

Pattern of collagen types and molecular structure of collagen in acute post-traumatic pulmonary fibrosis

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ABSTRACT Analyses of collagen types and their amino acid structures have been made with lungs obtained from eight patients who died from the adult respiratory distress syndrome after non-pulmonary trauma. Collagen in lungs from patients with the adult respiratory distress syndrome was twice as soluble as that in control lungs ($p < 0.01$). The proportion of type III collagen in the whole organ as well as in the pepsin solubilised fraction was slightly but significantly raised (net increase of about 5–10% type III collagen ($p < 0.05$)). Both type I and type III collagen from the patients contained less hydroxylysine than collagen from control lungs. The alterations in tissue composition described here and observed in normal wound healing support the notion that acute post-traumatic pulmonary fibrosis resembles a wound healing process in the lungs.

Introduction

The development of the adult respiratory distress syndrome is one of the most serious complications following severe systemic trauma and shock.^{1,2} A striking feature of late stage adult respiratory distress syndrome is a progressive tissue derangement leading to pulmonary fibrosis. This is characterised by the deposition of increased amounts of connective tissue, which has been analysed morphologically and biochemically.^{3–6} An increase of total lung collagen content to four to five times the normal level has been found in those patients who survived for more than two weeks.^{5,6} The molecular basis of this pathological process characterised by a rapidly enhanced collagen deposition is still only poorly understood.

Collagen, which is the major constituent of lung connective tissue, exists in various idiotypes, and 11 types have now been identified. Nine of these types are present in lung tissue, including pulmonary cartilage.⁷ The two most important interstitial collagen types are types I and III, which are thought to be important in pulmonary structure and function—type I collagen forming large fibre bundles with high

mechanical strength, while type III collagen seems to be more important for tissue flexibility.⁷ Fibre strength and hence tissue stability are furthermore influenced by the number of covalent cross links between collagen molecules. These cross links are particularly stabilised when the amino acid hydroxylysine takes part in this process.⁸

The aim of this study was to investigate alterations in collagen polymorphism and deposition in the lungs of patients dying from the adult respiratory distress syndrome.

Methods

STUDY POPULATION

We examined lung tissue from eight subjects who had suffered multiple injuries, resulting in severe haemorrhagic-traumatic shock, and who died after protracted respiratory failure (adult respiratory distress syndrome) (table 1). Excluded from the study were patients with previous disease of the lungs, those who had had major direct lung trauma, and those who had aspirated gastric contents. We selected only patients in the age range 18–45 years. All the subjects had similar clinical courses, with progressive deterioration of pulmonary function after an initial onset of respiratory insufficiency primarily characterised by pulmonary oedema. Lung function deteriorated progressively with time with a dramatic drop in the arterial oxygen tension (P_{aO_2}) despite an increase in the inspired oxygen fraction (F_{IO_2}) (the $P_{aO_2}:F_{IO_2}$

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Table 1 Lung weights* and collagen amounts in the study and control groups

	Age/sex	Survival time (d)	Total lung weight†		Total lung collagen	
			Wet (ww, g)	Dry (dw, g)	Concentration (mg/g dw)	Content (g)
Controls*			803 (70)	152 (21)	150.6 (39.5)	22.9 (5.2)
Patient No						
1	35/M	13	1775	282	75.3	21.2
2	18/F	14	1660	262	76.4	20.0
3	26/M	15	2310	366	34.4	13.3
4	36/M	16	3190	398	75.6	30.1
5	26/M	18	3170	442	57.3	25.3
6	45/F	19	2095	326	71.5	23.3
7	26/M	23	2855	454	78.9	35.8
8	44/M	29	2430	436	129.2	56.3

*Control data obtained from a previous study⁶ (n = 6), expressed as means (SD).

†Total lung weights represent the combined organ weights for both lungs.

ratio in all patients finally ranging from one fifth to one tenth of normal), a decrease of lung compliance of more than 50%, and an average increase in dead-space volume and shunt fraction of more than five times the normal. Postmortem analysis showed histological features of pulmonary fibrosis with fibroblast proliferation and deposition of large amounts of connective tissue. Ultrastructurally, a considerably enlarged interstitial space was observed along with interstitial oedema, cellular infiltration and proliferation and the deposition of bundles of collagen fibrils.

The collagen content of the lungs of all patients had been examined in a previous study.⁶ From five lungs we had sufficient material to perform the complete extraction procedure, while in the case of three additional patients only small amounts of parenchyma were available for the determination of the ratios of interstitial collagen type III to type I in whole tissue specimens.

Control lung samples were obtained at necropsy from previously normal victims of acute trauma (five men, one women). They showed no detectable pathological cardiopulmonary changes.

The lungs had been removed in a partial necropsy within two hours of death to avoid postmortem tissue autolysis. The tissue samples were stored at -80°C until analysis.

COLLAGEN EXTRACTION AND RATES OF SOLUBILITY

Collagen that contains a small number of cross links is more soluble in acidic and enzymatic collagen extraction procedures than normal collagen.^{8,9} For an analysis of the degree of intermolecular collagen cross linking, we thoroughly homogenised several lung tissue samples from each patient (weighing about 10 g wet weight) in 0.5% acetic acid. From this homogenate aliquots were withdrawn for quantifying

the collagen content by amino acid analysis as described.⁶ The homogenate was then stirred for 24 hours at 4°C . After centrifugation aliquots from the supernatant were used for the determination of the acetic acid soluble collagen fraction.

The acetic acid insoluble residue was then digested three times with pepsin according to the method of Layman *et al.*¹⁰ and the non-solubilised collagen was removed by centrifugation. Total pepsin soluble collagen was subsequently estimated by amino acid analysis of aliquots from the combined supernatants.

CHROMATOGRAPHIC PROCEDURES

The interstitial collagen types I and III from the pepsin solubilized collagen fraction were precipitated with 2.7 mol/l NaCl, 0.05 mol/l Tris-HCl, pH 7.4, and removed by centrifugation. Collagen types were separated by chromatography on Agarose A 1.5 under denaturing conditions as described by Piez *et al.*¹¹ Collagen type I was obtained as the α fraction of the eluate, while collagen type III was prepared by repeated chromatography of the γ fraction under reducing conditions.¹² Additionally, collagen type I/III ratios were calculated from slab gels¹³ using SDS-gel electrophoresis.¹⁴

QUANTITATION OF COLLAGEN TYPE I:III RATIOS BY CYANOGEN BROMIDE CLEAVAGE

Collagen type I: type III ratios in whole tissue samples were determined by the method of Laurent *et al.*¹⁵ with the modifications described by Kirk *et al.*¹⁶ Briefly, the lung tissue samples were homogenised five times in sodium dodecyl sulphate to remove non-collagenous proteins and the insoluble portion was obtained by centrifugation. This insoluble material was suspended in formic acid and subjected to treatment with cyanogen bromide. Since the cleavage of the collagen molecules with cyanogen bromide results in the formation of a distinct peptide pattern that

specific for each collagen molecule, this procedure can be used for an estimation of the ratio of collagen type I to type III. Thus the resulting peptides were separated by gel electrophoresis and reference peptides for collagen type I (α_1 (I)-CB 8) and type III (α_1 (III)-CB 5) were used for the estimation of the collagen type ratios.

AMINO ACID ANALYSIS

The molecular composition of pepsin soluble collagen was determined by amino acid analysis. Losses of threonine and serine were corrected by multiplication by the factors 1.08 and 1.21 respectively.¹⁷

STATISTICAL METHODS

For statistical analysis we used the two tailed unpaired *t* test, a level of $p \leq 0.05$ being regarded as significant.

Results

Postmortem analysis of lung tissue from all patients showed typical features of lungs in late stage shock: greatly increased lung wet and dry weights, interstitial oedema, hyaline membrane formation, proliferation of fibroblasts, cellular infiltration (patients with plasma cells and macrophages), and enhanced deposition of bundles of collagen fibrils (fig 1).

The biochemical quantitation of the solubilised amounts of collagen showed significantly increased collagen extraction after both acetic acid ($p \leq 0.01$) and enzymatic ($p \leq 0.01$) treatment of samples of lung tissue from the group of patients. Thus about two thirds of the total collagen was extracted from the shock lungs after pepsin treatment, whereas only 30% of the total collagen was extracted from control lung samples (fig 2). The ratio of type III to type I collagen in the solubilised pool of collagen was slightly, but significantly ($p \leq 0.05$) increased, a result that was further substantiated by analysis of total tissue collagen by cyanogen bromide cleavage (fig 3). A comparison of both determinations used showed a positive correlation ($r = 0.86$, $y = 0.64x + 1.05$). The ratio of α_1 (I) to α_2 (I) chains as determined by gel electrophoresis was normal in both groups (α_1 (I): α_2 (I) = 2:1). On routine slab gels only traces of type V collagen chains could be seen, with no apparent difference between patients and controls.

The ratios of hydroxyproline to proline in the chromatographically purified type I and type III collagen molecules were similar in patient and control lungs. By contrast, hydroxylysine to lysine ratios were significantly lower in both types of collagen obtained from patients' tissues than in tissue from controls (table 2).

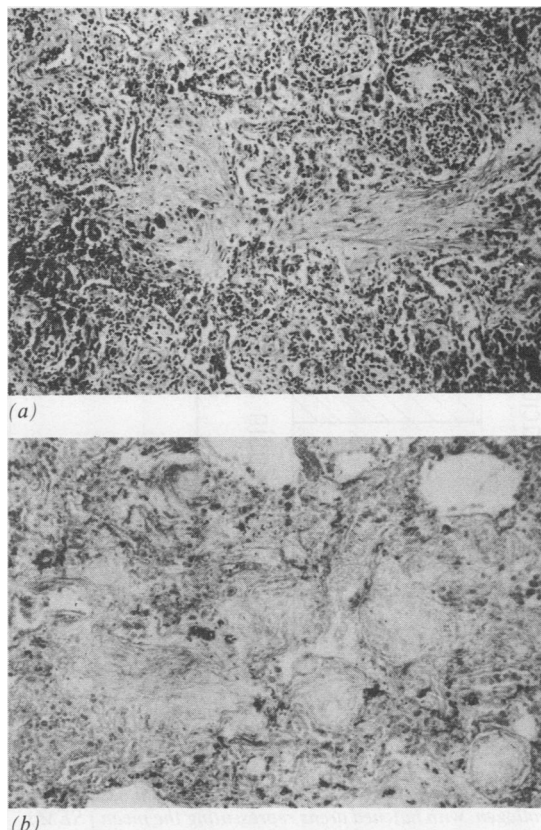


Fig 1 Photomicrographs of lung tissue obtained from patients with acute post-traumatic pulmonary fibrosis. (a) Patient No 6: Interstitial spaces thickened by oedema, cellular infiltration, and early fibrosis. (b) Patient No 8: severe interstitial fibrosis.

Discussion

The pathogenesis of acute pulmonary fibrosis occurring in the late stage of the adult respiratory distress syndrome is not clear.² There have been several morphological studies showing an increase in collagenous material.^{3,4} Although in several biochemical studies, including the present one, the apparent collagen concentration per dry weight in adult respiratory distress syndrome lungs showed reduced rather than increased values, the calculation of total lung collagen contents revealed significant increases, which were seen only after two weeks of survival.^{5,6} The reduction in collagen concentration may be due to an influx of products of haemorrhage and congestion during the inflammatory process. Proteins that exude into lung parenchyma because of the inflammatory response may reasonably be assumed to contribute to the dry weight mass. Thus, when lung dry weights

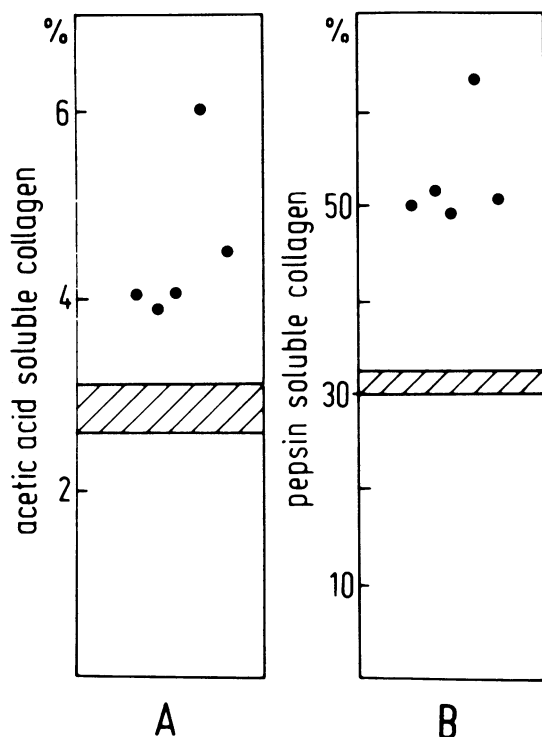


Fig 2 (A) Acetic acid and (B) enzymatic collagen solubility of lung samples from five patients with acute pulmonary fibrosis (data expressed as percentages of total tissue collagen, with hatched areas representing the mean (SEM) of lung samples from six control subjects).

were corrected for haemoglobin, collagen concentrations showed a gradual increase, exceeding the normal range.⁵ These observations provide evidence that the determination of the collagen concentrations per unit of dry weight does not reflect the actual changes.

Using sequential extraction of collagen from lung tissue, our study has shown that collagen from the

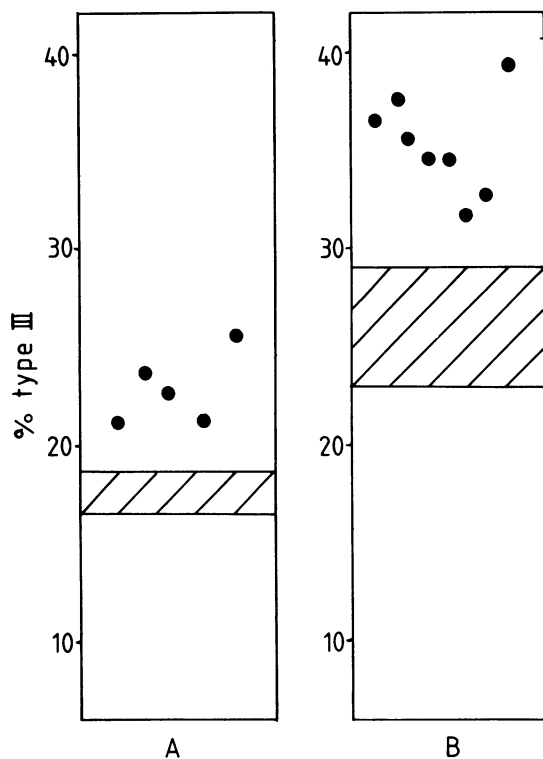


Fig 3 Ratios of collagen types III to type I in (A) pepsin solubilised collagen and (B) whole tissue collagen. The values represent collagen type III as percentages of total type I and III collagen. The hatched areas indicate control values ($n = 6$, mean (SEM)).

patients' lungs can be solubilised more readily than that from normal lungs, and that this is probably due to a smaller number of cross links.⁹ In normal lung tissue only minute amounts of collagen can be solubilised by conventional neutral or acidic extraction methods.¹⁸ Acid soluble collagen has a lower number of cross links.⁹ The use of enzymatic treatment (pepsin) generally solubilises a much larger amount of col-

Table 2 Percentage hydroxylation of prolyl and lysyl residues in type I and type III collagen

	% hydroxyproline/proline		% hydroxylysine/lysine	
	Type I	Type III	Type I	Type III
Controls* (n = 6)	49.2 (3.9)	51.0 (4.6)	36.3 (3.0)	43.0 (5.0)
Patient No				
4	50.3	50.9	31.3	37.2
5	48.2	56.7	30.2	30.9
6	46.3	55.4	27.9	31.8
7	41.7	51.3	28.8	36.9
8	46.7	51.4	30.4	34.8
Patients 4-8*	46.6 (3.2)	53.1 (2.7)	29.7 (1.4)	34.3 (2.9)

*Means (SD).

lagen by cleaving off the small non-helical parts of the collagen molecule, where intermolecular cross links are located.⁹ Both acid and enzymatically extractable collagen fractions were significantly increased, so we conclude that the degree of intermolecular cross linking is reduced. This notion is supported by the fact that both collagen type I and type III from patients' lungs contain less hydroxylysine residues than that from controls. The content of other amino acids is similar to that of controls. This may explain the increased collagen solubility, since hydroxylysine is essential for the formation of stable collagen cross links. The specific biological role of hydroxylysyl residues has not been fully elucidated,^{9,19} but the same type of collagen may contain various amounts of hydroxylysyl residues in different tissues or different stages of development. It is possible, however, that our preparation procedure, using pepsin treatment, may have caused a loss of hydroxylysyl residues from the non-helical parts of the molecules.

The presence of this amino acid is also required for the glycosylation of the molecule in addition to the formation of stable intermolecular cross links. Glycosylation may regulate the quaternary assembly of the collagen molecules and may interact with proteoglycan molecules or other components of the matrix by glycosidic binding.⁹ Thus it is possible that the linkage of proteoglycans to collagen, which is essential for macromolecular fibre arrangement, is regulated by collagen glycosylation. This may be disturbed when the amount of hydroxylysine is altered. Lysyl hydroxylation in adult respiratory distress syndrome lungs in this study was reduced by about 25% of that in control lungs. For the reasons stated therefore the macromolecular structure of the collagen molecules may be considerably altered in the lungs of patients with the adult respiratory distress syndrome.

Other collagen types, especially types IV and V, are unlikely to contribute to total tissue hydroxylysyl content, since we found no evidence for a dramatic increase in type V collagen on slab gel electrophoresis. A similar decrease in hydroxylation of lysine has been reported by Seyer *et al.*¹⁸ in patients with chronic idiopathic pulmonary fibrosis and lung tissue from these patients also has an increased collagen solubility.

We have shown a significantly greater type III collagen content in the lungs of the study group, in terms both of total material and of material solubilised by pepsin, than in the control lungs. This is substantiated by the fact that total type III collagen amounts (determined by cyanogen bromide cleavage) and type III collagen in pepsin solubilised material show a similar significant increase.

Recently it has been suggested that acute "early" fibrosis consists of enhanced type III collagen deposition while end stage fibrosis shows increased type I

collagen content⁷; but there exist conflicting observations on these changes in collagen type. These may be due to studies carried out at different stages of disease, with variable amounts of lung tissue damage. In chronic idiopathic pulmonary fibrosis an increase in type I collagen in relation to type III collagen has been reported in man¹⁸ and in experimental animal models (using paraquat, bleomycin, and ozone).²⁰ Similar results were obtained from an immunohistochemical study by Takiya *et al.*²¹ At the biochemical level this has also been confirmed by Kirk *et al.*^{16,22} who found increases in ratios of type I to type III collagen in lung tissue obtained at necropsy from patients with chronic pulmonary fibrosis. The latter study, however, suggested that there may exist a time dependent pattern of collagen type changes, since they found lower type I:III ratios in biopsy samples from patients with idiopathic pulmonary fibrosis than in those obtained from controls or postmortem samples. Similarly, Bateman *et al.*²³ using immunohistochemical techniques to study lung collagen types, suggested a relationship between the presence of type III collagen and the activity of the disease. Last *et al.*²⁴ however, analysing lung collagen from patients with the adult respiratory distress syndrome, found significant increases in type I:III collagen ratios. The same group reported increased type I:III ratios in neonates with respiratory distress requiring mechanical ventilation for up to 147 days.²⁵

Recently Laurent⁷ described the deposition of collagen as occurring as a "late event in the sequence of steps... described as the repair cascade," which, however, is obviously progressive and does not appear to be reversible. Our results for collagen amounts, solubility rates, and ratios of interstitial collagen types bears some resemblance to the normal progression in wound healing processes. Firstly, an initial drop in collagen concentration is followed by a return to normal of collagen concentrations in both the granulation tissue^{26,27} and early fibrotic lung tissue.^{5,6} Furthermore, the total collagen content of wound tissue gradually increases as in adult respiratory distress syndrome lung tissue, reaching almost normal values after the scar formation has finished.²⁶ In addition, collagen solubility rates for tissue obtained from granulating wounds are increased,^{9,28} in agreement with our observations. Finally there are numerous reports of increased type III:I ratios in early granulation tissue,²⁹⁻³¹ as we and others have reported for early pulmonary fibrosis.^{16,22} The report of increased type I collagen content in the lungs of patients with chronic pulmonary fibrosis¹⁸ seems consistent with the finding of the apparently increased type I collagen content in mature scars.^{28,31}

If this hypothesis of pulmonary fibrosis is true, could tissue remodelling be expected in this disease?

One might expect that the deposition of collagen molecules is reversible if the aetiological agent disappears in the early stages of the disease. There seems to be some evidence for this in a single case report, with sequential morphological analysis, of a patient who survived acute post-traumatic pulmonary fibrosis.³² In this instance the newly synthesised and only loosely cross linked collagen (with its increased solubility properties) may be removed more readily by degrading enzymes. On the other hand, in ongoing chronic pulmonary fibrosis recurrent "injuries" to the lung tissue may be assumed to result in a progressive and irreversible process. Finally, we cannot exclude the possibility that some patients react to a single pulmonary injury by a progressive deposition of collagen within the lung.

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