure. In the present paper we focus on documented and speculative changes in the quantity and/or the quality of lung collagen in animals exposed to toxicants under more or less controlled conditions.

A brief description of the complex pathways of biosynthesis leading to the structurally diverse members of the collagen family is in order before we discuss changes in lung collagen. This topic is periodically well reviewed, and several excellent recent sources at various levels of detail may be recommended (*1–4*). As far as we presently know, two of the seven or more collagen types that have been described (types I and III) have been shown to constitute more than 95% of the total lung parenchymal collagen. A simplified scheme for the biosynthesis of these interstitial collagens is indicated in Figure 1. It should be emphasized that most of the synthetic steps involving these two interstitial collagens are common, catalyzed by the same enzymes. In addition, it is known that, at least in tissue culture, fibroblasts can simultaneously synthesize both types of collagen (*5*, *6*). In terms of understanding toxicant effects on the lung, key biosynthetic steps in Figure 1 include hydroxylation of proline and lysine residues, secretion of triple-helical soluble procollagen from cells, and lysyl oxidase-catalyzed reactions leading to collagen crosslinking and fiber formation. We do not know the relative importance of the different cell types in the lung for collagen synthesis and deposition; fibroblasts are often assumed, on the basis of analogy rather than data, to be the important cell type for these steps.

Type II collagen, the characteristic collagen of cartilage, is also present in the lung; presumably it is found only in the cartilaginous rings supporting the trachea and large bronchi. Type IV, or basement membrane collagen, comprises less than 1% of the total lung collagen based on the lung content of 3-hydroxyproline, thought to be a marker for this collagen type (*7*, *8*). The extreme insolubility and low concentration of type IV collagen in lung have made it relatively difficult to study. Thus, we do not know a great deal about this collagen type, except by analogy with other, more accessible basement membrane collagens. Type V, also referred to as type AB collagen, is known to be present in lung parenchyma (*9*). Its exact role and the relative

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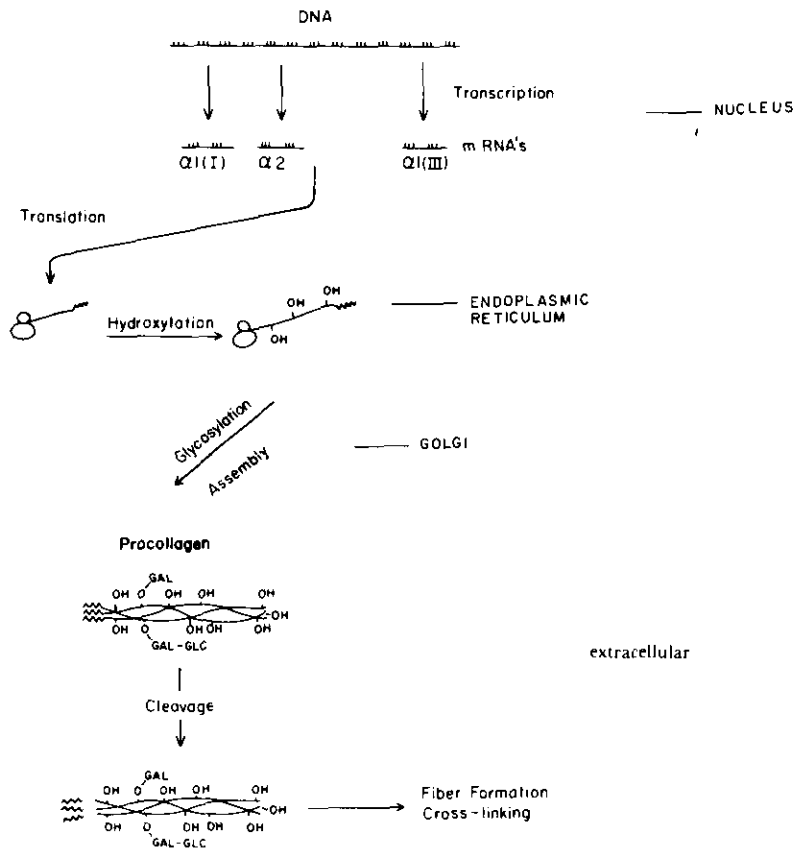

FIGURE 1. Schematic diagram illustrating the steps involved in biosynthesis of the monomer collagen molecule.

contributions of parenchyma, interstitium, and vasculature to its content are not well understood. Type I trimer, $\alpha_1(I)_3$, has not as yet, to our knowledge, been shown to occur naturally in the lung, although its occurrence in certain pathological states might be inferred from its synthesis by various cell lines, including fibroblasts, under certain conditions in tissue culture (*10,11*).

Biochemical studies on lung collagen may be crudely divided into three types. In the first type, the assay used for quantitation of total lung collagen is measurement of 4-hydroxyproline (a more or less specific marker for total collagen content). In the second type, some fraction of "soluble" (or extractable) collagen (usually less than 30% of the total lung collagen, often less than 5% of the total) is quantitated, usually by assay for hydroxyproline. In the third type, collagen types are investigated using methods that are rigorous (usually using the technique of CNBr peptide mapping to study total lung collagen or using tissue culture techniques to produce soluble procollagens for further study). It is important to note that only in the third type of assay is collagen, as such, identified and characterized; in the first two types of assay, hydroxyproline level is equated with collagen content. Physiological measurements related to lung collagen have all been crude, based on unproven assumptions about the relationship of lung collagen to various aspects of pressure versus volume curves generated with air- or saline-filled lungs. Histological and morphological evaluations have also generally been crude (based on the use of "specific stains" for collagen, the specificity of which is assumed to be the same in edematous, pathological lungs as in normal lungs), cruder (based on impressions from hematoxylin/eosin-stained preparations), and crudest (based on nonrandomly sampled lung sections evaluated descriptively by investigators with preconceived notions of what they were looking for). Quantitative histological or morphometric studies on randomly sampled lung sections are rare indeed.

Selected studies using lung biochemistry, physiology, and/or histology as endpoints will be discussed in detail. The focus throughout will be on how rigorous the data actually are.

Collagen Biosynthesis

Several genetically distinct collagens have been isolated from various vertebrate sources; presumably, all of them may be found in the lung (Table 1). As far as we know, the biosynthetic pathways for the protein component of all of these diverse collagens are the same. Their differences reflect differences in their primary structure (amino acid sequence), which, in turn, depends

Table 1. Collagen types and their structural features.

Collagen type	Tissue sources	Structure	Hydroxyproline: proline ratio	Level of glycosylation: % Glycosylation	Level of glycosylation: Hydroxylysine residues/1000	Other features
I	Skin, bone, organs, etc.	$\alpha_1(I)_2\alpha_2$	0.8	< 20	6–8	—
II	Cartilage	$\alpha_1(II)_3$	0.8	~50	20–25	—
III	Similar to Type I	$\alpha_1(III)_3$	1.2	15–20	6–8	Contains cysteine
IV	Basement membranes	$\alpha_1(IV)_3$	1.6	80	60–70	About 2% of total residues are 3-hydroxyproline
V	Basement membranes, endothelial cell-rich areas	$\alpha A\alpha B_2$	1.2	~60	~40	Not yet well characterized; similar to type IV
α_1(I) trimer	Tissue culture of various cell types, skin	$\alpha_1(I)_3$	0.8	20	~5	—
Others?	Basement membranes of various organs	?	—	—	—	Not yet characterized adequately to prove their uniqueness

upon the sequence of nucleotides in their messenger RNAs, i.e., in the information encoded in the DNA sequences constituting their structural genes. An additional source of difference between collagen types stems from variations in the complex series of post-translational modification steps that the various collagens undergo, as will be discussed below. As pointed out in Table 1, characteristic differences between the various collagen types include the level of hydroxylation of proline and the level of glycosylation of selected hydroxylysine residues in the collagen chains. The extent of these modifications, which are characteristic for each collagen type, is also presumably under genetic control, mediated by the primary structure of the peptide backbone of the collagens. The primary structure determines the folding of the peptide chains during their synthesis, which, in turn, controls the access of enzymes involved in post-translational modifications to the amino acid residues they might potentially modify.

Post-translational modifications of the collagens may be conveniently divided into three types, based on where they occur. Like all proteins destined to be secreted by a cell, the collagens are synthesized as precursor forms or procollagens containing an *N*-terminal extension (or "signal" peptide) on their peptide chains. The signal peptide is responsible for attachment of ribosomes upon which collagen is being made to the endoplasmic reticulum. Thus, we can examine post-translational modifications occurring as the nascent collagen chains are extruded through the endoplasmic reticular membranes into the lumen, changes occurring as the chains traverse the Golgi apparatus and are packaged for secretion, and changes occurring extracellularly after the collagen chains are secreted. During extrusion through the membrane of the endoplasmic reticulum, the most important modifications involve hydroxylation of specific residues of proline and of lysine in the nascent collagen by membrane-bound enzymes, prolyl and lysyl hydroxylases, respectively. Next, the nascent chains of interstitial collagens are selectively glycosylated with either galactose or glucosylgalactose at specific hydroxylysine residues. By analogy with other secretory proteins, collagen glycosylation presumably occurs in the Golgi apparatus. Basement membrane collagens are much more extensively glycosylated than other collagen types; these complex changes, which are not well characterized, take place at unknown locations within or outside the cells. Packaging of the collagen chains into vesicles to prepare them for secretion also occurs in the lumen of the endoplasmic reticulum. It is thought that alignment of the collagen α chains and subsequent formation of the characteristic triple-helix occurs within secretory vesicles. This process is thought to be guided by extension peptides (both N-terminal and C-terminal) that may aid in registration of the chains via formation of S-S bonds between cysteine residues.

Extracellular modifications of collagens that are of greater importance include proteolysis, crosslinking, and fiber formation. The important proteolytic steps, which involve removal of the N- and C-terminal extension peptides from collagen types I, II and III (less is known about the processing of types IV and V), are mediated by specific procollagen peptidases for each end of the collagen chains. The resultant triple-helical collagens are resistant to further digestion by proteases other than specific collagenases under physiological conditions. Crosslinking of collagen chains, both intra- and intermolecular, is an extraordinarily complex process both in terms of the chemistry involved and in terms of our understanding of how the process is controlled. For types III, IV and V collagen, persistence of intramolecular disulfide bonds between cysteine residues on separate α chains is an important source of intramolecular crosslinks. Types I and II collagen do not contain cysteine in their fully processed forms;

hence, disulfides are presumably not important crosslinkers in the mature collagen of these types (although S-S bonds do play a role in orientation of these procollagens to form triple-helical collagen prior to processing by procollagen peptidase). Of more importance is the role of lysine-based crosslinks in collagen structure. These crosslinks are the probable sole source of intermolecular covalent linkages; they play a role in intramolecular associations as well. The key step in formation of this type of crosslink is the oxidation of (hydroxy)lysine residues by an extracellular enzyme, lysyl oxidase, to give rise to reactive aldehydes of lysine. These reactive aldehydes can react with properly placed amino groups on other amino acids (usually of other lysine residues) to form Schiff bases, which are in themselves covalent crosslinks. They are relatively unstable, however, and may undergo further reactions to generate many other types of covalent crosslinks. Stabilized by various intermolecular interactions, including covalent crosslinking and interaction with noncollagenous matrix components, the mature interstitial collagen molecules can orient themselves into ordered fibers, with characteristic macroscopic structure and paracrystalline subunits. The characteristic interchain spacing in such fibers is responsible for the so-called segment long-spacing (SLS) periodicity, a particular pattern of uptake of negative stain during transmission electron microscopy often used to identify collagen fibers anatomically.

Lung Collagens

Our knowledge (such as it is) of lung collagen structure and diversity has been acquired very recently, in part because of the extreme insolubility of lung collagen. Less than 1% of adult lung collagen is soluble in salt or acetic solutions, the conventional extraction solvents used; even in very young animals fed lathyritic agents such as β-aminopropionitrile, it is difficult to solubilize more than 5% of the total lung collagen. Thus, the lung is a very difficult organ in which to study collagen synthesis and deposition. However, as techniques have become available to by-pass the problems of extraction and solubility, the scientific community has become attracted to the biochemical and toxicological study of collagen in the lung. While some workers still publish experiments dealing with qualitative or quantitative effects of toxic agents on soluble collagen extracted from animal lungs, their conclusions are of dubious validity; they are extrapolated from observations made on a pool of lung collagen that may constitute less than 0.1% of the total collagen present. A good rule of thumb when evaluating such studies is to check whether data are given that allow one to calculate if the sum of the soluble collagen and the insoluble collagen equals the total collagen. If so, is the amount of total collagen thus calculated a reasonable amount? Crystal (*7,8*) suggests that about 15 to 20% of the dry weight of lung parenchyma is collagen; thus, approximately 3 to 4% of the wet weight ought to be collagen. The total collagen content of the lung from a 100-g hamster is about 6 mg (11% of the dry weight) (*12*), as determined by an analytical method (autoclaving the lung and extracting the resultant gelatin) that probably underestimates the true collagen content. In our hands, the collagen content of the lung from a normal rat weighing 300 to 350 g is about 20 to 25 mg (about 10% of the lung dry weight). The lung of a 20-g mouse contains about 1.2 mg of collagen.

Types I, II and III collagen have been identified in the lung by direct isolation and by documentation of their biosynthesis *in vitro* by organ cultures of the lung (*7*). They have also been identified by immunological techniques *in vivo*, in which specific collagen antibodies bind to the chains *in situ* (*9*). Types IV and V collagen have been identified by immunological techniques in the lung, but their isolation and/or characterization as a product of collagen biosynthesis have proven much more difficult. Trelstad recently introduced a technique for isolation of types IV and V collagen involving removal of the bulk of the interstitial collagens by heat gelation, followed by analysis of the residual collagen that does not form fibrils at 37^{0}C (*13*). This technique has also been used for isolation of types IV and V collagen from the lung (*14*), but much work remains to be done before the material prepared by the heat gelation technique is rigorously characterized. This approach is hampered by the lack of appropriate standards to define types IV and V collagen from sources other than kidney, eye, and placenta, by possible organ-specific differences in these collagen types, and (as always with collagen) by the extreme insolubility of these collagen types in the lung.

Many workers who have studied collagen biosynthesis by lung *in vivo* or *in vitro* have seen radioactive bands upon polyacrylamide gel electrophoresis that have been tentatively identified as type IV or type V collagens based on their mobility (apparent molecular weight) on these gels. Given our lack of appropriate standards and the complexities of interpretation introduced by the potential presence of higher molecular weight precursor forms of types I and III collagen in such preparations, prudence dictates caution in the identification of putative collagen types based only upon their apparent molecular size on gel electrophoresis.

How does lung collagen change in fibrotic diseases? Intuitively, one would assume that a fibrotic lung would have a higher collagen content. However, early studies (*7,8*) on the collagen content of lung biopsies from patients dying of idiopathic pulmonary fibrosis showed no significant change. A potential source of error in collagen content determination of biopsy material arises when the collagen content is normalized to such parameters as biopsy dry weight, protein content, or DNA content. In certain disease states, these parameters may also increase. Indeed, recent data from animal models and from humans dying of adult respiratory distress syndrome (*15*) clearly document elevated levels of hydroxyproline in fibrotic lungs. On the other hand,

acute lung fibrosis (whether in animal models or in respiratory distress syndrome) may differ significantly from a chronic disease of insidious onset such as idiopathic pulmonary fibrosis with respect to lung collagen content. Further work is necessary to resolve this question.

The observation of normal collagen content in lungs from patients with idiopathic pulmonary fibrosis led to the postulate that the obvious changes in these lungs could be explained by a remodeling process; that is, a change in the relative ratios of the two primary collagen types (I and III). Kang and co-workers (*16*) initially observed that there was a significant increase in the ratio of type I collagen relative to type III collagen in the lungs of patients dying of this disease. Such a finding is consistent with mechanical and histological changes in these lungs. Type I collagen is less compliant than type III; hence, a higher percentage of type I collagen could result in a stiffer lung. Histologically, "collagen-specific" stains visualize only type I collagen, at least in normal lung tissue. Hence, we can also invoke collagen type switching to explain why more collagen is appreciated histologically (at least focally) in fibrotic lungs, without the necessity of invoking an increased collagen content.

Recently, we have demonstrated in several animal models of pulmonary fibrosis induced by ozone, paraquat, and bleomycin (*17*) that the increased amount of lung collagen being synthesized *in vitro* is indeed enriched for type I collagen; the type I/type III ratio shifts from the normal value of 66/33 to about 85/15. We have also shown a similar shift of collagen types being synthesized *in vivo* in lungs of mice exposed to the fibrotic agents butylated hydroxytoluene and oxygen (*47*). Thus, at least in acute pulmonary fibrosis, we feel that there occurs both increases in total lung collagen and shifts in the collagen types being synthesized by the lung. Until appropriate animal models are developed to test this hypothesis, this mechanism should not, perhaps, be generalized to chronic lung fibrosis.

How Not To Do It: Common Errors in Lung Collagen Research

As pointed out previously, one outstanding type of error is to study a small subfraction of the total lung collagen and to assume that changes observed in this pool are an accurate reflection of changes in total lung collagen. This is a major source of potential error when collagen extracted from a normal lung is compared to that extracted from an inflamed edematous lung replete with proteolytic enzymes, including collagenolytic activities imported with leukocytes, and other abnormal constituents of the pathological tissue. For example, Giri et al. (*18*) and Zuckerman et al. (*19*) impart significance (statistical and mechanistic) to changes in the solubility of lung collagen in 0.45 M NaCl. Such changes (apparent increases in collagen solubility from 1% to 2% of the total) are taken to indicate important underlying changes in lung collagen metabolism, rather than potential artifacts of isolation of a minute, ill-defined subfraction of the total collagen.

A more sophisticated version of the same type of error may occur when the subfraction chosen for analysis is soluble collagen after proteolytic digestion, usually with pepsin. Such treatment releases some of the otherwise insoluble collagen from matrix components by digestion of crosslinks and/or the terminal regions of α-chains, where the crosslinking residues are most prevalent. This fraction may amount to as much as 20-50% of the total lung collagen (*17,20*), based upon recovery of total lung hydroxyproline. It contains various peptide fragments in addition to intact collagen, the extent of degradation depending on the severity of the incubation conditions (temperature, duration, etc.) with pepsin. This is a particularly insidious potential source of error, since a substantial percentage of the total collagen (*20–50%*) is solubilized, and one is thus tempted to assume it is representative of the total. In fact, we ourselves have previously made this error (*21*). We have subsequently documented (*17*) that the type I/type III ratio of newly synthesized collagen shifts from the normal value of 66%:33% to values of 80–85%:15–20%, as determined by CNBr peptide mapping techniques in several animal models of pulmonary fibrosis. However, as shown in Table 2, analysis of pepsin-solubilized newly synthesized lung collagen either by electrophoresis on polyacrylamide gels or by column chromatography on carboxymethyl cellulose could lead one to the erroneous conclusion that the ratio of collagen types had not shifted, apparently due to preferential solubilization of type III (or preferential losses of type I) collagen in these assays. A further problem is that while 20 to 50%

Table 2. Collagen type ratios of pepsin-solubilized collagen synthesized in vitro by rat lung minces.

Lungs prepared from rats exposed to	% of collagen recovered that is type III	
	Carboxymethylcellulose chromatography	Polyacrylamide gel electrophoresis
Controls ($n=9$)	28.6 ± 2.7	—
Control	33	35
Paraquat	—	26
Paraquat	26	23
Ozone	27	32
Ozone	28	—

of lung collagen may be soluble in acetic acid after pepsin digestion, the buffers used for gel electrophoresis or especially for carboxymethycellulose column chromatography are not as good solvents for collagen. Thus, an unknown subfraction of the solubilized material is present in the gel band or column fractions in this type of analysis. Another potential problem that must be borne in mind is the possible preferential loss of intact type III collagen, which sometimes does not completely enter polyacrylamide gels, even under reducing conditions (conditions designed to prevent S-S bond formation, a type III collagen-specific crosslink).

Another variation of this type of sampling error is to find measurements of soluble collagen plus insoluble collagen that fail to add up to a reasonable value for total collagen (*18, 19*). Total collagen content of the lung from a normal hamster initially weighing about 100 g at the start of an experiment that lasts 30 days is about 6 mg/lung, equivalent to about 11% of the dry weight of these lungs (*12*). If we assume an average value of about 15% hydroxyproline for lung collagen content (14% for type I and 18% for type III) (*8*), 6 mg of total lung collagen would be equivalent to 0.9 mg (900 μg) of lung hydroxyproline. Reported total hydroxyproline values of 210 μg/lung (*18*) and 360 μg/lung (*19*) suggest some sort of error in either assay technique or in arithmetic. Thus, changes in these values may be due to analytical artifacts rather than to any experimental procedures performed.

A final type of sampling error involves the use of the differential salt solubility of various collagens as an analytical technique. Soluble collagens in large quantity (gram levels) can be partially purified, and the various collagen types can be partially separated by precipitation under carefully controlled conditions of pH and salt (NaCl) concentration. The key word to be noted in the previous sentence is partially. Differential salt precipitation is not a valid quantitative tool, especially when the quantities of soluble collagen obtainable from lungs of small animals are to be measured. For example, Huang (*22*) and Madri and Furthmayr (*9*) report the relative content of type III collagen in human lungs to be about 60% based on salt fractionation as an assay; these workers are clearly in error in that the correct ratio is about 33% type III (67% type I). Their error is due to the use of such invalid methods as salt fractionation of 20 to 30% of the total lung collagen, the maximal amount they could solubilize in the required buffers, and the erroneous scoring of some type I collagen as type III, presumably due to cross-contamination of the fractions obtained.

Another class of analytical error arises from ignoring the effects of inflammation/edema on the determination of lung hydroxyproline (collagen) levels. For example, precursor pool sizes may change in an inflamed/edematous lung, as we have discussed in detail elsewhere (*23*). Failure to account for such differences in the level of a precursor (e.g., proline) in damaged lungs could cause gross underestimates of the amount of collagen synthesized by lungs, evaluated either *in vivo* (*24*) or *in vitro* (*25–31*). Furthermore, while increases in unlabeled proline secondary to inflammatory edema will predictably cause underestimates of true collagen synthesis rates, a potential error in the other direction also can occur. If a larger percentage of the total bolus of labeled proline injected into the animal (usually IP or IV, so it is delivered to the lung essentially in a single pass via the vasculature) enters the lung (due to capillary leakage, edema, or changes in transvascular pressure) in an experimental animal than in a control, then collagen synthesis rates will be overestimated due to the elevated specific activity of the precursor in lungs of experimental animals. Thus, since the failure to account for possible changes in precursor pool size may cause errors (as high as three- to fourfold!) that give either false high or false low values for collagen synthesis rate, one cannot ignore this effect on the basis of assuming any error made is in the "correct" direction.

The problem of lung inflammation/edema also may affect the colorimetric assay for hydroxyproline in a manner independent of any effects upon the proline pool size in the lungs. Hydrolysis in 6 N HCl of lung tissue, especially tissue that is edematous or contaminated with blood, has given us false high values in this assay, possibly caused by colored impurities arising from heme in the samples that absorb light at the same wavelength as the pyrrole chromophore. For example, the apparent increased level of hydroxyproline we have reported (*32*) in lungs of rats exposed for three days to 0.5 ppm of ozone may be due to a false high value in these (slightly) edematous lungs, rather than to true increases in lung collagen content after these relatively mild exposures. Gross contamination of lung extracts with blood, on the other hand, interferes with the assay giving spurious low values. We find this assay to be completely reproducible for lung tissue only if we homogenize the perfused tissue and precipitate the proteins with trichloroacetic acid to remove soluble substances prior to assay.

A final class of errors in analysis of lung collagen may be collected under the rubric of inappropriate choice of technique. Due to the high insolubility and peculiar chemical and physical properties of collagen, the "quick and dirty" approach may, perhaps, be the worst possible choice in this field. Examples of this type of egregious error include the use of the Lowry procedure (vintage 1941) for gravimetric estimation of collagen by its conversion to gelatin after the entire insoluble protein fraction of lung is autoclaved (*31,33–35*). The problem with this approach is that the so-called collagen (gelatin) fraction is heavily cross-contaminated with degradation products of noncollagenous proteins (elastin, proteoglycans, and others) and that highly crosslinked insoluble collagens (especially those other than type I) may not be completely or reproducibly extracted. These problems are especially true for lung collagen. A popular variant technique, alleged to be "specific" for collagen, is extraction of insoluble lung proteins with

hot (90°C) 5 to 10% trichloroacetic acid (*25,27,36*) to prepare a soluble putative collagen fraction. In fact, noncollagenous proteins are extracted and extraction of collagen is not quantitive (*37*).

In short, then, two procedures should be followed when analyzing lung collagen. First, in any extraction or fractionation procedure the yield in both split fractions should be determined (usually by hydroxyproline assay) to ensure that the sum of the parts is indeed 100% of the whole (which, for whole lung, ought to be determined as a reference value for one's own animals and laboratory procedures). Second, any purification or extraction of putative soluble collagens should be monitored by gel electrophoresis for intactness of extracted collagens and confirmation of their identity. Since collagens in solution tend to aggregate into insoluble complexes, such bookkeeping and analyses are often important to avoid inadvertant discarding of desired material (*38*).

Effects Of Toxicants on Lung Collagen

Primarily because of the relative ease of doing the experiments, we know a lot more about changes in lung collagen after acute (days or weeks) exposure to lung toxicants than we do about experiments performed chronically (months or years). Upon exposure of the lung to high levels of a toxic agent via the airways (ozone, oxygen, bleomycin, silica, etc.) or the circulatory system (paraquat, bleomycin, ipomeanol, nitrofurantoin, etc.), the critical response seems to be an inflammatory influx of macrophages and leukocytes to sites of injury, with accompanying edema. If the animal survives this phase, then conventional wisdom suggests that fibroblasts are subsequently attracted to these sites, and that they in turn secrete the collagen that constitutes the fibrotic "scar." In point of fact, we know nothing about the relative roles of resident lung fibroblasts, fibroblasts recruited to the sites of injury and cells other than fibroblasts in the synthesis of collagen in either normal or injured lungs. We assume the importance of fibroblasts by analogy with studies of injuries to other tissues and organs, especially wound healing in the skin. To the best of our knowledge, the requisite careful cytomorphological studies to identify and quantitate fibroblasts (and other cells) at sites of lung injury over a specified time course in a well-defined animal model of pulmonary fibrosis have not yet been published. The difficulties of uniquely characterizing fibroblasts at the light microscopy level would make this a very difficult study to do, as electron microscopy or cell-specific antibodies would be required.

There are fascinating hints throughout the literature of factors produced by macrophages, by lymphocytes, and by neutrophils that may be positively chemotactic for fibroblasts, that may stimulate fibroblast proliferation, and that may specifically stimulate fibroblast collagen synthesis. These putative factors will be discussed in more detail below. Clearly, an essential component of our understanding of the lung inflammatory response that we presently lack is a comprehension of the qualitative and/or quantitative role(s) of such putative factors in mediating the transition from inflammation to fibrosis.

Special toxic agents reviewed in this volume, including bleomycin, ipomeanol, paraquat, oxidant gases (especially ozone and oxygen), silica, asbestos, and various radioactive materials, all may provoke a fibrotic response in the lung. Such a response is initially characterized by inflammatory edema, with accumulation of fluid and cells (pulmonary alveolar macrophages and various types of leukocytes) near the site(s) of injury. If overwhelming doses of the toxic substances are given, the animals die of drowning or systemic toxic effects in the first few days after administration of the toxicant. This early phase of the response usually peaks at 3 to 4 days after toxicant exposure and usually subsides at about 7 to 10 days if exposure is not continued or does not recur.

Conventional wisdom suggests that the influx of pulmonary alveolar macrophages to the site(s) of injury (or of particle deposition) is responsible for the release of chemotactic factors that, in turn, are responsible for the recruitment of fibroblasts to these sites (*39*). It has specifically been suggested that macrophages that have ingested silica particles may also release substances that stimulate fibroblasts to produce collagen, or to produce more collagen than their baseline synthesis levels (*39*). Other cells involved in various components of the inflammatory response also seem to be able to release substances chemotactic for one another, among them lymphocytes, granulocytes, and eosinophils (*40*). To complicate matters even further, some of the these same inflammatory cell types also seem to be able to release factors that stimulate fibroblast mitogenesis (*41*), which in turn increases the capacity for collagen synthesis at such sites. The interplay of chemotactic, mitogenic, and collagen synthesis-stimulating factors in teh etiology of organ fibrosis is a poorly understood area presently under very active investigation.

Conventional wisdom also suggests that collagen synthesis in an organ such as lung is the responsibility of fibroblasts, as is the abnormal deposition of collagen. In truth, however, we know little about which cell types are responsible for the synthesis of lung collagen, either in normal or in toxin-exposed tissues. We do know that (theoretically at least) the genetic information for the synthesis of all types of collagen resides in all eukaryotic cells. We also know that cultured cells of the vasculature, including endothelial cells (*42*), can synthesize various collagen types (*43*), that lung fibroblasts can synthesize collagen types I and III, and that airway cartilaginous rings can synthesize type II collagen (*8*). Various interstitial cells may also synthesize collagen. A prime candidate is the pericyte, which is morphologically similar to the myofibroblast. The myofibroblast is known to make both types I and III collagen and has been implicated in the increased collagen synthesis charac-

teristic of early stages of wound healing (*44*). Obviously other poorly characterized lung cell types may also contribute to the lung collagen content.

Perhaps we ought not to be asking why a given agent causes pulmonary fibrosis, but rather why all agents that cause lung inflammation/edema do not cause this disease? Certainly bacterial or viral pneumonia mimics the early stages of such a response without culminating in pulmonary fibrosis. On the other hand, cigarette smoke and other factors that ultimately can give rise to emphysema also can mimic this early inflammatory response, with a large-scale accumulation of pulmonary alveolar macrophages at local sites of injury. Clearly, our understanding of the term "inflammation" is not yet adequate to predict its end-stage result in the lung in cellular or molecular terms. A paradoxical example of our level of understanding of these processes (or the lack of understanding) is the case of ozone versus nitrogen dioxide effects on the lung. Chronic exposure of animals to relatively high levels of ozone causes pulmonary fibrosis, while exposures to relatively high levels of NO_2 reportedly result in emphysema (*45*). Yet, ozone and NO_2 are both gases with about the same molecular weight (and, hence, diffusibility and density) and the same potential as strong oxidants to generate free radicals at sites where they interact with cell membranes or the lung lining layer. Both gases penetrate to the deep lung. The only major difference between these substances (from a lung toxicant point of view) is their differing solubility in water: ozone is about ten times as water-soluble as NO_2. We can refine our question about why all agents that cause lung damage do not cause pulmonary fibrosis with respect to NO_2 and ozone: why do two very similar substances, both thought to have similar modes of action, selectively cause emphysema or fibrosis, respectively? The only difference we can discern between these two gases is their locus of interaction with the lung. Due to its lower solubility, NO_2 tends to penetrate deeper on the average, and more NO_2 than ozone (theoretically) passes through the small airways to interact directly with the alveolar epithelium. We simply cannot explain, either in cellular or molecular terms, how the inflammatory process might differ regionally within the lung so as to allow such different outcomes of apparently identical initial insults (lipid peroxidation?) to epithelial cells in different lung compartments. This argument is a bit oversimplified, in that NO_2 exposure may cause pulmonary fibrosis in addition to emphysema (*46*), but the basic paradox (lack of understanding?) remains that there exist apparent regional differences within the lung in what we think of as a stereotyped response to injury.

Conversely, we might also ask why we get the same result (pulmonary fibrosis) when we damage the endothelial cells with, for example, paraquat or high levels of oxygen as we do when we damage epithelial cells with, for example, ozone or butylated hydroxytoluene. Again, we really cannot explain, in cellular or molecular terms, why the end-stage response to damage of such diverse cell types is the same. The role of the integrity of the basement membrane and the resident population of reparative stem cells in determining the ultimate response of the lung to injury by a toxicant at a specific site is probably important in this context. Yet, again, except in descriptive terms, we understand essentially nothing about the factors that regulate the interplay of the different cell populations, either with each other or with the complex acellular matrix in which they are embedded. Whether the glue that holds cells to each other and to the basement membrane is an important target for lung toxicants is an unexplored area.

In summary, we know that diverse toxic substances can cause essentially the same fibrotic response of the lung to injury, even though their initial sites of damage may be either to epithelial or to endothelial cells. Conversely, we know that similar toxic substances such as ozone and NO_2 can cause diverse responses (fibrosis and emphysema, respectively) of the lung to injury, even though their initial sites of damage are to epithelial cells. We simply do not understand, except on the most superficial of levels, why the lung's responses are either the same or different to different toxic agents.

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