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Changes in Hyaluronan Deposition during Early Respiratory Distress Syndrome in Premature Monkeys

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ABSTRACT. Increased deposition of hyaluronan (HA) is part of the early response to fibrogenic stimulus in the lung exposed to bleomycin injury and has been associated with increased lung water in adult animals. Early respiratory distress syndrome (RDS) in premature infants is characterized by increased lung water, and late sequelae include fibrosis or bronchopulmonary dysplasia. We hypothesized that increased HA in the alveolar interstitium would be associated with increasingly severe RDS in prematurely delivered monkeys and that modes of therapy that affect severity of disease such as treatment with high-frequency oscillatory ventilation or exogenous surfactant would decrease this response. Thirty-four *Macaca nemestrina* monkeys were delivered at 134 ± 1 d (term = 168 d) and randomized to high-frequency oscillatory ventilation or conventional mechanical ventilation from birth. Sixteen of these animals received surfactant. At 6 h of age, the right lower lung was frozen *in situ* during inflation to 30 cm H_2O (approximately 2940 Pa) and then dehydrated and processed for microscopy. The presence and severity of RDS were evaluated by clinical and morphologic criteria. HA concentrations in lung extracts increased with progressively severe RDS ($p = 0.0003$). Treatment with high-frequency oscillatory ventilation decreased the lung injury score (1.69 ± 0.7 compared with 2.5 ± 0.9, $p = 0.05$), but changes in lung HA concentration did not reach significance (37.9 ± 22.7 compared with 44.8 ± 22.6). Surfactant treatment decreased lung HA concentration (29.6 ± 19.0 μg/wet lung) compared with non-surfactant-treated animals (54.7 ± 20.2 μg/g wet lung, $p = 0.0009$). Two fetal animals (144 and 163 d gestation) and seven additional premature animals ventilated for up to 96 h were compared with the animals killed at 6 h. HA concentrations increased with length of mechanical ventilation and severity of illness in these animals. HA was localized in freeze-dried lung sections using a biotinylated probe. Lung sections were blindly scored for the distribution of HA staining, and these scores were positively correlated with HA concentration measurements ($r = 0.75$, $p < 0.0001$). The quantity of HA in alveolar microvasculature correlated with severity of RDS ($r = 0.68$, $p = 0.0004$). We conclude that *1*) HA concentration in RDS lungs of prematurely delivered infant monkeys is increased relative to normal lungs at 6 h, *2*) increased HA is localized predominantly to the perivascular space of lung vasculature, and *3*) this response is decreased by surfactant treatment. (*Pediatr Res* 35: 238–243, 1994)

Abbreviations

CMV, conventional mechanical ventilation
HA, hyaluronic acid, hyaluronan
HABr, HA binding region
HFOV, high-frequency oscillatory ventilation
RDS, respiratory distress syndrome
PaO_2, arterial partial pressure of oxygen

HA is a major component of the extracellular matrix surrounding migrating and proliferating cells, and so is an important component of healing or regenerating tissue. One molecule of HA may contain as many as 50 000 repeating disaccharide units [glucuronic acid β and (1 →3) N-acetyl glucosamine] in a linear array. Extensive intrachain hydrogen bonding, mutual repulsion between the negatively charged carboxylate groups, and the β linkages between the sugar residues lend HA an inherent stiffness (1, 2). The large number of hydrophilic residues on the HA chain bind water, resulting in a viscous hydrated gel. Given no constraints, a hydrated HA molecule will occupy a volume 1 000 to 10 000 times the space of HA alone. The COO^- groups along the molecule also bind cations, increasing the osmotic pressure in the gel. Thus, large amounts of water are taken up in the presence of HA, increasing the turgor pressure within the matrix. HA also interacts or aggregates with other large proteoglycans forming even larger complexes. In addition to its space-filling role, HA can act as a specific ligand for HA receptors. Two such receptors have been identified: CD44 (3) and RHAMM (4). The interaction of HA with these receptors mediates several functions during normal cellular metabolism (5), as well as during developmental (6, 7) and repair processes (8).

In normal mature lung, HA is present at the basal surfaces of bronchiolar epithelium and in the adventitia of muscularized blood vessels, whereas little if any is present within the alveolar interstitium (9). Increased HA deposition has been associated with early injury in bleomycin-injured rat lungs (10), adult RDS (11), and emphysema (12), whereas increased HA synthesis has been reported in nonhuman primates with RDS (13). The large hydrodynamic volume of HA is thought to create a favorable environment for cell migration, an important component of early response to injury. The increase in HA seen in these disease states may promote pulmonary macrophage aggregation (14) and fibroblast migration (15) and may also contribute to alterations in lung fluid balance and pulmonary compliance (16, 17). It is interesting that not all pulmonary pathology is associated

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with increased HA, inasmuch as a decrease in lung HA has been reported in group B streptococcal pneumonia (18).

Because the intrinsic characteristics of HA could contribute to the increased lung water and decreased lung compliance seen in infants with RDS, we hypothesized that increased HA in the alveolar interstitium would be associated with increasingly severe RDS in prematurely delivered monkeys relative to normal control lungs and that modes of therapy that affect severity of disease such as treatment with HFOV or exogenous surfactant would decrease this response. Lung development in the primate *Macaca nemestrina* is analogous to human lung development (19) and as such this species has been used as an experimental model for the study of lung development (20, 21) and the pathogenesis of RDS (22). Increased synthesis of HA has been reported in animals with RDS (13), but it is not known whether increased HA deposition occurs in this disease or, if so, where this excess HA is localized. The early response to injury by this damaged tissue is critical to the subsequent healing and maturation of the lung.

MATERIALS AND METHODS

In vivo methods. Eighteen *Macaca nemestrina* monkeys were studied as a part of a related experiment comparing the effects of CMV and HFOV on the development of alveolar edema in monkeys at risk for RDS ($n = 9$ in each group) (22). An additional 16 surfactant-treated animals formed part of a second protocol extending the studies of mechanical ventilation and were included in this analysis ($n = 8$ randomized to HFOV and $n = 8$ randomized to CMV). Seven additional animals were delivered at 134 ± 1 d gestation and were mechanically ventilated (CMV) for varying lengths of time up to 96 h. Two fetal animals (aged 144 and 162 d) were also included for comparison.

Animals were delivered by hysterotomy at 134 ± 1 d after timed conception; term gestation in this species is 168 d. The infants were quickly dried, weighed, and positioned under a radiant warmer. A cuffed endotracheal tube (2.0 mm inner diameter) was immediately inserted and then connected to the assigned mechanical ventilator. Bovine surfactant supplied by Ross Laboratories (Division of Abbot Laboratories, N. Chicago, IL), as a liquid formulation of Survanta (beractant) intratracheal suspension (4 mL/kg) was given over the first several minutes of life to 16 of the 34 animals studied at 6 h. In HFOV animals, the mean airway pressure was kept at 15 cm H_2O (1470 Pa), and amplitude was adjusted to maintain a P_{CO_2} between 35 and 45 torr (4.7–6.0 kPa). In CMV animals, the mean airway pressure was raised from 8 cm H_2O (784 Pa) to 13 cm H_2O (1274 Pa) at 6 h to prevent hypoxia. One hundred percent oxygen was provided throughout the duration of the study for all 6-h animals. An umbilical artery catheter was placed in the lower abdominal aorta for administration of fluids and medication. A rectal temperature probe was inserted and a radiant heat source was servocontrolled to a skin thermistor to keep the rectal temperature between 36.5° and 37.5°C. Arterial blood gas tensions were measured at least hourly by the withdrawal of samples from the umbilical artery catheter (Corning 168, Corning Medical, Medfield, MA); all blood losses were replaced with maternal whole blood. Morphine sulfate at 24 μg/kg/h was administered for sedation through the umbilical artery catheter with dextrose 10% in water at a rate of 150 mL/kg/d. Blood pressure and heart rate were monitored continuously and recorded hourly. To maintain muscular paralysis, pancuronium bromide, 0.1 mg/kg, was given every 2 h. Anteroposterior and lateral chest radiographs were obtained in all animals at 2 and 5 h of age and graded by three blinded observers for presence and severity of RDS (1 = normal, 2 = mild, 3 = severe RDS). The two chest radiograph scores were added to give a possible range from 2 to 6. The presence and severity of clinical RDS were assessed by the following criteria: serial P_{aO_2} [1 = the majority of values of $P_{aO_2} \geq 400$ torr (53.3 kPa), 2 = P_{aO_2} of 300–399 torr (40–53.2 kPa), 3 = P_{aO_2} of 200–299 torr (26.6–39.9 kPa), 4 = P_{aO_2} of 100–199 torr (13.3–26.5 kPa), and 5 = $P_{aO_2} \leq 100$ torr (13.3 kPa)], chest radiograph score, and the gross appearance of lungs at the time of thoracotomy (1 = pale pink, well-inflated, normal appearing lungs; 2 = patchy areas of atelectasis, dark pink areas; 3 = dark pink lungs, large areas of atelectasis and/or hemorrhage, lungs sink in water). The presence and severity of RDS was graded 0 to 3+ on the basis of the cumulative score (0 = no RDS, range 4–6 cumulative points; 1+ = mild RDS, range 7–9; 2+ = moderate RDS, range 10–12; and 3+ = severe RDS, range 13–14). Ventilated, oxygenated animals with a score of 0 were used as controls ($n = 8$).

In the animals that were ventilated for longer than 6 h, ventilator settings and inspired oxygen were altered as needed to keep the P_{aO_2} between 50 and 100 torr (6.7–13.3 kPa) and the arterial P_{CO_2} between 35 and 45 torr (4.7–6 kPa). All these animals were ventilated with CMV.

Frozen tissue samples. At 6 h of life, monkeys were anesthetized with 30 mg/kg sodium pentobarbital i.v. A right intercostal incision was made. The exposed lung was sighed twice to 30 cm H_2O pressure (2940 Pa) and quickly frozen with Freon 22 (DuPont, Wilmington, DE) at −150°C. Samples of the frozen right lower lobe were removed with a small rotary saw while freezing continued. Samples were then dried for 2 d at 0.001 torr at −50°C and fixed with paraformaldehyde vapor (22). One sample from each animal was infiltrated and embedded with melted paraffin.

Radiometric assay. A radiometric assay was used to quantitate HA per g wet lung on at least one block of tissue obtained from the right lower lobe and as many as three blocks from the right upper and lower lobes of lung from 33 animals (tissue from one monkey was not available for assay). Lung tissue was consistently taken from peripheral parenchyma for assay purposes. Approximately 50 mg of lung were homogenized in 500 μL of 0.1 M Tris buffer and subjected to overnight Pronase (Sigma, St. Louis, MO) digestion (25 μg per 500 μL sample) at 45°C. The Pronase was inactivated by boiling for 2 min after digestion. Samples were centrifuged for 2 min at 14 000 rpm and the resulting supernatant was removed, weighed, and diluted 1:50. Duplicate aliquots were assayed for HA using a technique (Pharmacia Diagnostics, Uppsala, Sweden) based on the use of the specific binding region (HABr) from bovine nasal cartilage chondroitin sulfate proteoglycan core protein. HA from the sample (100 μL) was allowed to bind ^{125}I-labeled HABr in solution for at least 60 min. The unbound ^{125}I-labeled HABr was then quantified after interaction with HA covalently bound to AH Sepharose beads. Duplicate assays from at least one block of lung from all monkeys were done together with a standard curve. The standard curve was done in triplicate. Variability was less than 2%. This entire series of assays was duplicated with a standard curve. In addition, when available, up to three blocks of lung tissue were independently assayed for each experimental animal. All results from each animal were averaged. The entire series of samples was run in each independent determination.

DNA assay. Lung tissue DNA was assayed by a fluorometric procedure using Hoechst dye 33258 (Sigma) essentially as described by Labarca and Paigen (23). Briefly, 8–25 mg of frozen lung per sample were minced and sonicated at 60°C in 1% SDS in assay buffer composed of 0.05 M $NaPO_4$ buffer, pH 7.4, containing 2 M NaCl and 2 mM EDTA (to inhibit DNAse activity). Tissue homogenates were diluted 10-fold in the same buffer without SDS, and 1-μL triplicate aliquots were diluted into 1 mL of assay buffer containing 33258 Hoechst dye (1.5×10^{-6} M). Increased fluorescence was determined with a spectrophotofluorometer with excitation wavelength at 360 nm and emission recorded at 450 nm and compared with standard curve constructed using calf thymus DNA.

Localization of HA in tissue sections. Freeze-dried lung sections from 23 animals were available for study and were prepared for staining by hydration in PBS and incubation for 10 min each

of the following: 70% alcohol, methanol containing 0.3% hydrogen peroxide, 70% alcohol, and PBS. Biotinylated HABr of cartilage Aggrecan was prepared and kindly provided by Dr. Charles Underhill (Georgetown University, Washington, DC) for use as a histochemical probe for HA (9). Lung sections were incubated overnight at room temperature with a biotinylated probe (4 ng/L) for HA contained in 10% calf serum. The probe was localized after exposure to streptavidin-conjugated peroxidase using 3-amino 9-ethyl carbazole (Sigma) as substrate (red reaction product). Sections were counterstained with Mayer's hematoxylin for 5 min. Lung sections were examined by light microscopy at 100 to 400× and scored by two blinded observers using a scale from 1 (no HA in the alveolar walls or in the perivascular sheaths of medium- and small-sized vessels) to 4 (large amounts of HA in alveolar walls and surrounding medium and small pulmonary vessels). Selected lung samples prefixed with 1% cetylpyridinium chloride (Sigma), 3% paraformaldehyde, and 60 mM sucrose in PBS were compared with samples that were not prefixed. Prefixation of tissue with 1% cetylpyridinium chloride interfered with staining. To control for the presence of endogenous peroxidase, sections were incubated with 10% calf serum in PBS alone (no biotinylated HABr). Control sections were also pretreated with *Streptomyces* hyaluronidase (Sigma) (20 U/mL, 0.2 M sodium acetate buffer, pH 5.5, for 1 h at 37°C) or incubated with probe that had been preadsorbed with HA (100 ng/L overnight at 4°C) to insure specificity of the reaction. Each batch of slides was run with three negative control slides. The effect of surfactant on HABr is not known; however, control experiments done on sections from surfactant-treated animals were not different from those experiments done without surfactant. The HABr-HA interaction is highly specific and of high affinity, occurring even in the presence of 0.9 M guanidine hydrochloride.

Statistical methods. A two-tailed *t* test was done to compare HA lung concentration in animals with no RDS with that in those with any severity of RDS. Tukey's procedure for multiple comparisons was used with one-way analysis of variance to determine whether there was a difference in HA concentrations between RDS severity groups (0 to 3+). Two-tailed *t* tests were done on HA concentration from lung extracts of animals ventilated with HFOV and CMV and on lung extracts from surfactant-treated and nontreated animals. Spearman rank correlation test was done to determine whether a correlation between HA concentration from lung extracts and HA histologic scores (1+ to 4+) or between HA histologic score and RDS score existed. Data are presented as the mean ± SD. A p value of less than 0.05 was considered significant.

RESULTS

All animals evaluated at 6 h of life were treated with mechanical ventilation and 100% oxygen from birth until death. Twenty-six of 34 animals had evidence of RDS. Fifteen animals had mild (1+) RDS, nine animals had moderate (2+) RDS, and two had severe (3+) disease. Eight animals had no evidence of lung disease and served as controls. Of these eight controls, seven were ventilated with HFOV (six were surfactant treated, one was not) and one was conventionally ventilated (with surfactant treatment). The mean concentration of HA in lung extracts was significantly greater in all RDS animals (50.0 ± 21.0 μg/g wet lung, $n = 25$) than in animals without RDS (19.6 ± 11.3 μg/g wet lung, $n = 8$), $p < 0.001$ (Table 1).

As the severity of RDS increased, the quantity of HA in lung extracts increased ($p = 0.0003$), ranging from a mean of 19.6 ± 11.2 μg/g wet lung in the group without RDS to 86.3 ± 0.9 μg/g wet lung in the most severe (3+) RDS group (Fig. 1). Tukey's procedure for multiple comparisons used with one-way analysis of variance showed the group with no RDS to be different from the mild (1+), moderate (2+), and severe (3+) RDS animals ($p < 0.05$ for each comparison). When μg HA per μg DNA were determined for each group (13 600 ± 4550, 26 100 ± 4780, and 32 300 ± 6780 for 1+, 2+, and 3+ RDS, respectively), the same relationship held true ($p < 0.05$).

When all animals ventilated by HFOV ($n = 17$) were compared with those ventilated by CMV ($n = 16$), no difference in HA was noted (37.9 ± 22.7 and 44.8 ± 22.6 μg/g wet lung, respectively) despite somewhat lower scores for severity of RDS in the HFOV group (1.69 ± 0.7 and 2.5 ± 0.9; $p < 0.05$). Irrespective of mode of ventilation, HA concentration in lung extracts was lower in surfactant-treated animals ($n = 16$) compared with untreated animals ($n = 17$) (29.6 ± 19 and 54.7 ± 20.2 μg/g wet lung, respectively; $p = 0.0009$). The effect of surfactant treatment on severity of RDS and on concentration of HA is seen in Figure 1, with mean severity score for the surfactant-treated animals equal to 1.63 ± 0.72 and the RDS score for untreated animals equal to 2.56 ± 0.82 ($p < 0.05$). The lowest concentration of HA in lung extracts was noted in the surfactant-treated lungs without RDS, regardless of mode of ventilation (Table 1).

Quantitation of HA extracted from the lungs of prematurely delivered animals maintained with prolonged CMV (up to 96 h) and supplemental oxygen revealed HA content that varied with severity of RDS and length of mechanical ventilation. Table 2 shows the relationship of severity of illness and length of mechanical ventilation to quantity of HA. Thus, in general, the sicker the animal and the longer it was mechanically ventilated, the higher the HA content. The two fetal animals were included for comparison.

Histochemical staining of lung sections was done to determine the location of HA in control and RDS animals. Histologic scores (1 through 4) were given for HA staining by two blinded observers, with staining for HA in progressively smaller vessels and in the alveolar walls receiving higher histochemical scores. Histologic HA scores and HA quantity determined from lung extracts were correlated ($r = 0.75$, $p < 0.00001$). There was a positive correlation between the clinically determined RDS score and the histologic HA score ($r = 0.68$, $p < 0.0004$).

HA was localized to the adventitia of large muscularized blood vessels in normal and RDS lung sections with no differences noted as a result of RDS (data not shown). Similarly, the basal surfaces of bronchiolar epithelium and the surrounding cartilage were positively stained for HA. The perivascular sheaths of small- and medium-sized muscular arteries became thickened and were stained heavily for HA as severity of lung disease progressed (Fig. 2*B–D*). As the severity of RDS increased, HA was increasingly associated with the microvasculature in the interalveolar spaces as determined by histologic scoring of HA. In the most severely affected cases, HA was present in the alveolar walls (Fig. 3*B–D*). Increased accumulations of HA were also noted in the interlobar and subpleural regions of lung (Fig. 3*D*), possibly corresponding to lymphatic drainage. It should be noted that all lungs were inflated to 30 cm H_2O (2940 Pa) before fixation; thus, the difference in alveolar inflation reflects the disease process in these animals. Consistent with the somewhat patchy distribution of RDS, there was some heterogeneity of staining for HA within lung sections. No staining for HA was noted in hyaline membranes or proteinaceous debris present in the alveolar space of affected animals. None of the lung sections that were stained to insure specificity of the reaction showed any positive reaction product (Fig. 4*A–C*). The control sections incubated with serum in PBS alone demonstrate that the red reaction product is not the result of endogenous peroxidases (Fig. 4*A*). Pretreatment with *Streptomyces* hyaluronidase eliminated the red reaction product, demonstrating specificity of the probe (Fig. 4*B*), and incubation of sections with probe that had been preadsorbed with HA also eliminated the red reaction product, demonstrating specificity of the HABr-HA interaction (Fig. 4*C*).

DISCUSSION

The quantity of HA in lungs of premature primates demonstrated to have RDS at 6 h of life is greater than that found in

Table 1. *Six-h experimental groups**

	CMV	CMV-SURF	HFOV	HFOV-SURF	Total
RDS					
n	8	7	8	2	25†
HA (μg/g wet lung)	60.2 ± 19.5	32.3 ± 12.4	51.5 ± 21.2	65.1 ± 18.6	50.0 ± 21.0
Normal					
n	0	1	1	6	8
HA (μg/g wet lung)		13.2	39	17.5 ± 9.4	19.6 ± 11.3

* CMV-SURF, CMV with surfactant treatment; HFOV-SURF, HFOV with surfactant treatment. HA values are mean ± SD.
† Tissue from one animal was unavailable for assay.

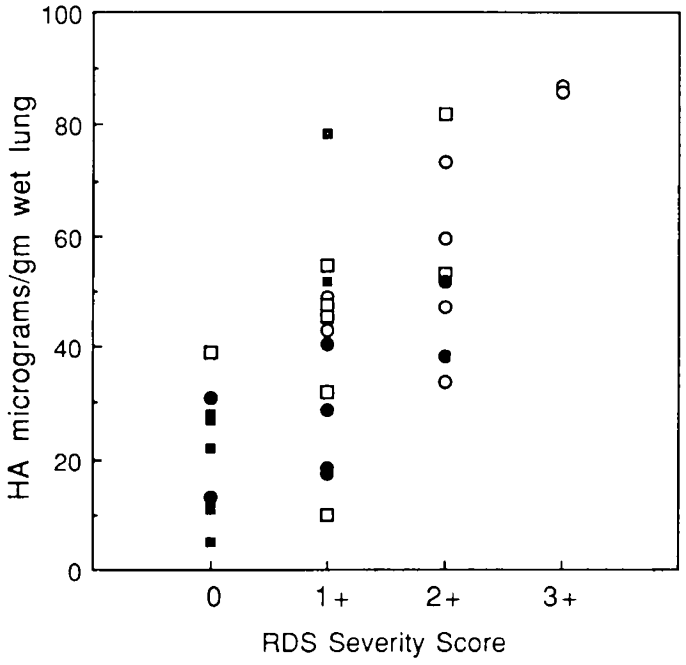

Fig. 1. Concentration of HA (μg/g wet lung) as determined by radioassay from lung extracts is shown as a function of severity of RDS. 0 indicates no RDS, and 3+ indicates severe RDS. Animals treated with CMV alone are shown by *open circles* ($n = 8$), those treated with HFOV alone are shown by *open squares* ($n = 8$), those treated with CMV with surfactant ($n = 8$) are shown by *filled circles*, and those treated with HFOV with surfactant ($n = 8$) are shown by *filled squares*.

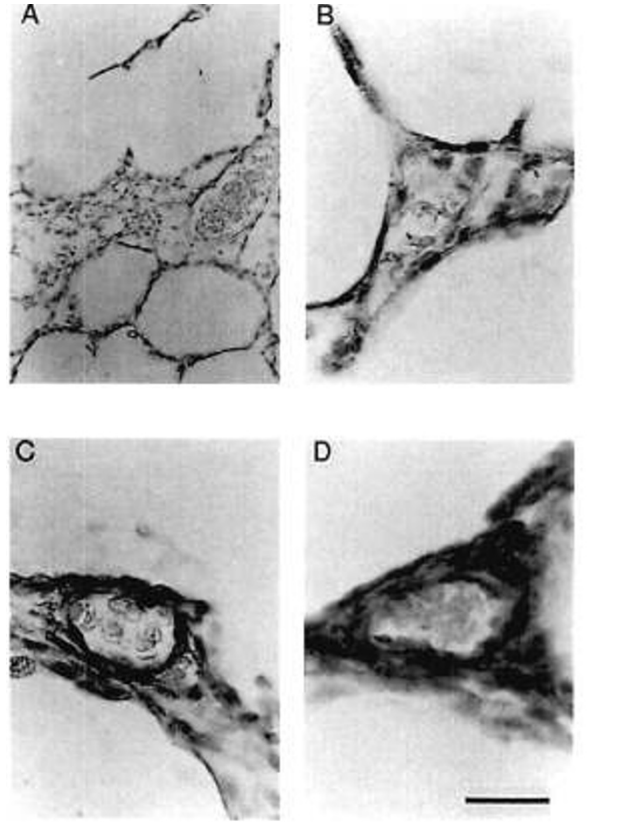

Fig. 2. Freeze-dried lung sections, stained for HA (red) using an indirect avidin-biotin-peroxidase technique to localize biotinylated HA probe. The sections were counterstained with hematoxylin. *Panel A* shows the control level of staining in the absence of the biotinylated HA probe. Original magnification 100×. *Panels B* through *D* show medium-sized muscular arteries from normal lung (*B*) and lungs with mild RDS (*C*) and severe RDS (*D*). Original magnification 400×. Bar = 100 μm for *A* and 30 μm for *B* through *D*.

Table 2. *Duration of mechanical ventilation and HA content in premature monkeys*

	HA content (μg/g wet lung)		
Ventilation (h)	Well*	Intermediate*	Sick*
0	33.4		
0	41.6		
1		46.3	
6†	19.6 ± 11.2	46.1 ± 12.1	85.6/86.9
18			392.4
24			274.5/202.2‡
30		175.7	
64.5			272.2
96	170.9		
96			837.0

* Monkeys were classified as well, intermediate, or sick on the basis of clinical and pathologic criteria.
† Average values taken from 6-h animals. Individual values given when there were less than three animals.
‡ These values were from the right and left lower lobes of one animal. The higher value was from the lung that appeared more atelectatic.

lungs of animals without RDS, as determined by both radiometric assay and histochemical staining with a biotinylated HABr probe. Because all animals in this study were ventilated with supplemental oxygen from birth, this study does not address the effects of mechanical ventilation on lung HA, but rather the difference between premature ventilated animals with and without RDS. Inasmuch as data are reported in μg/g wet lung, this tends to minimize the differences between the RDS and control groups, because RDS animals are known to have more lung edema and therefore heavier lungs. When lung HA per μg DNA was studied as a function of severity of RDS, the same relationship held, with increasingly severe RDS associated with increased lung HA. It is not clear from these data whether the increase in lung HA in early RDS reflects a relative inhibition of the normal decrease in HA accompanying birth, mediated through a marked increase in hyaluronidase activity (24), or an increase in HA synthesis, or both; however, previous work from our laboratory

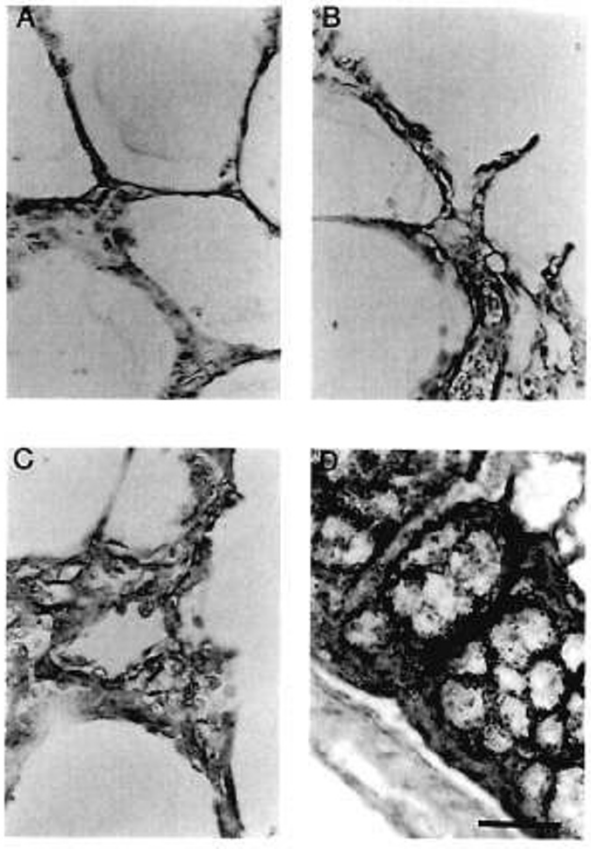

Fig. 3. Freeze-dried lung sections stained for HA (red) showing subpleural lung parenchyma. *Panel A* shows the control level of staining in the absence of the biotinylated HA probe. *Panel B* demonstrates normal lung, *panel C* shows mild RDS, and *panel D* shows severe RDS. Original magnification 100×. Bar = 50 μm.

suggests that there is an increase in HA synthesis (13). It is likely that HA accumulation in acute RDS is just beginning at 6 h and that the peak excess relative to normal lungs would occur at approximately 3 d of life, concomitant with maximal clinical severity of disease. This is supported by data collected from three animals ventilated up to 96 h. Although there were too few animals in this group to make statistical comparisons, clearly HA content is just beginning to rise in sick animals at 6 h. It is remarkable, however, that by just 6 h HA has increased as much as 3-fold in the most severely affected animals compared with the healthy animals. This highlights the dynamic nature of the extracellular matrix. This increased HA could contribute to increased lung water, decreased lung compliance, and increased pulmonary vascular resistance that occur with RDS (17). Treatment with HFOV alone, despite a decrease in severity of RDS by the scoring criteria used (1.69 ± 0.7 compared with 2.5 ± 0.9, $p < 0.05$), did not have a statistically significant effect on HA concentration, although mean values were decreased (37.9 ± 22.7 compared with 44.8 ± 22.6). This may be caused by inadequate sample size. Alternatively, it is possible that HA *per se* is not a marker of severity of lung disease. Interestingly, surfactant treatment decreased both the severity of RDS in these animals and the concentration of HA present in lung extracts, suggesting that less extensive tissue damage and cellular response have occurred. It is also possible that surfactant had a direct effect on HA metabolism independent of its effect on tissue damage.

The combination of biochemical and histochemical techniques allowed both quantification and localization of lung HA. The distribution of HA in bronchial cartilage, bronchial epithelium, and large arterial adventitia was the same in all lungs studied, whereas the distribution of HA in smaller interstitial vessels was increased in animals with RDS. In animals without RDS, minimal HA was detectable in the medium and small vessels of the lung. In animals with mild disease, the medium-sizes muscular arterioles were most affected, and as the severity of disease increased, progressively smaller pulmonary vessels accumulated perivascular HA. HA did not appear to be a component of intraalveolar hyaline membranes, suggesting that the increased HA noted in these lungs was the result of a specific cell response to injury rather than nonspecific leakage of intravascular contents into the interstitium and alveolar space.

It is not known which cells in the lung are responsible for the increase in HA accumulation during acute RDS or what the specific stimulus for increased production or decreased degradation is. It is known that exposure of lung fibroblasts in culture to transforming growth factor-β markedly stimulates HA synthesis (25). HA synthetase induction and increased HA synthesis by human lung fibroblasts occurs after exposure of these cells to recombinant interferons, tumor necrosis factor, and lymphotoxin (26). Stretch alone may cause a change in the extracellular matrix produced by different cell types, as has been shown in pulmonary artery endothelial cells (27) and smooth muscle cells (28). Undue stretch on the immature matrix of the premature lung may be an important component of injury during acute RDS.

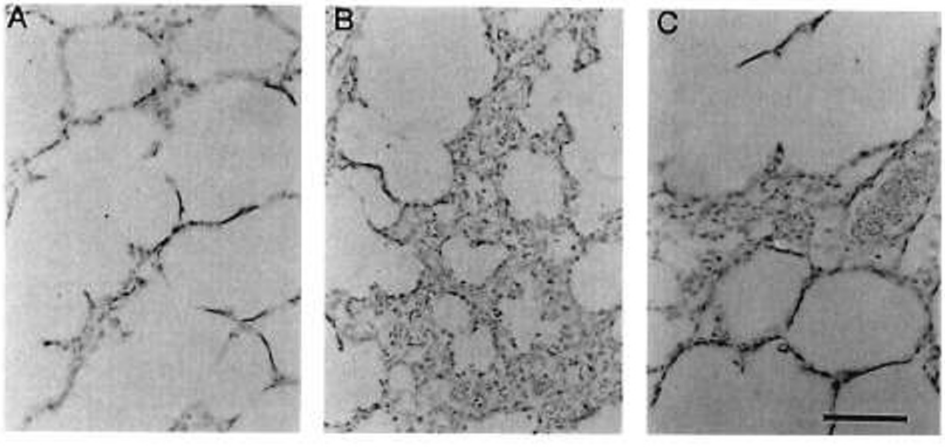

Fig. 4. Control lung sections showing specificity of red reaction product. *A*, Lung parenchyma from animal with moderate to severe RDS showing the control level of staining in the absence of the biotinylated HA probe. *B*, Lung parenchyma from animal with mild RDS, hyaluronidase-treated before incubation with probe (see Materials and Methods). *C*, Bronchial epithelium from normal lung, probe preadsorbed with HA. Original magnification 100×. Bar = 100 μm.

The presence of HA at the basal surfaces of bronchiolar epithelium, in the surrounding cartilage, and in the adventitia of large muscularized blood vessels has been shown in normal adult hamster lungs (9). We additionally found positive staining for HA in the perivascular region of alveolar walls in animals with RDS. Nettelbladt *et al.* (29) detected increased alveolar wall HA in histochemical studies of bleomycin-injured rat lung, although not in the perivascular sheath. These workers noted that the increase in HA coincided with the maximal increase in lung wet weight at 4 d postinjury and that some pulmonary macrophages were filled with HA (30). A complementary study in bleomycin-injured rats showed the HA content in bronchoalveolar lavage to peak at d 5, returning to baseline by 21, with neutrophil counts following a similar course (31). This pattern of early HA accumulation in response to injury may be very important in effecting a normal healing process. During the development of acute RDS in this premature monkey model, there is a several-fold increase in neutrophils noted as early as 11 to 24 h, continuing into the third day of disease. These cells are initially evenly distributed in the alveolar, interstitial, and vascular spaces, but with time the majority of the neutrophils are found in the interstitial space. As recovery occurs (d 4), an increase in alveolar macrophages is noted (32). The CD44 HA receptor has been demonstrated on pulmonary macrophages in significantly greater concentration than in macrophages from other tissues. Blocking this receptor using an MAb inhibits macrophage aggregation (33, 34). AH has also been shown to be chemotactic for neutrophils (35). It is possible that the HA secreted by injured lung cells may participate in the chemotaxis and aggregation of inflammatory cells, and that with a normal healing response, the HA is degraded by invading neutrophils and macrophages, inhibiting further influx of these cells.

The early response of the lung to injury may determine the nature and extent of the resulting dysfunction. Because changes in HA are associated with this early response to injury, experiments that manipulate the rate of synthesis or degradation should contribute new information about the pathophysiology of this early response and how it relates to normal and abnormal healing.

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