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A Molecular Marker for Fibrotic Collagen in Lungs of Infants with Respiratory Distress Syndrome

KAREN M. REISER AND JEROLD A. LAST

Department of Internal Medicine and California Primate Research Center, University of California, Davis, California 95616

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The hallmark of pulmonary fibrosis is abnormal deposition of collagen in the lungs of affected individuals. It is known that the relative ratios of the two major collagen types of the lung, types I and III, are altered in patients that died of idiopathic pulmonary fibrosis (1) or of the adult (2) or infant (3) respiratory distress syndromes. Whether these changes in collagen type ratios represent the result of extensive remodeling of the lung (4) or of deposition of an excess of newly synthesized collagen (5) of a particular type has not yet been proven definitively in human lung disease. In animal models of acute lung fibrosis it has been established unequivocally that comparable shifts in collagen type ratios are the result of excessive deposition of newly synthesized type I collagen in affected lungs (6, 7).

These observations of pulmonary fibrosis in rodent models have led us to search for molecular markers of abnormal collagen being synthesized in acutely damaged "prefibrotic" lungs, and being deposited in fibrotic lungs. We have previously reported (7, 8) that there are changes in the collagen difunctional crosslinks in lungs from silicotic rats. Specifically, there is an increase in the dihydroxylysinonorleucine:hydroxylysinonorleucine (DHLNL:HLNL) ratio. These observations led us to examine the crosslinks in collagen from lungs of infants dying of complications after treatment for respiratory distress syndrome of the newborn. In this study we attempted to test the hypothesis that an increase in the relative amount of DHLNL serves as a molecular marker of the deposition of fibrotic collagen.

MATERIALS AND METHODS

Lung Tissue

Lung lobes (generally the intact left lower lobe or pieces thereof) were obtained at autopsy. Lungs were frozen on dry ice, then stored at −20°C for up to several years before the analyses reported herein. Patients had various neonatal (and prenatal) diseases, which have been described in detail elsewhere (3). This protocol was approved by the University Committee on Ethics of Human Experimentation

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and informed consent was obtained from parents for acquisition of tissue. Adult lungs were also obtained at autopsy; informed consent was obtained from next of kin. These patients have been described previously (2).

Preparation of Tissue for Analysis

Lung tissue was minced into fine pieces and washed with 5 mM phosphate buffer containing 0.9% NaCl, pH 7.4, to remove blood and soluble proteins. After removal of the wash fluid with a Pasteur pipet, about 40 mg wet wt of tissue was incubated in 3 ml of 0.1 M sodium phosphate, pH 7.4, for 4 hr at room temperature (about 25°C). NaB^3H_4 (142 Ci/mole; Amersham, Arlington Heights, Ill.) was then added at a ratio of 1 part per 30 parts (dry weight) of the sample. After 1 hr the reduction was stopped by the addition of about 1 ml of glacial acetic acid (to pH 3–4). The tissues were then thoroughly rinsed with distilled water, hydrolyzed in 6 N HCl for 18 hr at 110°C, rotary evaporated to remove HCl, and filtered using a Rainin (Emeryville, Calif.) microfiltration apparatus. Hydroxyproline content of the hydrolysates was determined by a colorimetric assay (9).

Analysis of collagen crosslinks: Chromatography. Crosslinks were analyzed using a modification of an HPLC program described by us in detail previously (10). The reduced difunctional crosslinks DHLNL and HLNL, and also hydroxylysine, were analyzed by chromatography of aliquots of the lung hydrolysate containing 50 μg of hydroxyproline on a C_{18} reverse-phase column (Ultrasphere 0.4 × 5 cm; Altex, Berkeley, Calif.). An isocratic elution system was used; the buffer consisted of 22.5% *n*-propanol in 0.1 M phosphate buffer, pH 2.83, containing 0.3% sodium dodecyl sulfate. Flow rate was 0.8 ml/min. Amino acids and crosslinks in the effluent were visualized by their fluorescence (excitation filter cutoff, 360 nm; emission filter cutoff, 455 nm; Gilson-Spectra-Glo, Gilson, Middleton, Wisc.) after postcolumn derivatization with *o*-phthalaldehyde (10). In this system the difunctional crosslinks are eluted well after arginine and thus can be directly visualized even in an unfractionated lung hydrolysate. The difunctional crosslinks are completely separated from each other in this system. Fractions (1.3 ml) were collected from the fluorometer effluent every minute for determination of radioactivity by liquid scintillation counting. Samples were counted in 8 ml of Instagel (Packard, Downers Grove, Ill.) at an efficiency of about 33%.

For analysis of the trifunctional crosslink hydroxypyridinium (OHP), we used a slightly different solvent system that required a shorter elution time (OHP can also be determined at the same time as the difunctional crosslinks if two fluorometers are hooked up in sequence; OHP elutes just after HLNL). An aliquot of lung hydrolysate containing about 5 μg of hydroxyproline was chromatographed on a 0.4 × 10-cm C_{18} reverse-phase column (Accupak Short-One, Rainin). The elution solvent was the same as that described above except that it contained 24% *n*-propanol. A Hitachi 2000 adjustable wavelength fluorometer was used (excitation = 295 nm, emission = 395 nm) with a 12-μl flow cell to detect OHP in the eluate by its intrinsic fluorescence. A purified standard was prepared from bovine achilles tendon as described by us previously (10) and was used for calibrating the system.

Statistical analysis of data. Data are presented where appropriate as mean values ± SD. Comparisons of group means was by one-way analysis of variance using the Minitab program on a Burroughs Prime Computer.

RESULTS

Eight lungs (four controls, three of which were from stillborn infants; four from infants that required ventilator support for treatment of respiratory distress due to immaturity) were available for this study. Two of the Infant Respiratory Distress Syndrome (IRDS) infants (B.G. and B.C.) and all four of the controls have been described in detail previously (3). Salient clinical data are summarized in Table 1. Mean age at death of controls was 36 ± 9 weeks, while for the IRDS infants mean age was 38 ± 10 weeks. Obviously, more time was spent on average *ex utero* by the IRDS groups. Mean birth weight for the control group was about 2220 ± 980 g, while for the IRDS babies it was 1630 ± 1310 g.

We analyzed difunctional collagen crosslinks in hydrolysates of washed lungs after reduction with NaB^3H_4 to stabilize and radioactively label these compounds. The results of these studies are presented in Table 2. We also estimated the content of hydroxypyridinium (OHP), a nonreducible trifunctional crosslink derived from DHLNL, in these lungs, as quantified by its intrinsic fluorescence (Table 2). We did not have enough lung tissue to perform duplicate determinations for many of the subjects studied. Thus, only single determinations are shown in Table 2. However, in other studies of human and rat lung tissue we have found that variability between runs for the same sample is less than 5%. Variability between samples taken from different parts of the same lung is less than 10% in normal human lungs for analysis of either difunctional crosslinks or OHP. The most striking difference between control and IRDS lungs was in their total amount of reducible crosslinks, which was elevated 5- to 10-fold, and in their ratio of DHLNL to HLNL. Control lungs had a ratio of 2.1 ± 0.4, as compared with the corresponding value of 4.6 ± 0.4 in the IRDS lungs. This difference was highly significant ($P < 0.01$) despite the relatively small size of the groups

TABLE 1
Clinical Features of Patients in Study

Group and patient	Sex	Estimated gestational age (weeks)	Weeks on ventilator	Estimated age at death (weeks)	Birth weight (g)
Controls					
B.D.	M	29	—	29	1460
B.Ha.	F	44	—	44	2920
B.La.	F	28	—	28	1300
B.Ep.	F	43	—	44	3200
IRDS					
B.C.	M	28	21	49	1100
B.G.	M	27	5	32	880
B.J.	F	26	2	28	950
B.W.	M	44	1	45	3590

TABLE 2
Collagen Crosslinks in Patients' Lungs

Group and patient	Difunctional crosslinks (cpm/50 μg hydroxyproline) DHLNL	HLNL	DHLNL:HLNL	OHP content (mole/mole of collagen)
Controls				
B.D.	6,490	4,240	2.6	Not detected
B.Ha.	3,440	1,750	2.0	0.10
B.La.	2,380	1,480	1.6	0.01
B.Ep.	5,740	2,680	2.1	0.10
IRDS				
B.C.	44,800	10,900	4.6	0.05
B.G.	20,700	4,290	4.8	0.01
B.J.	17,800	3,530	5.0	0.01
B.W.	23,100	7,340	4.1	0.10

Note. DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; OHP, hydroxypyridinium.

being compared. We also observed an apparent difference in the OHP content of some of the lungs, which appeared to be age related. Since the value of 0.10 mole of OHP per mole of collagen is at about the lowest level of accurate determination of this crosslink in unfractionated lung tissue, the lower values should be viewed as approximate. Higher values of OHP were strongly associated with gestational ages in excess of 40 weeks. For the group of actual age (i.e., gestational age plus time on respirator) at death 29 ± 2 weeks (B.D., B.La., B.G., B.J.), OHP content was approximately one-tenth or less of the values in the group of gestational age 44 ± 0.6 weeks (B.Ha., B.Ep., B.W.). B.C., of gestational age 28 weeks, but of actual age at death 49 weeks, had intermediate values of OHP content in his lungs.

Four lungs (10 separate analyses) from adults (average age about 45 years) dying of diseases or trauma not related to the lung were also analyzed for OHP content. We found 0.32 ± 0.02 mole OHP per mole of collagen in these samples.

DISCUSSION

Our working hypothesis when we began this study was that we would see an increased ratio of DHLNL:HLNL in lungs from infants with the "prefibrotic disorder," infant respiratory distress syndrome. In fact, we found such an increase (Table 2), from a control ratio of about 2.1:1 to a ratio of 4.6:1 in the IRDS lungs. These changes are similar to shifts in DHLNL:HLNL ratios we have previously observed in lungs of rats with experimental silicosis (7, 8) and in rats intratracheally instilled with bleomycin 4 weeks previously (11).

Although there have been few studies in which crosslinking in human pulmonary fibrotic disorders has been directly analyzed, crosslinking in tissues other than lung has been investigated in various diseases associated with derangements in collagen metabolism. Increased DHLNL:HLNL ratios have been reported in hypertrophic scarring (12), early wound healing (13), palmar fascia from patients

with Dupuytren's disease (14), and hip joint capsules from patients with congenital dislocation of the hip (15).

What sort of mechanism might underlie this alteration in crosslinking? Many of the diseases associated with changes in DHLNL:HLNL ratios are also associated with alterations in collagen type ratios, including IRDS (3). However, it is unlikely that there is a simple relationship between type ratios and crosslinking patterns. In fact, Barnes *et al.* (13) observed that the increased levels of DHLNL seen in guinea pig scar collagen were present on both type I and type III collagen chains. Rather, the increase in DHLNL:HLNL must represent a difference in the structure of the collagen α chains, such as an increase in the level of lysine hydroxylation. Indeed, it has been suggested (13) that rapidly proliferating tissue, such as might occur after injury, is characterized by a return to a more "embryonic" type of collagen with higher levels of lysine hydroxylation.

We also observed increased levels of the "mature" collagen crosslink, OHP, which is thought to arise from condensation of three hydroxylysine residues (16), in lungs of gestationally older infants. DHLNL is believed to be a precursor of OHP. It is interesting that term infants already seem to have about one-third of the OHP content per collagen molecule as do adult lungs, suggesting relatively rapid synthesis of this mature crosslink in human lung collagen. While our studies in animal models of fibrotic lung disease suggest that there are increased levels of OHP expressed on a per mole of collagen basis in collagen of fibrotic lungs (17,18), there seems to be a greater correlation with gestational age than with IRDS in the infant lungs in the present study. This may well be a consequence of the acute nature of IRDS, as we are only able to appreciate increased lung content of OHP in rat lungs several months after the onset of fibrosis. However, we also observed a rapid increase (within a few weeks of injury) in the DHLNL:HLNL ratio of lung collagen in these animal models, suggesting that our observations in the IRDS lungs (Table 2) may presage such an increase in the IRDS patients were they to survive long enough to allow for the maturation of the difunctional crosslink DHLNL to OHP. It would be very interesting to examine OHP content of lungs from patients with chronic lung disease (idiopathic pulmonary fibrosis or sarcoidosis) of long duration to examine whether OHP content is elevated.

In summary, then, our findings with regard to lung collagen crosslinks in IRDS patients are consistent with our previous observations in two different animal models of pulmonary fibrosis. Differences in crosslinks observed in fibrotic lung collagen suggest a key role for changes in patterns of hydroxylation of lysine in the collagen accumulating in such diseased lungs. These data suggest that derangement of a normal control mechanism for modulation of collagen synthesis (or maturation into fibers) may accompany this shift in crosslinking. If that is indeed the case, then treatment of lung fibrosis after the stage of acute lung damage might prove feasible, a philosophical departure from current clinical procedure.

SUMMARY

Lung samples from four infants who died of respiratory complications of prematurity (Infant Respiratory Distress Syndrome, IRDS) were analyzed for

their content of various collagen crosslink amino acids by newly developed techniques of high-performance liquid chromatography. Comparable analyses were performed with tissue from stillborn infants with apparently normal lungs (control group) and from adults without apparent lung disease. We observed increased amounts of the difunctional crosslink dihydroxylysinonorleucine (DHLNL) in the IRDS lungs. Gestational age seemed to be the most important determinant of total lung content of the trifunctional crosslink hydroxypyridinium (OHP). Term infants had about one-third of the OHP content in their lung collagen as was found in the adult lungs. These observations suggest that there are important changes in the molecular structure of collagen in a human fibrotic lung disease, changes that are paralleled in various animal models of experimental pulmonary fibrosis, and in various human diseases involving abnormalities of skin or bone collagen metabolism.

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