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Glycosaminoglycan and Collagen Synthesis in *N*-Nitroso-*N*-Methylurethane Induced Pulmonary Fibrosis (40814)[1]

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Interstitial pulmonary fibrosis is a term used to describe a group of heterogeneous lung disorders characterized by diverse, but related, morphological changes (1–5). The name was derived from studies which revealed an apparent histological increase in interstitial connective tissue, and particularly in collagen (6, 7). However, the distribution and synthesis of connective tissue components in this disease remain poorly understood.

To better characterize the changes in connective tissue in one form of pulmonary fibrosis, an animal model of the disease was employed. This model was first introduced by Herrold (8) and further characterized by Ryan (9) and was induced by the administration of *N*-nitroso-*N*-methylurethane (NNNMU) to Syrian hamsters. The morphological changes produced ultimately resemble the human form of chronic, idiopathic pulmonary interstitital fibrosis. In the present study, synthesis of glycosaminoglycans and collagen in the lungs of diseased and control animals was studied at 1 and 3 months following administration of NNNMU. Alterations in glycosaminoglycans were studied because little is known of these changes in pulmonary fibrosis and because of the possible importance of these connective tissue components in lung function. In addition, it was of interest to determine if the histologically apparent increase in connective tissue seen in this model was corroborated by biochemical analysis.

Materials and methods. Induction of pulmonary fibrosis. One-month-old Syrian Golden hamsters (outbred females) were divided into experimental and control groups. A total of 36 animals (18 experimental, 18 control) were used for the biochemical studies. An additional number of animals (15 experimental, 14 control), employed in studies not described here, are included in regard to wet lung weight only (see below). The experimental animals received 0.1-ml interscapular, subcutaneous injections weekly of a 2.0 mg/ml solution of *N*-nitroso-*N*-methylurethane (NNNMU) (Aldrich Chemical Co., Milwaukee, Wis.) dissolved in a 0.5% solution of ethanol in water. Injections were continued for up to 16 weeks, depending on a particular animal's respiratory status. The control group received weekly 0.1-ml injections of 0.5% ethanol in water for 16 weeks.

Labeling of lung connective tissues. At 1 and at 3 months following cessation of NNNMU injections, both experimental and control animals were given intraperitoneal injections of either ^{35}S (in the form of carrier-free sulfuric acid) or [^{3}H]proline (both isotopes from New England Nuclear, Boston, Mass.) The isotopes were used to label the glycosminoglycans and collagen, respectively. Such labeling procedures have been shown to reflect turnover rates of molecular substances both *in vivo* and *in vitro* (10, 11). Injections of the isotopes were given daily for 4 consecutive days to obtain adequate labeling (1.5 mCi/kg/day).

Tissue preparation. The animals were sacrificed following the labeling procedure by intraperitoneal injection of barbiturate. The lungs were resected from the thorax. Trachea, mainstem bronchi, and major pulmonary vasculature were dissected free and discarded. The lungs were then weighed and tissue sections (one from each lung) were taken for histology. The wet tissues, minus these sections, were again weighed for later comparison with dry lung weight. The lung tissues were then minced into small fragments, washed free of blood, and homogenized. The homogenates were dialyzed free of salts and lyophilized. The

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lyophilates were weighed and portions were used for the biochemical studies.

Analysis of labeled glycosaminoglycans. Weighed portions of the lyophilized lung tissue were delipidated in acetone and air dried. The lipid-extracted tissue was suspended in 0.1 *N* sodium acetate buffer, pH 5.5, and digested by papain (Sigma Chemical Co., St. Louis, Mo.) activated with 5 m*M* EDTA and 5 m*M* cysteine hydrochloride. Digestion was performed at 57°C for 48 hr. Ten percent TCA was then added and the undigested proteins were precipitated at 4°C. Following centrifugation (10,000*g* for 30 min.), the precipitates were discarded. The supernatants were dialyzed free of TCA and smaller polypeptides. Carrier chondroitin sulfate was added to the dialysates, which were then lyophilized and resuspended in a small volume of water (2–3 ml). The glycosaminoglycans were precipitated by addition of 3 vol of sodium acetate-saturated ethanol. Both precipitate and supernate were measured for radioactivity; over 90% of the counts were found to be in the precipitated material. The remaining, unprecipitated counts may be associated with a glycosaminoglycan precursor and/or some degradation product.

The isolated glycosaminoglycans were then digested with chondroitinase ABC and, in some cases, with chondroitinase AC-II (both from Miles Laboratories, Elkhart, Ind.) according to the method of Saito *et al.* (12). Analysis of enzyme purity was not performed in our laboratory. The method of purification and measurement of specificity are extensively discussed by Saito *et al.* (12) and by Hiyama and Okada (13). According to the manufacturer (Seikagaku Kogyo Co.) the enzymes were free of protease, RNase, and DNase as well as glucuronidase. They also stated that chondroitinase ABC contained small amounts of sulfatase activity, which might affect results only with large excesses of the enzyme. Tests of the manufactured enzymes were conducted by Shapiro *et al.* (10).

The digests were chromatographed on Whatman No. 3 filter paper in a system of butanol, acetic acid, and 1 *N* ammonium hydroxide (2:3:1). This yielded three separate products: (i) enzyme-resistant glycosaminoglycans (heparin and/or heparan sulfate); (ii) disaccharides of chondroitin-4-sulfate and dermatan sulfate (with chondroitinase AC-II, dermatan sulfate remained undigested and chromatographed with the enzyme-resistant material); (iii) disaccharides of chondroitin-6-sulfate. The developed chromatograms were cut into small strips, each of which was placed in 10 ml of scintillation fluid and measured for radioactivity in a liquid scintillation spectrometer.

Total incorporation of label into glycosaminoglycans was expressed as counts/milligram dry tissue. Incorporation of label into individual glycosaminoglycans was calculated as a percentage of the total labeled material in the three pools yielded by chromatography.

Collagen analysis. Portions of the ^{3}H-labeled lung tissue were weighed and suspended in 0.01 *M* Tris buffer, pH 7.4, supplemented with 10 m*M* $CaCl_2$, 10 m*M* KCl, and 1.5 m*M* $MgCl_2$ (14). A purified preparation of bacterial collagenase (form III, from Advance Biofactures, Lynbrook, N.Y.) was added to the suspension and incubated overnight at room temperature. Digestion with the collagenase was performed a total of three times, with washing of the residue following each digestion. The collagenase was tested for the presence of contaminating proteases with [^{3}H]-tryptophan-labeled chick embryo protein (15). Virtually no digestion was found to occur and the collagenase was presumed to be free of extraneous protease activity. Lung tissue digests, including washes, were then analyzed for radioactivity. Incorporation of label into the digestible collagen was expressed as counts/milligram dry tissue.

The progress of the collagenase digestion was assayed by the ninhydrin colorimetric procedure of Moore and Stein (16) which detects free amino groups. Leucine was used as a standard and the total free amino groups in the three collagenase digests of

each lung sample were finally expressed as μM leucine/milligram dry tissue.

The digested residues were tested for remaining hydroxyproline (as a measure of undigested collagen). The context of hydroxyproline remaining in the residue was 2.6–4.6 μg hydroxyproline/milligram. This corresponds to 2.6–4.6% collagen remaining in the residue and is less than or equal to 10% of the collagen originally present in the tissue sample.

Determinations of extent of lung disease. Generous biopsies were taken from the left and right lungs of each experimental animal. At least one biopsy of each pair of control lungs was also obtained to ascertain if any incidental pathological changes had occurred. The biopsies were inflated under negative pressure to a pressure of −20 cm H_2O and fixed in formalin. They were then processed for histological examination (hematoxylin and eosin staining), coded, and graded by a pathologist (JOC) for extent of disease according to the following scale: 0 = absence of any pathological change; 1 = only minimal, focal disruption of normal architecture; 2 = more diffuse changes involving discrete areas of epithelial hyperplasia and fibrosis; 3 = moderate damage to the lung architecture involving large areas of epithelial cell hyperplasia and fibrosis; 4= obliteration of the major portion of alveolar parenchyma with broad areas of alveolar collapse and microcyst formation but with occasional areas of normal tissue remaining; 5 = almost total destruction of normal architecture.

Results. Lung histology. Animals receiving injections of NNNMU showed sequential morphological changes in the pulmonary parenchyma over a period of 4 to 5 months. The initial stages of the disease were marked by formation of hyaline membranes in alveoli and by a widening of the interalveolar septa due to a combination of edema, hemorrhage, and inflammatory cell infiltrates. The latter consisted of a mixture of lymphocytes, plasma cells, neutrophils, and macrophages. Many macrophages were seen within the alveolar spaces, especially as the disease progressed. Swelling of alveolar epithelial lining cells occurred in the more affected areas along with proliferation of type II cells in the interstitium. As the disease progressed, entire clusters of alveoli underwent collapse, while adjacent air spaces became dilated and distorted (Fig. 1).

By the fourth month, the inflammatory infiltrates had receded and the disease entered a predominantly healing phase. This was marked by a histologically apparent increase in interstitial collagen.

Though the lungs of all animals receiving NNNMU underwent this sequence of pathological changes, the severity of the disease showed individual variation from animal to animal. This permitted correlations between biochemical changes in the connective tissue and histologically determined extent of disease.

Lung weight. Both wet and dry weights were obtained on lung tissues. There was a considerable spread in lung weights in both the experimental and control groups (Table I). However, at 1 month and again at 3 months post-NNNMU treatment, the experimental animals showed a statistically significant increase in wet lung weight. Dry weight/wet weight percentages were also calculated (Table I). They did not reveal a significant difference between the experimental and control groups at either time point, suggesting that the increased wet weights of the experimental lungs were not due to edema, but rather to increases in tissue mass.

Uptake of ^{35}S into lung glycosaminoglycans. Experimental and control animals were compared for uptake of ^{35}S into lung glycosaminoglycans. At 1 month following cessation of the NNNMU regimen, the diseased lungs showed a statistically significantly greater incorporation of isotope than did control tissues (1064 ± 329 vs 677 ± 303 cpm/mg; $P < 0.05$). At 3 months post-NNNMU, experimental lungs again showed a greater mean uptake of label, but the difference was not statistically significant, due, in part, to the small number of animals tested (Table II).

Portions of the labeled lung glycosamino-

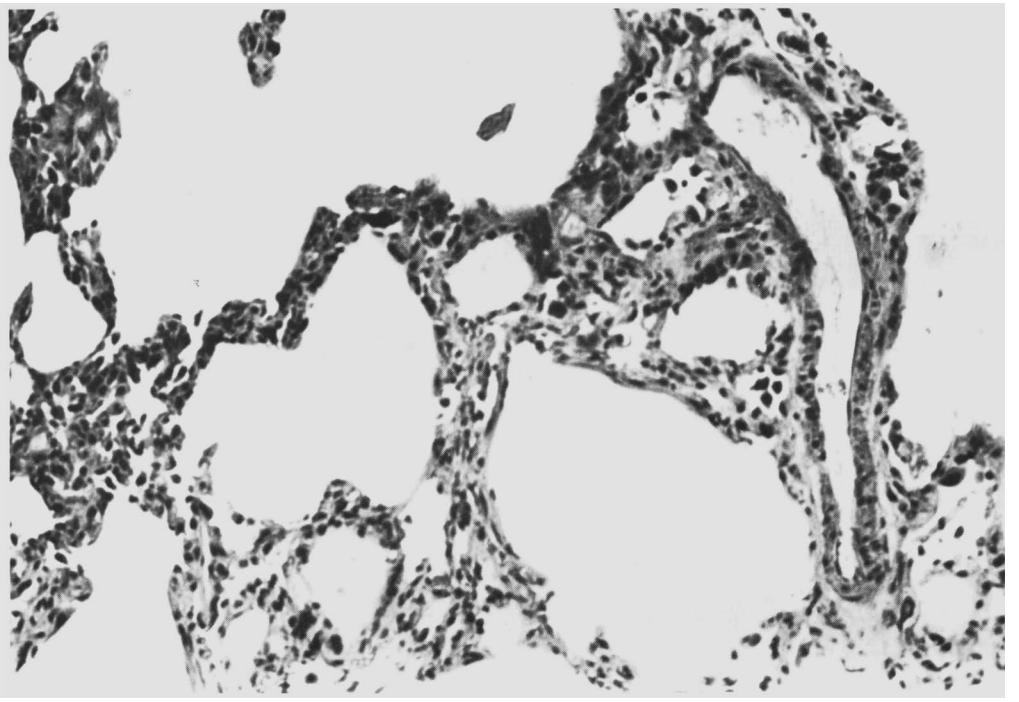

FIG. 1. Photomicrograph of experimental hamster lung 1 month post-NNNMU. Lungs were inflated under negative pressure and fixed in formalin. Alveoli appear distorted, with areas of marked dilatation. The pulmonary interstitium is widened and contains a mixture of inflammatory cells, type II pneumocytes, and fibroblasts (190×).

glycans which were degraded with chondroitinase ABC revealed that the experimental lungs contained a significantly higher percentage of labeled disaccharides of chondroitin 4-sulfate and/or dermatan sulfate than did controls. At 1 month post-NNNMU, the mean percentage of label associated with this disaccharide in experimental lungs was 57.6% as compared to 47.0% for controls. Three months post-NNNMU, the mean value for treated animals was 46.7%, whereas for controls the value was 35.9% (Table II).

Conversely, the NNNMU-treated lungs showed significantly lower percentages of labeled heparin and/or heparan sulfate than did control tissues. At 1 month post-NNNMU, the mean value for experimental animals was 28.7% as compared to 39.0% for untreated animals. Similarly, at 3

TABLE I. COMPARISON OF WET LUNG WEIGHTS AND DRY/WET WEIGHT PERCENTAGES IN CONTROL AND NNNMU-TREATED LUNGS[a]

Group	Month post-NNNMU	Lung weight	
		Wet wt. (mg)	Dry/Wet (%)
Control	1	851 ± 141 (27)*[b]	8.98 ± 1.97 (9)
Expt	1	920 ± 166 (26)*	7.78 ± 2.16 (9)
Control	3	822 ± 88 (6)**	10.18 ± 1.48 (6)
Expt	3	1183 ± 117 (6)**	10.44 ± 1.15 (5)

[a] Results are shown as mean values ± SD.
[b] Figures in parentheses refer to numbers of animals tested.
* $P < 0.05$ two-tailed analysis of variance.
** $P < 0.002$ two-tailed analysis of variance.

TABLE II. UPTAKE BY LUNG GLYCOSAMINOGLYCANS

Group	Month Post-NNNMU	Total Uptake (cpm/mg)	Percentage Ch'ase ABC Resistant	Percentage ΔDi-4S[a]	Percentage Derm-S	Percentage ΔDi-6S[b]
Expt	1	1064 ± 329(8)*,[c]	28.7 ± 6.0(9)†	57.6 ± 10.4(9)**	29.0 ± 4.6(3)††	14.7 ± 6.0(9)
Cont.	1	677 ± 303(7)*	39.0 ± 5.6(9)†	47.0 ± 8.7(9)**	19.3 ± 1.5(3)††	14.2 ± 5.4(9)
Expt	3	1493 ± 398(3)	24.7 ± 3.1(3)††	46.7 ± 1.3(3)††	—	28.7 ± 2.5(3)
Cont.	3	1101 ± 150(3)	38.5 ± 0.9(3)††	35.9 ± 2.3(3)††	—	25.6 ± 2.1(3)

[a] Di-4S = disaccharides of chondroitin 4-sulfate and dermatan sulfate.
[b] Di-6S = disaccharides of chondroitin 6-sulfate.
[c] Figures in parentheses refer to number of animals tested.
* $P < 0.05$ one-tailed analysis of variance.
** $P < 0.05$ two-tailed analysis of variance.
† $P < 0.01$ two-tailed analysis of variance.
†† $P < 0.05$ Mann–Whitney–Wilcoxon rank sum test.

months post-NNNMU, experimental lungs had a mean value of 24.7%, while controls averaged 38.5% (Table II).

The percentage of labeled disaccharides of chondroitin 6-sulfate was similar for both groups at 1 and at 3 months following NNNMU treatment; however, the relative amount of labeled chondroitin 6-sulfate was greater for both experimental and control lungs at the 3-month point (Table II).

Lung glycosaminoglycans were additionally degraded with chondroitinase AC-II. This enzyme digests chondroitin 4-sulfate, but not dermatan sulfate, thus permitting determination of ^{35}S uptake into each of these components separately. At 1 month post-NNNMU, nearly all the increased percentage of uptake of label by this group of glycosaminoglycans in experimental lungs (as seen with ABC'ase digestion) was associated with dermatan sulfate, rather than with chondroitin 4-sulfate (Table II). Problems encountered with enzyme stability prevented repetition of this experiment at 3 months post-NNNMU.

Correlation of morphological severity of lung disease and ^{35}S labeling of glycosaminoglycans. Lung disease in the treated hamsters was graded by light microscopy following coding of the histological sections. The microscopic slides were graded on a scale of 0–5, with 0 representing absence of disease. Uptake of label by individual glycosaminoglycans (ABC'ase digests) was compared to the extent of lung lesions. A positive correlation existed between severity of lung disease and uptake of label by disaccharides of chondroitin 4-sulfate and dermatan sulfate at 1 month post-NNNMU (Fig. 2). A negative correlation existed between percent of label incorporated into chondroitinase-resistant heparin and/or heparan sulfate and the severity of disease in the hamster lungs one month after NNNMU treatment (Fig. 3).

Lung collagen. Bacterial collagenase was used as a means of extracting collagen from the lung tissues. Uptake of [^{3}H]proline into collagenase-digestible collagen was determined as a measure of newly synthesized collagen. At 1 month post-NNNMU, the experimental lungs had significantly fewer collagenase-digestible counts than did con-

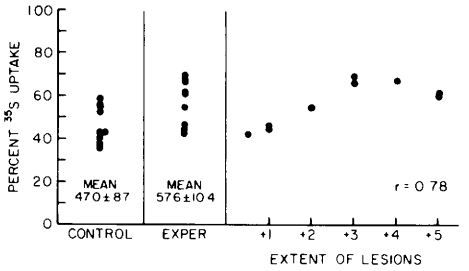

FIG. 2. Histogram (left side of Fig.) comparing uptake of ^{35}S by disaccharides of chondroitin-4 sulfate and dermatan sulfate in control and experimental lungs at 1 month post-NNNMU ($P < 0.05$). Graph (right side) comparing extent of disease in treated lungs with percentage of label incorporated into these disaccharides. Correlation coefficient is statistically significant at 0.05 level.

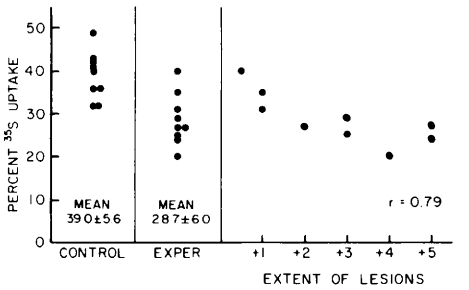

FIG. 3. Histogram (left side of Fig.) comparing uptake of ^{35}S by chondroitinase-resistant glycosaminoglycans (heparin and/or heparan sulfate) in control and experimental lungs at 1 month post-NNNMU ($P < 0.01$). Graph (right side) comparing extent of disease in treated lungs with percentage of label incorporated into these glycosaminoglycans. Correlation coefficient is statistically significant at 0.05 level.

trols (2610 ± 551 vs. 3687 ± 245 cpm/mg; $P < 0.05$). Again 3 months after the NNNMU regimen, treated lungs had a smaller mean number of radioactive counts than did control tissues, but the difference between the groups was not statistically significant at this time point (Table III).

The collagenase digests were also analyzed for free amino groups as a measure of total digestible lung collagen. The experimental animals contained less degradable lung collagen than did controls at 1 and at 3 months following NNNMU treatment. The difference between the groups was statistically significant at the 1-month point (Table III).

Discussion. The purpose of this study was to quantify certain connective tissue components in this experimental model of anatomically apparent interstitial pulmonary fibrosis. Possible changes in lung glycosaminoglycans were of special interest because they have not been adequately characterized in this disorder. While representing less than 1% of the total weight of lung parenchyma, glycosaminoglycans may nevertheless play an important role in lung function. They are a significant part of the extracellular matrix and may influence interactions among cells, including such events as cell differentiation (17). Furthermore, their close association with other connective tissue components, such as collagen (18), suggests that changes in glycosaminoglycans may affect the fiber skeleton of the lung.

Among the changes noted in glycosaminoglycans in the lungs of NNNMU-treated animals were increased uptake of a labeled precursor by these components as a whole, as well as changes from control in label uptake by individual glycosaminoglycans. Presumably, this indicates alterations in the pattern of turnover of glycos-

TABLE III. LUNG COLLAGEN (COLLAGENASE DIGESTIBLE)

Group	Month Post-NNNMU	Labeled (cpm/mg)	Total (μM/mg)[a]
Expt	1	2610 ± 551 (3)*[b]	0.94 ± .02 (3)*
Cont.	1	3687 ± 245 (3)*	1.11 ± .05 (3)*
Expt	3	3717 ± 1138 (3)	1.27 ± .35 (3)
Cont.	3	3989 ± 369 (3)	1.41 ± .41 (3)

[a] Micromoles leucine equivalents/mg dry tissue [ninhydrin photometric assay (16)].
[b] Figures in parentheses refer to number of animals tested.
* $P < 0.05$ Mann–Whitney–Wilcoxon rank sum test.

aminoglycans in this model of pulmonary fibrosis. The extent to which these changes in turnover varied from normal were related, in some cases, to the morphological severity of the lung disease.

In utilizing a labeled substrate to measure potential changes in diseased versus normal lungs, the problem arises of possible differences in distribution of isotope in various tissue compartments between experimental and control animals. Such differences in pool sizes may conceivably alter uptake of label, leading to difficulties in interpreting the meaning of the changes in total labeling of glycosaminoglycans and collagen which were found in fibrotic lungs as compared to controls. Nevertheless, the data regarding relative uptake of label into individual glycosaminoglycans, including correlations with severity of disease, should not be affected by possible pool size differences.

The etiology of the observed changes in glycosaminoglycan labeling is not clear at present. Little is known of glycosaminoglycan synthesis by individual lung cells and the dramatic shifts in lung cell populations in this disease may in part be responsible for these changes. Other explanations for these abnormalities include changes in the state of differentiation of certain lung cells and alterations in the rates of synthesis and/or degradation of individual glycosaminoglycans. The role of the specific etiological agent cannot be adequately assessed because so few studies exist of changes in glycosaminoglycans in other animal models of pulmonary fibrosis or in the human forms of the disease (19, 20).

In regard to lung collagen, the authors found that the uptake of radiolabeled proline into the collagenase susceptible portion of lung tissue was significantly lower in NNNMU-treated animals at 1 month post-treatment. Consistent with this observation, total collagenase-digestible lung collagen was also lower. Since, as pointed out above, the bacterial collagenase used in this study degrades at least 90% of lung collagen, these findings are unlikley to be an artifact of the method employed. While this result is paradoxical in view of the conspicuousness of collagen on histological examination of the diseased lungs, disparities between biochemical and histological evidence of collagen deposition have been observed before. In studies of human specimens of interstitial lung disease, no increase occurred, on the average, in synthesis or in total amount of lung collagen (21). This does not mean that localized increases in collagen in lungs involved with the disease do not take place. Collagen may in fact be accumulating in certain areas of the lung while it is simultaneously being degraded elsewhere at similar or greater rates. Some support for this notion is found in the recent work of Gadek *et al.* (22) which has shown that active collagenases are present in the lower respiratory tract of patients with idiopathic pulmonary fibrosis. The histological impression of increased lung collagen in pulmonary fibrosis may result from architectural derangement of the parenchyma, with compression of some areas as a result of collapse. In addition, alterations in the collagen fibril itself or in closely adherent molecules may sufficiently alter staining characteristics so as to give the histological appearance of increased interstitial collagen.

It is not surprising that in other models of interstitial fibrosis changes in collagen composition different from those of the present study have been found (23, 24). It may be predicted that different agents used to induce the disease (which can result in dissimilar morphological changes) would produce different biochemical alterations. Furthermore, the time points at which the connective tissue is analyzed differ among these studies and this may also lead to disparate findings. In this study, connective tissue analysis was performed at an advanced stage of the disease, at a point when the inflammation had receded and repair of the lung was in progress. Despite the difficulties of comparing changes in connective tissue in these various animal models of pulmonary fibrosis and in extrapolation to the human form of the disease, such models do provide a means of studying reproducible changes in tissue morphology and in corresponding biochemistry which may eventually reveal underlying pathogenetic mechanisms.

Summary. Glycosaminoglycan and collagen synthesis were studied in a hamster

model of interstitial pulmonary fibrosis, induced by weekly subcutaneous injections of *N*-nitroso-*N*-methylurethane (NNNMU) for up to 16 weeks. Experimental and control animals were injected intraperitoneally with ^{35}S or [^{3}H]proline to label glycosaminoglycans and collagen, respectively. The labeling studies were performed at 1 and at 3 months following completion of NNNMU treatment. Uptake of ^{35}S into lung glycosaminoglycans was higher in diseased animals than in controls at both 1 and 3 months post-NNNMU. The experimental lungs had a significantly increased percentage of labeled dermatan sulfate and/or chondroitin 4-sulfate compared to controls at both time intervals. Additional studies performed only on the 1-month samples showed that this increase in percentage labeling was primarily in dermatan sulfate. NNNMU-treated lungs also had a significantly lower percentage of labeled heparin and/or heparan sulfate than did controls at 1 and 3 months post-NNNMU. Regarding collagen synthesis, both ^{3}H-labeled and total collagenase-digestible collagen was lower in diseased lungs than in controls at 1 and 3 months post-NNNMU. The differences at 1 month post-NNNMU were statistically significant. These results suggest that, in this model, changes from normal in glycosaminoglycans occur during the repair process. However, the histologically apparent increase in lung collagen is not corroborated by biochemical analysis.

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