Collagen polymorphism in idiopathic chronic pulmonary fibrosis.

J M Seyer, …, E T Hutcheson, A H Kang

*J Clin Invest.* 1976; 57(6): 1498-1507. [https://doi.org/10.1172/JCI108420](https://doi.org/10.1172/JCI108420).

Collagens in normal human lung and in idiopathic chronic fibrosis were investigated in terms of their covalent structure and compared for possible alterations in the diseased state. Collagens were solubilized by limited digestion with pepsin under nondenaturing conditions, and after purification they, were fractionated into types I and III. Carboxymethylcellulose and agarose chromatography of both types I and III collagens, and amino acid and carbohydrate analyses of the resulting alpha-chains indicated that the alpha 1 (I), alpha 2, and alpha 1 (III) chains of normal human lung were identical with the human skin alpha-chains in all respects examined except that the normal lung chains contained higher levels of hydroxylysine. Examination of collagens obtained from the diseased lung revealed that the content of hydroxylysine of the alpha 1 (I) and the alpha 1 (III) chains appeared to be diminished as compared to the normal lung chains. The values, expressed as residues per 1,000 residues, are 7.1 and 8.3 for the alpha 1 (I) and the alpha 1 (III) chains, respectively, as compared to 10.0 and 11.1 for the alpha-chains from the normal tissue. The chromatographic properties and amino acid and carbohydrate composition of the alpha-chains from the diseased tissue were otherwise indistinguishable from those of normal lung. In addition, isolation and characterization of the CNBr peptides of alpha 1 (I), alpha […]

Find the latest version:

[http://jci.me/108420/pdf](http://jci.me/108420/pdf)
Collagen Polymorphism in Idiopathic Chronic Pulmonary Fibrosis


From the Veterans Administration Hospital and the Departments of Biochemistry and Medicine, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104

Abstract

Collagens in normal human lung and in idiopathic chronic fibrosis were investigated in terms of their covalent structure and compared for possible alterations in the diseased state. Collagens were solubilized by limited digestion with pepsin under nondenaturing conditions, and after purification, they were fractionated into types I and III. Carboxymethylcellulose and agarose chromatography of both types I and III collagens, and amino acid and carbohydrate analyses of the resulting α-chains indicated that the α1(I), α2, and α1(III) chains of normal human lung were identical with the human skin α-chains in all respects examined except that the normal lung chains contained higher levels of hydroxylysine. Examination of collagens obtained from the diseased lung revealed that the content of hydroxylysine of the α1(I) and the α1(III) chains appeared to be diminished as compared to the normal lung chains. The values, expressed as residues per 1,000 residues, are 7.1 and 8.3 for the α1(I) and the α1(III) chains, respectively, as compared to 10.0 and 11.1 for the α-chains from the normal tissue. The chromatographic properties and amino acid and carbohydrate composition of the α-chains from the diseased tissue were otherwise indistinguishable from those of normal lung. In addition, isolation and characterization of the CNBr peptides of α1(I), α2 and α1(III) from the diseased lung revealed no significant differences from the CNBr peptides from other human tissues reported previously.

Normal and diseased lungs were also digested with CNBr, and the resultant α1(I) and α1(III) peptides were separated chromatographically. The relative quantities of these peptides indicate that type III collagen constitutes 33% of the total collagen in normal human lung, with the remainder being type I, whereas in idiopathic chronic pulmonary fibrosis, the relative content of type III collagen is markedly diminished, ranging from 12 to 24% in different patients.

These results indicate that an alteration in tissue collagen polymorphism as well as subtle variations in the collagen structure accompany the fibrotic process in the diseased state, and suggest that these alterations may have possible pathogenetic implications.

Introduction

Connective tissue of lung is fundamental to the structural integrity and function of the organ (1, 2). Knowledge of the chemical nature and the properties of the connective tissue matrix elements in normal lungs, and their alterations in diseased organs is therefore essential to our understanding of the mechanisms involved in pathologic processes. Lung connective tissue is composed of collagen, elastin, and ground substances (3–5). Of these, collagen is the most abundant protein, comprising 11% of adult lung (6); it has the greatest tensile strength and probably plays an important role in the mechanical properties of lung (7, 8). Recent studies from several laboratories have elucidated at least four genetically distinct collagens from various tissues (9). The most completely characterized and widely distributed in tissues is type I collagen. It is present in nearly all connective tissues, including skin, bone, tendon, aorta, and most parenchymal organs, and it is composed of two α1(I) chains and one α2 chain, or [α1(I)]2 α2 (10, 11). Type II collagen, consisting of [α1(II)]2, is present solely in cartilage. Type III collagen, with the chain composition of [α1(III)]3, has recently been isolated from human skin, aorta, and leiomyoma (12–14). In addition, basement membranes...
of renal glomeruli and anterior lens capsules have been shown to contain type IV collagen, composed of [α1-
(IV)]3 (15, 16).

The discovery of the existence of several genetically
different collagens in the same tissue has made the ques-
tions concerning collagen polymorphism of both the nor-
mal and the diseased organs more cogent. In chronic
pulmonary fibrosis, histologic evidence suggests that
there is an increase in collagen in affected regions of
the lung (17), but to our knowledge there has been no
biochemical characterization of the protein in this
fibrotic state. Recent work by Bradley et al. (6, 18, 19)
has indicated that explants from peripheral portions of
normal lung synthesize type I collagen and explants of
trachea and the bronchial tree synthesize type II collagen
in tissue culture. In addition, type I collagen has
been isolated and characterized from normal rabbit lungs (6).

In this study, we report the isolation and characterization
of type III and type I collagen from normal adult human
lung and their behavior in chronic pulmonary fibrosis.

Our results clearly indicate that in fibrotic lung the rela-
tive content of type III is markedly diminished, while
the content of type I is increased. The degree of lysine
hydroxylation seems to be also reduced in the α1 chains
of type I and type III collagen in the diseased lung.

METHODS

Lung samples. Human lung was obtained from adults at
the time of autopsy. Diseased lungs were taken from five
males (ages 50-70) with idiopathic pulmonary fibrosis. The
diagnosis was confirmed by histologic examination of au-
topsy samples. The normal lungs were obtained from male
patients of similar ages as the diseased subjects, who had
died of unrelated causes. Only the lungs without gross
and microscopic evidence of acute or chronic disease proc-
esses were used in this study. All pathologic examinations
were carried out by Dr. J. M. Young, Chief, Pathology
Service, Memphis Veterans Administration Hospital. The
tracheobronchial tissues were removed as much as pos-
sible by dissection. The remaining peripheral lung was cut
into pieces, ground in a mechanical meat grinder, and ho-
mogenized briefly with a Waring blender at 4°C (Waring
Products Div., Dynamics Corp. of America, New Har-
tford, Conn.) in 0.05 M Tris, pH 7.4, to remove relatively
large amounts of noncollagenous substances, and finally
with cold distilled water. The insoluble tissue residue, which
contained over 99% of the original collagen as determined by
hydroxyproline analysis (20), was collected by cen-
trifugation and either used directly for pepsin digestion or
CNBr cleavage.

Solubilization of collagen by limited pepsin digestion and
isolation of molecular species. The washed lung homoge-
nate residue was suspended in cold 0.5 M acetic acid, and
the pH of the suspension adjusted to 2.5 by the addition of
formic acid. All operations were performed at 4°C. Pepsin
was then added (1 g/50 g wet weight) and digestion was
allowed to proceed for 72 h with gentle shaking. The un-
digested residue was separated by centrifugation for 1 h
at 10,000 g, and digested two more times with pepsin under
identical conditions. Collagen present in the three pepsin
extracts was precipitated by dialysis against 0.01 M Na-
HPO4, and harvested by centrifugation. The precipitate was
redissolved in 0.5 M acetic acid and precipitated again by
the addition of NaCl to a final concentration of 1 M. The
final precipitate was resolubilized in 0.05 M Tris/1 M NaCl,
pH 7.4. Type III collagen was then precipitated by a care-
ful addition of NaCl to a concentration of 1.5 M and
collected by centrifugation (12, 14). Remaining collagen in
the supernatant solution (predominantly type I) was pre-
cipitated by increasing the NaCl concentration to 2.5 M.
Each type of collagen was then dissolved in 0.1 M acetic
acid, dialyzed exhaustively against the same solution, and
lyophilized.

Cleavage with cyanogen bromide. Samples of washed
lung homogenates (500-1,000 mg) were suspended in 50-
100 ml of 70% formic acid. Samples of purified collagen
chains (50-200 mg) were dissolved in 20-50 ml of 70%
formic acid. The suspension or solution was flushed with
nitrogen, and a weight of CNBr (Pierce Chemical Co.,
Rockford, III.) equal to two times the weight of the sample
was added. Digestion was allowed to proceed for 4 h at
40°C with gentle stirring. Insoluble material, if any, was
separated by centrifugation for 10 min at 40,000 g, and the
supernate containing the liberated peptides was lyophilized
after a 10-fold dilution with cold distilled water. The
fraction of solubilization of collagenous protein by CNBr di-
gestation was determined by hydroxyproline analysis of por-
tions of the supernate and the residue.

Ion exchange chromatography. Separation of the con-
stituent α-chains of pepsin-solubilized collagen was achieved by chromatography of a heat denatured collagen solution on a column (2.5 x 10 cm) of carboxymethylcellulose (CM-
cellulose).1 (CM-52, Whatman, Inc., Clifton, N. J.) at 43°C
(21). Samples were solubilized in 0.02 M sodium acetate/1
M urea, pH 4.8, denatured by warming the solution at 43°C
for 20 min, and applied to the column. The collagen chains
were eluted with a linear gradient of NaCl from 0 to 0.1
M over a total volume of 1,200 ml. The effluent was
monitored continuously at 248 nm in a Gilford recording
spectrophotometer (model 2400S, Gilford Instrument Lab-
oratories, Inc., Oberlin, Ohio) equipped with a flow cell,
and collected in fractions of 10 ml. The fractions corre-
sponding to each peak were pooled, desalted on a column
(4 x 60 cm) of Sephadex G-25 (Pharmacia Fine Chemicals,
Inc., Piscataway, N. J.) using 0.1 M acetic acid as the eluant,
and lyophilized.

Peptides generated by CNBr digestion were chromato-
ographed on a column (0.9 x 15 cm) of CM-cellulose at 43°C
(22). Samples (50-100 mg) were dissolved in 5 ml of start-
ing buffer (0.02 N sodium citrate/0.16 M NaCl, pH 3.8). A
flow rate of 48 ml/h was used and the effluent was continu-
ously monitored at 230 nm. The fractions representing each
peptide peak were pooled, lyophilized, desalted on Bio-Gel
P-2 (Bio-Rad Laboratories, Richmond, Calif.) (100-200
mesh) using 0.1 M acetic acid, and relyophilized.

Certain of the CNBr peptide fractions obtained from the
initial CM-cellulose chromatography at pH 3.8 required
further fractionation at pH 4.8. A column (0.9 x 10 cm)

1 Abbreviation used in this paper: CM-cellulose, carboxy-
methylcellulose.
of CM-cellulose was equilibrated with starting buffer (0.02 M sodium acetate, pH 4.8) at 43°C, and after application of samples was eluted with a concave gradient from 250 ml of the starting buffer and 415 ml of limiting buffer (0.02 M sodium acetate, 0.16 M NaCl, pH 4.8) in a two-chamber constant level device (23, 24).

Other peptide fractions were rechromatographed on a column (0.9 x 7 cm) of phosphocellulose (Whatman Inc., P-11). Samples were dissolved in 2 ml of starting buffer (0.001 M sodium acetate, pH 3.8) and applied to the column equilibrated with the same buffer at 43°C. Peptides were then eluted with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 600 ml.

**Molecular sieve chromatography.** Separation and molecular weight determination of denatured collagen chains and CNBr peptides were achieved on calibrated columns (2 x 120 cm) of agarose beads, A 15m or A 1.5m (200-400 mesh, Bio-Rad Laboratories), or Sephadex G-50 SF (Pharmacia Fine Chemicals, Inc.) in 0.05 M Tris/1 M CaCl₂, pH 7.4, at room temperature as described previously (25). All samples were heated to 43°C to ensure denaturation before application to the column. A drop of tritiated water was added to each sample to mark the column volumes (25).

**Amino acid and carbohydrate analyses.** For amino acid analysis, samples were hydrolyzed in doubly distilled constant boiling HCl under an atmosphere of nitrogen for 24 h at 110°C. Analyses were performed on an automatic analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif., model 121) using a four-buffer elution system previously described (26).

Samples for analysis of hydroxylysine glycosides were hydrolyzed in 2 N NaOH in borosilicate-free test tubes at 110°C for 24 h. Analyses were performed on a Beckman 121 analyzer employing the methods described by Askenasi and Kefalides (27).

**RESULTS**

Attempts to extract native collagen directly from normal or fibrotic lungs using 1 M NaCl or 0.5 M acetic acid were unsuccessful due to extremely low yields. Much greater quantities of collagen, however, could be extracted by limited digestion with pepsin. The results are presented in Table I. Generally, the first pepsin extract contained lower yields of solubilized collagen than the second and third extracts. The yields of collagen from pepsin-digested fibrotic lung were higher than for normal lungs (Table I).

After purification of the pepsin-extracted collagen by dialysis against 0.01 M NaHPO₄, and NaCl precipitation from acetic acid solution, the collagen fraction precipitating at 1.5 M NaCl at pH 7.4 was chromatographed on CM-cellulose. The elution pattern obtained from fibrotic lungs is shown in Fig. 1. The peaks were identified by their behavior during agarose chromatography (not shown) and amino acid analysis (Table II). The peak labeled α1(III) (Fig. 1), eluted from agarose A 15m as a homogenous peak at the position corresponding to the γ-component (mol wt 280,000). Thus, the predominant species of collagen in the 1.5 M NaCl precipitate was type III.

The pepsin-extracted collagen fraction precipitating at the NaCl concentration between 1.5 and 2.5 M at pH 7.4 was also fractionated on CM-cellulose. A representative chromatogram is illustrated again in Fig. 1. The peak labeled α1(I) was identified by its elution position on agarose A 15m chromatography (figure not shown, mol wt 95,000), amino acid analysis (Table II), and chromatography of a CNBr digest (see below). Significant amounts of the type III chains were also present in the 2.5 M NaCl precipitate, reflecting incomplete precipitation of type III collagen at 1.5 M NaCl (14). However, gel filtration on agarose A 15m allowed clear-cut separation and purification of [α1(III)]₆ (mol wt 280,000) and α2 (mol wt 95,000). Amino acid analysis (Table II) and chromatography of CNBr digests of these materials (see below) confirmed their

**TABLE I**

<table>
<thead>
<tr>
<th>Extractants</th>
<th>Normal</th>
<th>Fibrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagen</td>
<td>Protein</td>
</tr>
<tr>
<td>0.05 M Tris HCl, pH 7.4</td>
<td>0</td>
<td>6.3</td>
</tr>
<tr>
<td>First pepsin digestion</td>
<td>4.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Second pepsin digestion</td>
<td>7.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Third pepsin digestion</td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Total extracted</td>
<td>16.1</td>
<td>31.1</td>
</tr>
</tbody>
</table>

* The results are expressed as percent of collagen or protein solubilized by each extractant.
‡ Collagen content based on hydroxyproline analysis.
§ Total protein content was determined from amino acid analysis of a portion of each extract and converted to protein assuming a mean residue weight of 100.
FIGURE 1 CM-cellulose chromatography of pepsin-solubilized human lung collagen. The lower curve represents the elution pattern obtained from the fraction precipitating at 1.5 M NaCl at pH 7.4 (indicated by an arrow), and the upper curve, the fraction precipitating between 1.5-2.5 M NaCl (indicated by an arrow). The peaks were identified by their behavior during agarose A 15m chromatography and subsequent analyses of their amino acid compositions and the CNBr peptides. The presence of the α1(III) chains in the 1.5-2.5 NaCl precipitate (upper curve) is due to incomplete precipitation of type III at 1.5 M NaCl.

### Table II

**Amino Acid Composition of Human Lung Collagens; Values Expressed as Amino Acid Residues per 1,000 Amino Acids**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal</th>
<th>Fibrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α1(I)</td>
<td>α2</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>107</td>
<td>103</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>45</td>
<td>44</td>
</tr>
<tr>
<td>Threonine</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Serine</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>78</td>
<td>74</td>
</tr>
<tr>
<td>Proline</td>
<td>116</td>
<td>111</td>
</tr>
<tr>
<td>Glycine</td>
<td>335</td>
<td>340</td>
</tr>
<tr>
<td>Alanine</td>
<td>108</td>
<td>96</td>
</tr>
<tr>
<td>Cysteine‡</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Methionine§</td>
<td>7.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Leucine</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10</td>
<td>8.6</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>10.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>27.0</td>
<td>20.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.7</td>
<td>10</td>
</tr>
<tr>
<td>Arginine</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>Glc-Gal-Hyl‖</td>
<td>0.85</td>
<td>0.83</td>
</tr>
<tr>
<td>Gal-Hyl‖</td>
<td>0.12</td>
<td>0.35</td>
</tr>
</tbody>
</table>

*Average of three analyses each.
‡Calculated as cysteic acid.
§Includes methionine sulfone.
‖Analysis performed after 2 N NaOH hydrolysis. Expressed as residue per 1,000 amino acids.
The amino acid composition and the carbohydrate content of the α-chains isolated from normal and fibrotic lungs are listed in Table II. The values obtained for normal and fibrotic collagens, both type I and type III, are identical within experimental error except possibly for the degree of lysine hydroxylation. The content of hydroxylysine was consistently observed to be lower in the α1(I) and the α1(III) chains isolated from the diseased lungs than that obtained from normal lungs (Table II). The composition obtained for the lung collagen chains in the present study is also in agreement with the composition reported previously for type I and type III collagens of human skin (14, 28) aorta (12, 13) and leiomyoma (12), except that the lung collagens contain elevated amounts of hydroxylysine.

To further characterize the covalent structure of type III collagen of lung, the α1(III) chain obtained by CM-cellulose and agarose chromatography was cleaved at the methionyl residues by treatment with CNBr, and the resulting peptides were fractionated on CM-cellulose at pH 3.8 (Fig. 2). Each of the peptides was further purified as outlined below. Peptides 1 and 2 were rechromatographed on phosphocellulose and molecular weights determined by Sephadex G-50 SF gel filtration. Sephadex G-50 SF likewise was used for isolation and molecular weight determination of peptides 3, 6, and 7. The larger peptides 4, 5, and 8 were further purified by rechromatography on CM-cellulose at pH 4.8 (24). Their molecular weights were determined on agarose A 1.5m (25). Peptide 9 could only partially be eluted by the gradient used. Application of 1 M NaCl solution, however, removed the bulk of the peptide. On agarose A 1.5m gel filtration, peptide 9 eluted as two peaks; one with a mol wt of 23,000 and the other with a mol wt of 45,000. Both, however, had an identical amino acid composition. Since the peptide contains a residue of cysteine, presumably the larger peptide represents a dimer of the peptide linked together through a disulfide bridge. A similar polymerization of the peptide was noted by Chung et al. (29) in the CNBr peptides from uterine leiomyoma. The amino acid composition and molecular weight of the purified CNBr peptides are presented in Table III. The sum of the composition of the peptides agree within experimental error, with the observed composition of α1(III). The data are also in agreement with the values obtained on the CNBr peptides of uterine leiomyoma (29).

The lung α1(I) and α2 chains were also further characterized by cyanogen bromide digestion, and separation of the resulting peptides by CM-cellulose chromatography and agarose A 1.5m gel filtration. The elution patterns from CM-cellulose chromatography are shown for comparison in Fig. 2. Each of the peptides was further purified by the procedures described for the α1(III) peptides. No differences were noted from the results obtained from analyses of human skin α1(I) and α2 with respect to molecular weights and amino acid composition (data not shown) (30).

Next, the question of collagen polymorphism in normal and fibrotic lungs was investigated. Because of the fact that pepsin solubilization yielded relatively small portions of the total collagen present in the lung, determination of the α1(I) : α1(III) ratio in the extracted collagen would not have reflected their distribution in the tissue. Thus, the washed total lung homogenates were subjected to CNBr digestion directly. Prior extraction of the homogenates with 0.05 M Tris, pH 7.4, and distilled water was essential to remove large quantities of non-collagenous proteins and other substances. Under the experimental conditions used in the present study, 88% or more of the total hydroxyproline was solubilized by CNBr digestion. CNBr digests were then fractionated on CM-cellulose. As shown in Fig. 3, the chromatographic pattern was complex due to the presence of peptides from both type I and type III collagens, with the

---

**Figure 2** CM-cellulose elution patterns of CNBr digests of human lung α1(I), α1(III), and α2. Chromatography was performed under identical conditions of column size, elution gradient, flow rate, and recorder chart speed (see Methods). The peaks were identified by their behavior during subsequent ion exchange and (or) molecular sieve chromatography and amino acid analyses.

---

TABLE III
Amino Acid Composition* and Molecular Weight of CNBr Peptides from Human Lung α1(III)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total  α1(III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxyproline</td>
<td>2.18</td>
<td>7.71</td>
<td>12</td>
<td>19</td>
<td>30</td>
<td>11</td>
<td>3</td>
<td>3.0</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.05</td>
<td>1.33</td>
<td>2</td>
<td>2.1</td>
<td>9</td>
<td>9.1</td>
<td>9</td>
<td>8.9</td>
<td>4.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.2</td>
<td>0</td>
<td>1.2</td>
<td>5.7</td>
<td>3</td>
<td>2.9</td>
<td>1</td>
<td>1.4</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Serine</td>
<td>0.5</td>
<td>3</td>
<td>4.6</td>
<td>0.3</td>
<td>9.8</td>
<td>3.2</td>
<td>3</td>
<td>0.4</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>1.0</td>
<td>3.4</td>
<td>7</td>
<td>6.7</td>
<td>10</td>
<td>9.9</td>
<td>6</td>
<td>6.0</td>
<td>5</td>
<td>4.8</td>
</tr>
<tr>
<td>Proline</td>
<td>1.0</td>
<td>5</td>
<td>3.3</td>
<td>13</td>
<td>17</td>
<td>26</td>
<td>9</td>
<td>8.7</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.3</td>
<td>17</td>
<td>60</td>
<td>81</td>
<td>31</td>
<td>31</td>
<td>15</td>
<td>49</td>
<td>77</td>
<td>365</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.0</td>
<td>1.4</td>
<td>7</td>
<td>6.9</td>
<td>15</td>
<td>19</td>
<td>7</td>
<td>7.0</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>Cysteine†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1.6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Valine</td>
<td>0</td>
<td>1.0</td>
<td>0.6</td>
<td>5</td>
<td>5.2</td>
<td>2</td>
<td>1.6</td>
<td>2</td>
<td>2.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0</td>
<td>3</td>
<td>2.6</td>
<td>0</td>
<td>4</td>
<td>3.6</td>
<td>2</td>
<td>1.6</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>0</td>
<td>2</td>
<td>2.0</td>
<td>6</td>
<td>5.7</td>
<td>6</td>
<td>5.7</td>
<td>2</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
<td>2</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0</td>
<td>1</td>
<td>1.1</td>
<td>0</td>
<td>3</td>
<td>2.6</td>
<td>1</td>
<td>0.6</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.7</td>
<td>1</td>
<td>0.6</td>
<td>1</td>
<td>0.6</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>2</td>
<td>1.7</td>
<td>6</td>
<td>6.3</td>
<td>8</td>
<td>7.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>1</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>0</td>
<td>0.4</td>
<td>2</td>
<td>2.2</td>
<td>4</td>
<td>3.5</td>
<td>3</td>
<td>3.2</td>
<td>7</td>
<td>7.3</td>
</tr>
<tr>
<td>Homoserine††</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

Total: 10 40 91 152 238 87 41 134 227 997 1,012
Molecular Weight‡‡: 1,200 4,000 9,000 15,500 22,000 8,000 4,000 12,000 23,000 96,700

* Residues per peptide rounded off to the nearest whole number. Actual values are given where less than 10 residues occur.
+ Calculated from α1(III) amino acid analyses using an average residue mol wt of 91.2 and assuming a mol wt of 92,400 for α1(III) when obtained after solubilization with peptatin.
† Determined as cysteic acid after reduction of peptide 9.
‡ Includes homoserine lactone.
¶ Peptides 1, 2, 3, 6, and 7 were analyzed on Sephadex G-50 SF, and 4, 5, 8 and 9 on Agarose A 1.5m (25).

characteristic pattern of type I being more evident. The presence of peptide 4 of α1(III) preceding α1(1)-CB3 was a useful qualitative indicator for type III collagen. For quantitative purposes, however, peptide 8 of type III collagen coeluted with α1(1)-CB8 and α1(1)-CB(8-3), and because of the difference in their molecular weight, these peptides could be separated by agarose A 1.5m chromatography (Fig. 4). Three peaks were consistently observed. The first eluted at a position corresponding to a mol wt of 37,000 and was identified as uncleaved α1(1)-CB(8-3) (22). This was generally present as the shoulder on the next major peak identified as α1(1)-CB8 with a mol wt of 24,000. The uncleaved α1(1)-CB(8-3) usually represented 10–15% of the total α1(1)-CB8 and was added to it to obtain quantitation of type I collagen. The last peak eluting at a position corresponding to a mol wt of 12,000 was identified as peptide 8 of type III collagen by amino acid analysis. The molar ratio of type I and type III collagens present in lung was then calculated from the molar ratio of α1(1)-CB8 plus α1(1)-CB(8-3) and peptide 8 of type III. Quantitation was based on the hydroxyproline assay of each of the peaks. Table IV summarizes the relative abundance of each collagen type in three normal and five fibrotic lungs. Significantly lower levels of type III collagen were consistently observed in the diseased lung.

To assess the reliability and the reproducibility of these determinations under our conditions, several experiments were performed:

(a) Known amounts of purified α1(1) and α1(III) (CM-cellulose and agarose chromatography) were mixed to yield standard mixtures containing 10, 20, 30, and 40% α1(III), and each mixture was separately digested with CNBr, and the resulting peptides were separated by CM-cellulose chromatography. A pattern obtained from a mixture consisting of 30% type III and 70% type I is shown in Fig. 3. The material indicated

---

**Table IV**

Percentage of Type III Collagen in Normal and Fibrotic Human Lung; Values Expressed as Percent Type III Collagen with Respect to Type I

<table>
<thead>
<tr>
<th></th>
<th>Normal lung*</th>
<th>Fibrotic lung*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31 ± 2 (3)</td>
<td>20 ± 2 (3)</td>
</tr>
<tr>
<td></td>
<td>33 (1)</td>
<td>15 ± 3 (3)</td>
</tr>
<tr>
<td></td>
<td>28 (1)</td>
<td>18 (1)</td>
</tr>
<tr>
<td></td>
<td>12 (1)</td>
<td>24 (1)</td>
</tr>
</tbody>
</table>

* Each value represents an individual lung. Numbers in parentheses indicate the number of times each sample was analyzed starting from the whole lung. The variation from the average value is indicated by ± designation.
The chemical nature of collagen in lung has never been fully investigated and represents the aim of this investigation. Type I and type III collagens which are present in peripheral lung were made soluble by limited pepsin digestion under conditions which maintain the helical conformation of the molecule. Pepsin, under these conditions, cleaves only the nonhelical terminal segments which contain the lysine-derived interchain cross-links (31, 32). The bulk of the collagen molecule is resistant to pepsin digestion so long as the native triple helix remains intact. Pepsin treatment of the total tissue, therefore, has been used to solubilize collagen with similar physical-chemical properties as the intact tropocollagen molecule.

The preservation of the triple helical structure enables extracted lung collagens to be purified by low salt precipitation (0.01 M NaHPO4), acid precipitation (0.5 M acetic acid with 1 M NaCl), and finally selective precipitation of types III and I collagens at 1.5 and 2.5 M NaCl (0.05 M Tris, pH 7.4), respectively. CM-cellulose chromatography of both the 1.5 and 2.5 M NaCl precipitates indicates that quantitative separation of the two types of collagen was not achieved, with a considerable amount of type III remaining in the 1.5–2.5 M NaCl fraction. Nevertheless, homogeneous a1(I), a2, and a1(III) chains could be obtained by further fractionation of the CM-cellulose fractions on agarose. The reason the observed a1(I):a2 ratio being greater than 2:1 is not clear. A possible explanation might be greater susceptibility of the a2 chain to pepsin digestion with a corresponding apparent increase in the a1(I):a2 ratio.

**DISCUSSION**

Histopathologic studies indicate that the collagen content of human lung increases in chronic pulmonary fibrosis (17). The chemical nature of collagen in lung has never been fully investigated and represents the aim of this investigation. Type I and type III collagens which are present in peripheral lung were made soluble by limited pepsin digestion under conditions which maintain the helical conformation of the molecule. Pepsin, under these conditions, cleaves only the nonhelical terminal segments which contain the lysine-derived interchain cross-links (31, 32). The bulk of the collagen molecule is resistant to pepsin digestion so long as the native triple helix remains intact. Pepsin treatment of the total tissue, therefore, has been used to solubilize collagen with similar physical-chemical properties as the intact tropocollagen molecule.

The preservation of the triple helical structure enables extracted lung collagens to be purified by low salt precipitation (0.01 M NaHPO4), acid precipitation (0.5 M acetic acid with 1 M NaCl), and finally selective precipitation of types III and I collagens at 1.5 and 2.5 M NaCl (0.05 M Tris, pH 7.4), respectively. CM-cellulose chromatography of both the 1.5 and 2.5 M NaCl precipitates indicates that quantitative separation of the two types of collagen was not achieved, with a considerable amount of type III remaining in the 1.5–2.5 M NaCl fraction. Nevertheless, homogeneous a1(I), a2, and a1(III) chains could be obtained by further fractionation of the CM-cellulose fractions on agarose. The reason the observed a1(I):a2 ratio being greater than 2:1 is not clear. A possible explanation might be greater susceptibility of the a2 chain to pepsin digestion with a corresponding apparent increase in the a1(I):a2 ratio.

**FIGURE 3** CM-cellulose chromatography of a CNBr digest of washed fibrotic lung residue (lower panel) and of a standard mixture consisting of 70% purified a1(I) and 30% purified a1(III) (upper panel). The fractions indicated by the horizontal bar were pooled, desalted on Sephadex G-25, lyophilized, and subjected to agarose A 1.5m chromatography.

by the horizontal bar was collected and after desalting and lyophilization, subjected to gel filtration on agarose A 1.5m (Fig. 4). The percent of type III calculated from hydroxyproline analyses of the resulting peptide fractions was 28%. By identical maneuvers, values of 37, 22, and 9% a1(III) were obtained from the mixtures containing 40, 20, and 10% type III, respectively.

(b) Standard mixtures of purified [a1(III)], and type I collagens [a1(I)]_2 were made by mixing known amounts of each, and analyzed by identical procedures described above. Quantitation of the mixtures containing 40, 30, and 20% [a1(III)], yielded corresponding values of 38, 30, and 21%, respectively.

(c) Triplicate lung samples were taken from each of the following: one normal and two different patients. Each sample was separately digested with CNBr. The resulting peptides were separated by CM-cellulose and agarose chromatography in a manner described above. The percent of type III present in the triplicate samples agreed within 3 percentage points as indicated in Table IV.

**FIGURE 4** Agarose A 1.5m gel filtration of the fractions collected from CM-cellulose chromatography (Fig. 3). Lower panel, fibrotic human lung. Upper panel, standard mixture of 70% a1(I) and 30% a1(III).
The possibility must also be considered that α1(1) may be contaminated with α1(II) from the remaining small bronchial tree or α1(IV) from the remaining vasculature. Both of these might have been solubilized by pepsin digestion and eluted from CM-cellulose in a position similar to α1(1) (15, 33). The relatively high levels of hydroxylysine observed in this study might also be consistent with this supposition. The presence of significant amounts of type II collagen may, however, be ruled out from the following considerations. Human α1(II) contains 14 residues/1,000 of hydroxylysine (34). This would necessitate a large amount of contamination to account for the high level of the amino acid found in human lung α1(1). At that level of contamination, the α1(II) specific CNBr peptides would have been detected during isolation of the α1(1) peptides. No such peptides were detected in the CNBr digests of lung α1(1).

Type IV collagen has not been characterized as well as the other types. However, available data indicate that it is distinguished by its high content of 3-hydroxyproline and carbohydrate (15, 16). The lung α1(1) in the present paper contained about one residue each of 3-hydroxyproline and hydroxylsinglycosides, and thus a significant contamination by α1(IV) is unlikely.

The amino acid composition of the lung α-chains revealed no significant differences between the normal and fibrotic lung collagen except for a possible decrease in lysine hydroxylations (Table II) in the diseased organ. The observed values for hydroxylysine are 7.1 and 8.3 for α1(1) and α1(III) of the pathologic organ as compared with 10.0 and 11.1 for α1(1) and α1(III) of normal organ, respectively. These values should be interpreted with some caution, since slight variations were also observed in the total lysine content (lysine plus hydroxylysine) of various chains. The magnitude of the decrease in hydroxylysine, however, appears to be greater than accountable by variations in the measurement.

Proline hydroxylation was not altered in the diseased organ. This is interesting in view of the fact that the posttranslational enzymes, i.e., prolyl and lysyl hydroxylases have been shown to increase during experimentally induced fibrosis (35). The fact that there is no increase in hydroxyproline or hydroxylysine content suggests that these increased enzymatic activities reflect an overall increased collagen synthesis incident to the fibrotic process. The levels of hydroxylysine in both normal and fibrotic lungs are higher when compared to human skin where the hydroxylysine content has been reported to be 5 or 6/1,000 residues (14, 29). This must, therefore, represent a tissue-specific increase in lysine hydroxylation. The functional significance of this amino acid in collagen is not understood at the present time. The occurrence of tissue-specific differences in the content of hydroxylysine, and its alterations in certain diseased states, such as rickets (36), however, do suggest its involvement in some aspect of collagen function.

Glycosylation of both fibrotic and normal lung collagen, types I and III, was found to be identical. α1(1) contained approximately 0.8 residue of O-glucosylgalactosylhydroxylysine and 0.3 residue of O-galactosylhydroxylysine per chain. Type III collagen contained approximately the same amount. No correction factors were used to account for partial destruction due to hydrolysis. The lack of change in the degree of glycosylation of hydroxylysine in chronic pulmonary fibrosis is in variance with the data obtained from scarred rabbit cornea in which substantial decreases in glycosylation were noted in the scar tissue as compared to the control (37). That data, however, was obtained from collagenase digests of total tissue, rather than from purified collagen, and must therefore be regarded with a certain degree of uncertainty.

The CNBr peptides from lung α1(1), α2, and α1(III) were isolated and characterized with respect to molecular weight and amino acid composition. Nine peptides were obtained from CNBr digestion of type III human lung collagen, and were purified by a combination of ion exchange and molecular sieve chromatography. The amino acid compositions were in agreement with those obtained from human skin (29), with the exception of peptides 1, 3, and 6. In the present study no leucine or isoleucine was found in peptide 1, while one each of the amino acids was previously found (29). Whether these differences represent tissue-specific variations is not known. Peptide 3 contained 91 amino acids as opposed to 109 which was previously reported for skin (29). Although the sequence of these CNBr peptides within the α-chain has not been established, peptide 3 most likely represents the amino terminus since two tyrosine residues are present. In type I collagen, tyrosine is present only in the amino or carboxy terminal peptide. Variable pepsin digestion would have therefore yielded discrepancies in the length of this peptide and explain the difference. The partial amino acid sequence of peptides 4, 5, and 6 has already established their analogy with CB3, CB7, and CB8 of α1(I) polypeptide chain (38). Peptide 9 contained the two cysteine residues. It was isolated as a monomer and dimer with mol wt of 23,000 and 45,000, respectively, with the same amino acid composition. This peptide was reported to be the carboxy terminal peptide, and therefore analogous to α1(1)-CB6. The position of peptides 1, 2, 7, and 8 in relation to α1(1) chain has not yet been determined. No significant differences were observed in the CNBr
peptides of human lung α1(I) and α2 as compared to those of corresponding human skin chains (30).

The specific nature of the CNBr cleavage and the resulting peptides allowed investigation of collagen polymorphism in normal and diseased lung. Peptide 8 of type III collagen eluted from CM-cellulose with α1(1)-CB8 and the partially cleaved CB(B-8-3). Since the latter peptides are larger in mol wt (24,000 and 37,000, respectively) as opposed to peptide 8 of α1(III), they were readily separated by agarose A 1.5cm chromatography. Because of the reproducible elution pattern in this area of the CM-cellulose chromatogram, no difficulties were encountered in identifying and pooling consistent fractions, even in CNBr digests of the total tissue. Since quantitation was based on hydroxyproline assay, possible contamination by noncollagenous proteins would not have produced errors. In reality, however, quantitation by planimetry of the area under the chromatographic peaks yielded nearly identical results. The reproducibility of the procedures used in this study was supported by experiments using artificial mixtures of purified type I and type III collagens as well as by good agreement obtained in triplicate analyses of several lung samples (Table IV).

Normal lung contained an average of 31% type III collagen. Variable results were obtained from fibrotic lungs, depending on the patient, with a range of 12-24% type III collagen. This is presumably related to the varying severity among different patients, but no attempt was made to quantitatively correlate the biochemical findings with histopathologic findings. The consistent depression in type III collagen indicates that the fibrotic response of this tissue to injury, whatever it may be in chronic pulmonary fibrosis, is to result in a relative increase in the amount of type I in relation to type III collagen. Whether this is due to a preferential increase in the synthesis of type I collagen or due to a preferential increase in the degradation of type III collagen, or both, cannot be decided at present.

An additional possibility must also be considered that the relative diminution in the content of type III may be a result of a reduction in the relative amount of blood vessels in the diseased lung and not due to any changes in the collagen composition of lung scar per se. It was previously reported that blood vessels are relatively rich in type III collagen (13). To our knowledge, there have been no studies designed to quantitate the blood vessel content of lung in normal or diseased states. Microscopic examination of pathologic lung slides might suggest that the blood vessel content may be diminished. Our preliminary results obtained from studies of scleroderma lungs from one patient, however, suggest that such a possibility is less likely, at least as an entire explanation of the observed collagen distribution. Although the scleroderma lungs showed much of the same pathologic features as the idiopathic fibrosis, the relative distribution of type I and III collagens was unaltered.

Thus, it is not yet possible to precisely relate the biochemical findings to specific anatomic or pathologic loci in lung. Nevertheless, the results obtained in the present investigation do define biochemical changes in collagen in this disorder and provide basis for further investigation.

ACKNOWLEDGMENTS

The authors are grateful to Dr. J. M. Young, of this hospital, for obtaining tissue samples and for valuable discussions. We also acknowledge the excellent technical help of C. Dickey, G. Randall, and T. Marshall.

This study was conducted under Veterans Administration Research Projects 4826-01 and 4826-02, and was supported in part by U. S. Public Health Service grant AM 16586.


REFERENCES


Collagen Polymorphism in Idiopathic Chronic Pulmonary Fibrosis