The Effect of Hyaluronan on Elastic Fiber Injury *In Vitro* and Elastase-Induced Airspace Enlargement *In Vivo* (44553)

JEROME O. CANTOR,*·†·[1] BRONISLAVA SHTEYNGART,* JOSEPH M. CERRETA,‡ MING LIU, GERARD ARMAND, AND GERARD M. TURINO*

St. Luke's—Roosevelt Institute for Health Sciences, New York, New York 10019; †Maimonides Medical Center, Brooklyn, New York 11219; and ‡St. John's University School of Pharmacy, Jamaica, New York 11439

Abstract. **This laboratory has previously described a method of preventing air-space enlargement in experimental pulmonary emphysema using aerosolized hyaluronan (HA). Although it was found that HA preferentially binds to elastic fibers (which undergo breakdown by elastases in emphysema), it remains to be shown that such attachment actually prevents damage to the fibers. In the current study, cell-free radiolabeled extracellular matrices, derived from rat pleural mesothelial cells, were used to test the ability of low molecular weight (≈ 100 kDa) streptococcal HA to prevent elastolysis. Coating the matrices with HA significantly decreased elastolysis ($P<0.05$) induced by porcine pancreatic elastase (43%), human neutrophil elastase (53%), and human macrophage metalloelastase (80%). Concomitant *in vivo* studies examined the ability of an aerosol preparation of the streptococcal HA to prevent experimental emphysema induced by intratracheal administration of porcine pancreatic elastase. As seen with earlier studies involving bovine tracheal HA, a single aerosol exposure significantly decreased elastase-induced airspace enlargement, as measured by the mean linear intercept (107.5 vs 89.6 μm; $P < 0.05$). Furthermore, repeated exposure to the HA aerosol for 1 month did not reveal any morphological changes in the lung. The results provide further evidence that aerosolized HA may be an effective means of preventing pulmonary emphysema and perhaps other lung diseases that involve elastic fiber injury.**

[P.S.E.B.M. 2000, Vol 225:65–71]

Human pulmonary emphysema is believed to result from the degradation of parenchymal elastin by elastases from inflammatory cells (1, 2). Whereas most attempts at developing a treatment for the disease have focused on the use of elastase inhibitors to reduce elastic fiber damage and loss of alveoli, this laboratory has reported a method of preventing such injury by intratracheal administration of hyaluronic acid (HA) (3). Animals receiving HA in either liquid or aerosol form prior to induction of experimental emphysema with pancreatic or neutrophil elastase have been shown to develop significantly less airspace enlargement than controls treated with the enzyme alone (4, 5).

In those studies, HA was found to have no elastase inhibitory activity (3), and the mechanism responsible for its protective effect in experimental emphysema remained unclear. HA was shown to bind to lung elastic fibers (4, 5), but its ability to actually prevent the breakdown of these fibers has yet to be determined.

The current investigation specifically addresses this issue by examining the ability of HA to prevent elastic fiber injury *in vitro*. Radiolabeled elastin-rich matrix, prepared from cultured rat pleural mesothelial cells, was used to determine the effect of HA on elastase-induced elastolysis. Matrix treated with HA was exposed to several different types of elastase, including human neutrophil elastase and

This work was supported by the Ned Doyle Foundation, the Charles A. Mastronardi Fund, the Franklyn Bracken Fund, and the James P. Mara Center for Lung Disease at the St. Luke's—Roosevelt Hospital Center.

[1] To whom requests for reprints should be addressed at the Department of Medicine, Antenucci Research Building, St. Luke's—Roosevelt Hospital Center, 1000 Tenth Avenue, New York, NY 10019. E-mail: JOCANTOR@pol.net

Received October 8, 1999. [P.S.E.B.M. 2000, Vol 225]
Accepted April 26, 2000.

0037-9727/00/2251-0065$15.00/0

human macrophage metalloelastase (MMP-12), both believed to play a role in pulmonary emphysema (1, 2).

Whereas previous experiments from this laboratory used commercially available bovine tracheal HA, the present studies were performed with low molecular weight streptococcal HA (100 kDa average mol wt). Unlike bovine tracheal HA, streptococcal HA can be produced readily in large quantities by fermentation and is therefore more suitable for use in studies designed to determine therapeutic efficacy.

In addition to testing the streptococcal HA for prevention of elastolysis *in vitro*, an aerosol preparation of the material was examined for its ability to prevent experimental emphysema in hamsters. Animals treated with the aerosol were instilled intratracheally with porcine pancreatic elastase to induce emphysema, then examined for differences in pulmonary airspace size compared with untreated controls.

Since pulmonary emphysema often results in only a gradual loss of lung function, the ability to limit the destructive effects of elastolysis by even a small amount could significantly alter the natural history of the disease.

Materials and Methods

Preparation of Low-Molecular-Weight HA. Low-molecular-weight (≈ 100 kDa), streptococcal HA was obtained from Glycomed Research (Hastings-on-Hudson, NY). The average molecular weight of the material was determined by measuring viscosity, using a Cannon semimicro dilution viscometer (Cannon Instruments Co, State College, PA). Intrinsic viscosity (η) was determined by extrapolating viscosity measurements to zero concentration (6). Average molecular weight was calculated by using intrinsic viscosity data in the Mark-Houwink equation (i.e., $\eta = K(M)^a$ where a and K are constants for HA in saline solution (6).

The purity of the HA preparation was determined by measuring the content of uronic acid, hexosamine, and protein. Uronic acid was measured with the carbazole reaction method (7). The content of hexosamine was determined by a modification of the Elson-Morgan procedure (8). Protein was measured by the method of Lowry *et al.* (9).

Fluorescein-Labeled HA. Fluorescein labeling of the low-molecular-weight HA was performed according to previously published techniques (10). A solution of 100 mg of HA in 80 ml water was diluted with 40 ml dimethyl sulfoxide and combined with acetaldehyde (50 μl), cyclohexyl isocyanide (50 μl), and fluorescein amine (50 mg). The mixture was incubated at 22°C for 5 hr, and the resultant fluorescein-labeled HA was purified by alcohol precipitation and gel filtration on Sephacryl S-500, using a 1 × 135 cm column equilibrated with 0.2 *M* pyridine-acetate buffer at pH 6.2. As previously demonstrated, the fluorescein labeling procedure does not significantly degrade HA (4).

Preparation of a Radiolabeled Cell-Free Tissue Culture Matrix. Rat pleural mesothelial cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in 75 cm^2 plastic flasks using Nutrient Mixture Ham's F-12 medium supplemented with 15% fetal bovine serum, 1% glutamine, 20 units/ml streptomycin, and 20 units/ml penicillin G. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells and extracellular matrix were radiolabeled for 6 weeks with ^{14}C-lysine (6.25 μCi per flask). At the end of the labeling period, the cultures were washed with phosphate-buffered saline (PBS), and the cells were lysed with 0.5% sodium deoxycholate and EGTA. Following removal of the cellular material, the matrix was rinsed with PBS and allowed to air dry. The plastic surface containing the radiolabeled matrix was then cut into 2 × 2-cm squares.

Determining the Effect of HA on Elastase Digestion of the Matrix. The matrix squares were incubated with 0.5 mg of low-molecular-weight HA in 0.5 ml PBS for 30 min at room temperature. Controls were treated with PBS alone. Following removal of the liquid, the matrices were dried, then incubated for 3 hr at 37°C with 0.5 ml of either (i) 10, 1.0, or 0.1 μg/ml of porcine pancreatic elastase (Elastin Products Co., Owensville, MO) in 0.1 *M* Tris buffer, pH 8.0; (ii) 1.0 or 0.1 μg/ml of human macrophage metalloelastase (11) in 0.05 *M* Tris buffer, pH 7.5, with 0.01 *M* $CaCl_2$ and 0.15 *M* NaCl; or (iii) 10 μg/ml of human neutrophil elastase (Elastin Products Co., Owensville, MO) in 0.1 *M* Tris buffer, pH 8.0. An additional set of controls was treated with Tris buffer alone under the same conditions. The liquid was then removed, combined with a single 0.5 ml PBS wash of the matrix, and measured for radioactivity in a liquid scintillation spectrometer.

Release of radioactivity was standardized to the matrix squares themselves, which were of uniform size (2 × 2 cm). Total digestible radioactivity per square was determined by treating the matrix with relatively high concentrations of pancreatic elastase (up to 1 mg/ml). The elastase concentration used for the measurements of the protective effect of HA was then adjusted to insure that the experiments were performed within a range that yielded linear release of radioactivity with increasing concentrations of enzyme.

Identification of Matrix Elastic Fibers. Immunohistochemical identification of elastic fibers within the matrix was performed, using a primary goat anti-rat lung α-elastin antibody (Elastin Products Co, Owensville, MO) and a secondary, fluorescein-labeled rabbit anti-goat IgG antibody (Zymed Laboratories, San Francisco, CA). Matrix samples, prepared from cells grown on glass slide coverslips, were fixed in acetone, treated with goat serum for 30 min, and washed with PBS. The samples were then incubated with goat anti-rat lung elastin antiserum for 1 hr, and again washed with PBS. After treatment with rabbit serum for 30 min, the secondary, fluorescein-labeled rabbit anti-goat IgG antibody was applied for 1 hr. The matrix samples

were then washed with PBS, mounted on glass slides, and examined with a fluorescence microscope.

The Verhoeff-Van Gieson stain was also used to determine the presence of collagen and elastic fibers. Matrix samples, prepared from cells grown on cover slips, were fixed in 10% neutral-buffered formalin, mounted on glass slides, then stained and viewed with a light microscope.

To determine the relationship between HA and the elastic fiber network in the matrix, samples were treated with fluorescein-labeled HA (1 mg/ml) for 30 min, washed with PBS, and examined with a fluorescence microscope. The resulting pattern of fluorescence was compared with that observed with the immunohistochemical studies of the matrix elastic fibers.

Aerosol Exposure to Fluorescein-Labeled HA. Syrian hamsters, weighing ≈ 100 g, were placed inside a dual-port plexiglass chamber and exposed to aerosolized fluorescein-HA (20 mg in 20 ml water) for 50 min *via* a Whisper-Jet nebulizer (Marquest Medical Products, Englewood, CO) attached to a compressed air source.

Approximately 30 min following exposure to the aerosol, the animals were sacrificed, and their lungs were fixed *in situ* by inserting a catheter into the trachea and instilling 10% neutral-buffered formalin at a pressure of 20 cm H_2O. After 2 hr, both the lungs and the heart were removed from the chest as a single block and additionally fixed in 10% formalin for several days. The lungs were then dissected free of extraparenchymal structures, sectioned randomly, and histologically processed. Unstained slide sections were examined with a fluorescence microscope and compared with ones treated with bovine testicular hyaluronidase (Poly Scientific, Bay Shore, NY) to determine if the fluorescence was due to labeled HA.

Exposure of Elastase-Treated Animals to Aerosolized HA. Hamsters, weighing ≈ 100 g, were exposed to an aerosol solution of 20 mg HA in 20 ml water for 50 min, as described above. Control animals were exposed to 20 ml water alone for 50 min. Approximately 30 min following aerosol exposure, the animals were anesthetized with ketamine and instilled intratracheally with 40 units of porcine pancreatic elastase (Elastin Products Company, Owensville, MO) dissolved in 0.2 ml normal saline solution. The elastase was delivered into the trachea *via* a 26-gauge needle mounted on a 1-ml syringe.

Determination of Mean Linear Intercept. One week following intratracheal instillation of elastase, the animals were sacrificed by intraperitoneal injection of sodium pentobarbital. Their lungs were then fixed and histologically processed as described above. Slide sections stained with hematoxylin and eosin were coded, and mean linear intercept measurements were made by an experienced morphologist (JMC), according to published procedures (12).

Long-Term Aerosol Exposure Studies. Hamsters, weighing ≈ 100 g, were exposed to aerosolized HA (10 mg in 10 ml water) for 25 min, three times per week, for 4 weeks. Then 72 hr following the last aerosol exposure, the animals were sacrificed, and their lungs were fixed *in situ* as described above. Slide sections of the lungs were then examined with a light microscope to determine the presence of pathological changes.

Data Analysis. The two-sample t test was used to determine statistically significant differences between treatment groups ($P < 0.05$).

Results

Characterization of Low-Molecular-Weight HA. The current studies employed a low-molecular-weight preparation of streptococcal HA. In contrast to the bovine tracheal HA used in previous investigations (3–5), streptococcal HA can be readily produced in large quantities by fermentation and is therefore more amenable to further studies designed to determine therapeutic efficacy.

The streptococcal HA had an average molecular weight of 101 kDa, which is similar to that of the bovine preparation. Protein content was less than 0.1%. The ratio of hexuronic acid to hexosamines was 1:1, which is characteristic of HA.

***In Vitro* Studies.** Cell-free matrix was prepared from cultures of rat pleural mesothelial cells, which have previously been shown to synthesize elastin (13). Both the histochemical and immunofluorescence studies demonstrated that the matrix contains a complex network of elastic fibers (Fig. 1). Relatively little collagen was present, based on the absence of positive (red) staining for this component with the Verhoeff-Van Gieson stain. Fluorescein-labeled HA bound to the matrix, producing a pattern of fluorescence that resembled the staining pattern of elastic fibers (Fig. 2).

Radiolabeled cell-free matrix was used to determine the effect of HA on elastase-induced elastic fiber injury. The concentration of HA (1 mg/ml) used in these experiments was the same as that used for the *in vivo* studies described below. Each matrix sample was incubated with HA for 30 min, then exposed to varying concentrations of elastase for 3 hr. Release of radioactivity from the matrices was used to measure the degree of elastolysis.

Treatment of the matrices with HA reduced the amount of radioactivity released by exposure to pancreatic elastase (Fig. 3). Although there was only a small difference between HA-treated and untreated matrices with 10 μg/ml of pancreatic elastase (3536 vs 3423 cpm), the reduction in release of radioactivity was much larger with application of lower concentrations of the enzyme. A 35% decrease in radioactivity was observed with 1 μg/ml of elastase (2819 vs 1844 cpm; $P < 0.001$), and a 43% reduction was seen with 100 ng/ml (1257 vs 715 cpm; $P < 0.01$). Background counts from matrix treated with Tris buffer instead of elastase averaged 190 cpm.

Washing the HA-treated matrices with PBS prior to elastase treatment did not reduce the protective effect. Matrix samples treated with HA and rinsed with PBS prior to incubation with 1 μg/ml of pancreatic elastase showed a

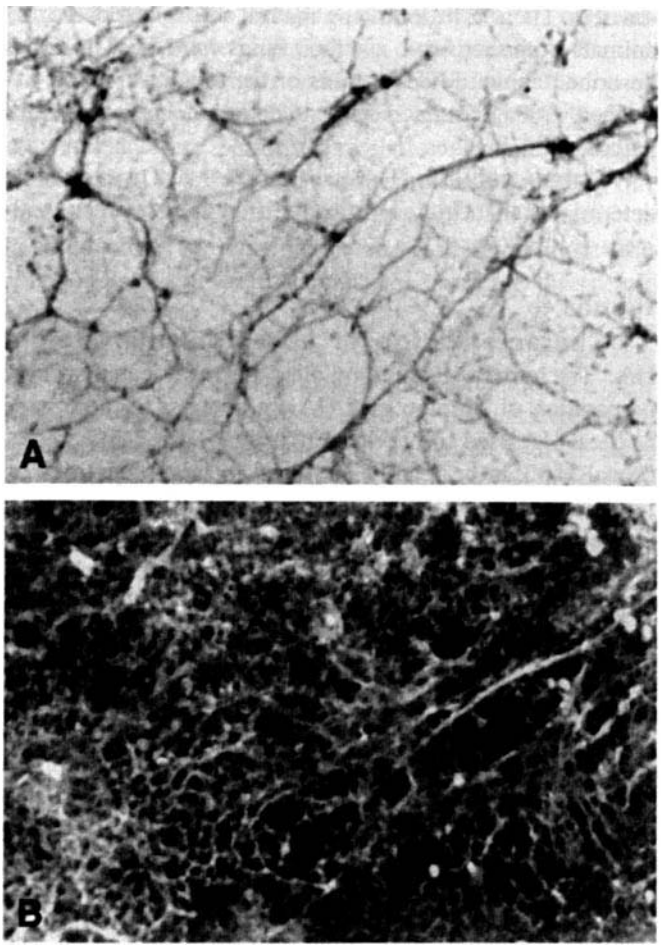

Figure 1. (A, upper) The Verhoeff-Van Gieson stain reveals a complex network of elastic fibers within the matrix. Original magnification: ×2000. (B, lower) Immunofluorescence, using anti-rat lung elastin antibodies, further demonstrates the abundance of elastic fibers within the matrix. Original magnification: ×600.

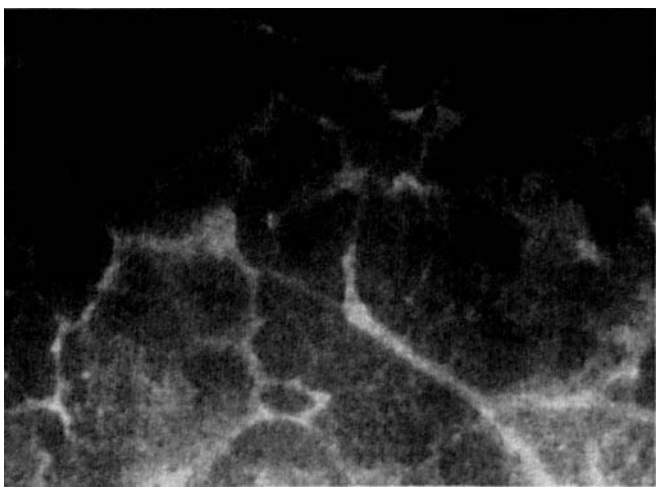

Figure 2. Fluorescein-labeled HA binds to the matrix, producing a pattern similar to that seen with both histochemical staining and immunofluorescence. Original magnification: ×2000.

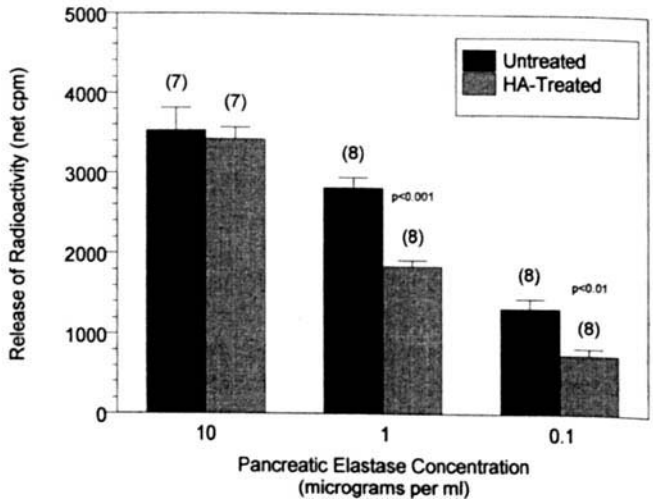

Figure 3. Treatment of the matrix with HA reduces release of radioactivity induced by pancreatic elastase. T-bars indicate SEM. Figures in parentheses refer to number of samples tested.

60% reduction in release of radioactivity compared with controls (2091 vs 833 cpm; $P < 0.05$).

A similar protective effect was seen with human metalloelastase (Fig. 4). Again, the lower concentration of enzyme was associated with a greater reduction in release of radioactivity from the HA-treated matrices. A 46% decrease in radioactivity was seen with 1 μg/ml of enzyme (855 vs 465 cpm; $P < 0.01$), whereas an 80% reduction was seen with 100 ng/ml (128 vs 26 cpm; $P < 0.05$).

HA treatment also reduced the release of radioactivity by human neutrophil elastase (Fig. 5). A 53% decrease in radioactivity was observed at an enzyme concentration of 10 μg/ml (990 vs 464 cpm; $P < 0.001$).

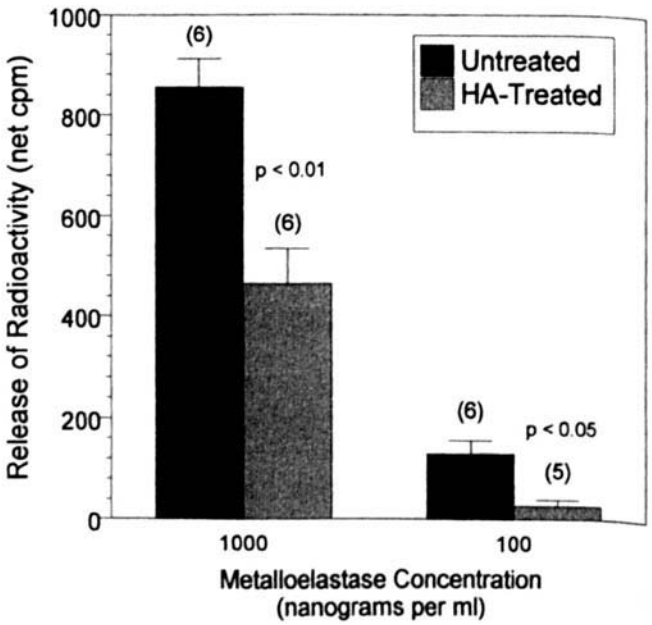

Figure 4. HA significantly decreases human macrophage metalloelastase-induced release of radioactivity from the matrix. T-bars indicate SEM. Figures in parentheses refer to number of samples tested.

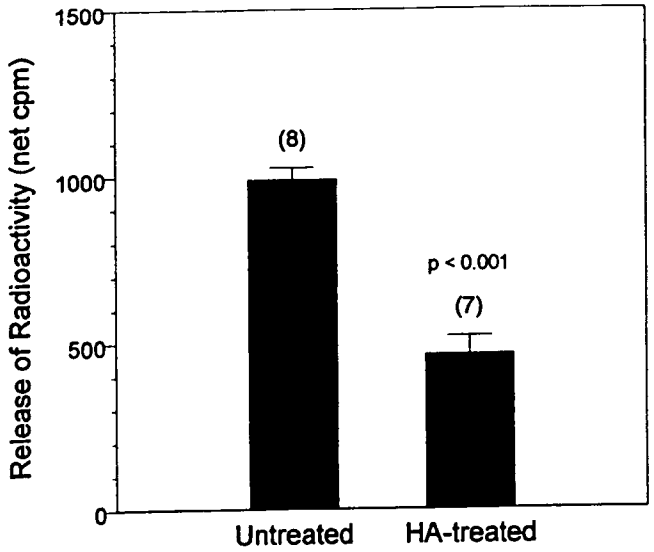

Figure 5. HA reduces elastolysis resulting from exposure to 10 μg/ml of human neutrophil elastase. T-bars indicate SEM. Figures in parentheses refer to number of samples tested.

***In Vivo* Studies.** HA was labeled with fluorescein to determine its anatomic location within the lung following aerosolization. Prominent fluorescence was observed after a 50-min exposure to a 0.1% solution of the labeled HA. There was preferential adherence of the fluorescein-HA to interstitial, vascular, and pleural elastic fibers (Fig. 6A). These fibers were previously identified as elastic in nature based on comparison with tissue sections treated with the Verhoeff-Van Gieson stain (4). Hyaluronidase treatment of the tissue sections abolished the fluorescence (Fig. 6B).

Hamsters exposed to aerosolized HA (0.1% solution) for 50 min prior to intratracheal instillation of porcine pancreatic elastase had a significantly lower mean linear intercept at 1 week compared with elastase-treated animals exposed to aerosolized water alone (107.5 vs 89.6 μm; $P < 0.05$; Fig. 7).

To determine the potential pulmonary toxicity of HA, hamsters were exposed to the aerosol for a 4-week period (three times per week for 25 min). Such treatment produced no morphological changes in the lung. This study supplements earlier work indicating that a single 50-min exposure to the aerosol did not increase the percentage of neutrophils present in lung lavage fluid at 24 hr (5).

Discussion

The concept that pulmonary emphysema is caused by an imbalance between proteinases and their inhibitors has served to focus research on the role of elastases with the hope that inhibiting the activity of these enzymes will prevent lung injury (14). However, such a treatment strategy assumes that emphysema is caused by a single abnormality, namely, excess elastase activity. If the disease represents a more general response of the lung to a variety of insults (with elastases playing a variable role), then enzyme inhibition may have only limited efficacy, and other forms of treatment may be required.

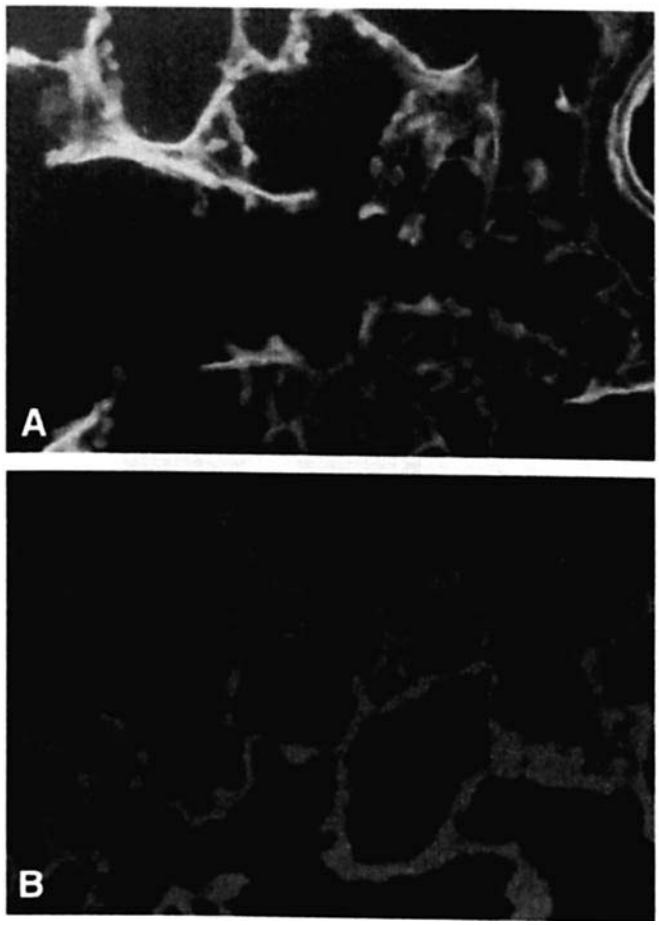

Figure 6. (A, upper) Aerosolized fluorescein-labeled HA binds to lung elastic fibers. Original magnification: ×600. (B, lower) Fluorescence is abolished following hyaluronidase treatment. Original magnification: ×600.

As studies from this laboratory indicate, an alternative approach to preventing alveolar destruction may involve the use of HA to protect lung elastic fibers directly from injury. HA preferentially binds to elastic fibers, prevents elastolysis, and has been shown to limit airspace enlargement in experimental models of emphysema induced by either pancreatic or neutrophil elastase (3–5). Since elastic fiber breakdown may be a final common pathway in the disease process, this form of treatment might be effective against a number of agents capable of causing emphysema, including various oxidants present in air pollutants and cigarette smoke.

In the extracellular matrix, HA has been shown to be a versatile molecule with multiple functions. It acts to stabilize proteoglycans and retain water, and it may also contribute to tissue growth and repair (15–18). A number of studies indicate that HA and other glycosaminoglycans may form a network that surrounds elastic fibers (19–21). Therefore, degradation of HA might be necessary for elastases and cells, such as monocytes or neutrophils, to gain access to these fibers. As shown by this laboratory and other investigators, pretreatment of the lung with hyaluronidase to

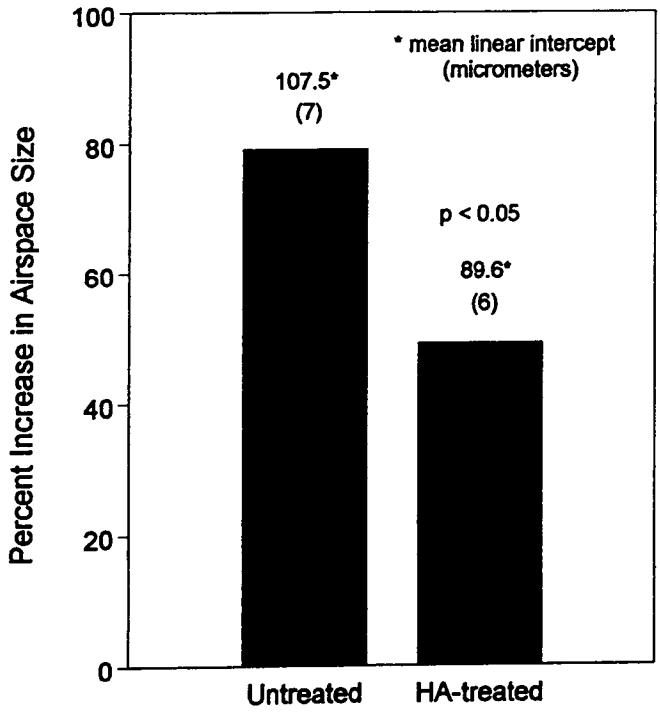

Figure 7. Aerosolized HA significantly reduces pancreatic elastase-induced airspace enlargement. Measurements were made 1 week after intratracheal instillation of the enzyme. The percentage increase in alveolar diameter (mean linear intercept) is based on a normal value of 60 μm (24). Figures in parentheses refer to number of animals tested.

reduce its HA content results in a significantly greater elastase-induced airspace enlargement compared to lungs with normal HA content (3, 22). HA is also significantly reduced in the lungs of patients with pulmonary emphysema (23).

Whereas several studies, including one from this laboratory, indicate that the concentrations of elastase used in the current investigation generally exceed that found in the lung (24, 25), local elastase concentration may be much higher when a neutrophil or macrophage is in contact with elastic fibers. Therefore, the ability of HA to reduce such contact might be especially important in preventing direct cell-mediated elastic fiber damage. Compared with elastase inhibitors, which might not gain access to the area of contact between cells and elastic fibers, HA would be more likely to decrease this type of elastolysis.

The mechanism responsible for the interaction between HA and elastic fibers may possibly involve formation of electrostatic or hydrogen bonds between these two components. Such binding sites may not be situated on the elastin protein itself, but may instead involve surrounding structures such as the microfibrillar component or other glycoproteins. The self-aggregating nature of HA also suggests that both exogenously administered and native HA may combine to form larger molecular complexes (26).

Furthermore, the ability to bind to elastic fibers may not be limited to HA. Recent studies from this laboratory indicate that lysozyme preferentially binds to lung elastic fibers in human emphysema (27). It may be speculated that lysozyme interferes with the attachment of HA to elastic fibers, thereby adversely affecting the progression of the disease.

In addition to providing a physical barrier, HA may protect elastic fibers by virtue of its ability to retain water. It has been shown that a loss of HA can decrease extravascular water content in the lung interstitium (16). Negatively charged carboxyl groups attached to the saccharide moieties of HA repel one another, enlarging the domain of HA and enhancing its ability to entrap water (18). This process may cause an increase in viscosity that reduces the movement of surrounding molecules, including elastases, thereby limiting injury to elastic fibers.

The observation that HA is effective *in vitro* against both human neutrophil elastase and human macrophage metalloelastase raises the prospect that it could be useful in treating human emphysema. The metalloelastase, in particular, may be responsible for emphysematous changes associated with cigarette smoking (28). Since increased activity of these enzymes is noted in a variety of lung inflammatory reactions, HA may be effective against other forms of pulmonary injury as well.

It should be emphasized that the experimental model of emphysema employed in these studies involves the use of exogenously administered elastase whereas human emphysema is believed to result from the release of elastases by cells residing within the lung interstitium. This difference imposes limits on predicting the effect of HA on protease-induced injury in the human lung. Nevertheless, the fact that aerosolized HA permeates the lung interstitium and becomes adherent to elastic fibers suggests that it would provide protection against endogenous elastase injury as well.

Although it is unclear how a large molecule like HA crosses the alveolar barrier, other high molecular weight compounds, such as dextran, are known to be transported rapidly to the lung interstitium following aerosolization (29). Moreover, pulmonary injury can significantly enhance the movement of aerosolized substances across the alveolar barrier (29). In the case of intratracheal elastase administration, severe injury to the alveoli would greatly facilitate the transport of HA into the lung interstitium.

With regard to the duration of protection by HA, earlier studies from this laboratory have indicated that intratracheally instilled HA remains attached to elastic fibers for at least 4 hr following administration and is largely cleared from the lung by 24 hr (4). Furthermore, the instilled HA limits elastase-induced lung injury when given either 2 hr before or 1 hr after the enzyme (4). The inability of HA to protect the lung when given 2 hr following elastase administration may be due to the fact that much of the injury to elastic fibers has already occurred within this period.

Since the magnitude and duration of the protective effect of HA may be dependent on both the amount of intratracheally instilled elastase and the conditions of exposure to HA, it is difficult to extrapolate the data from these experiments to the human disease process. However, it is

possible that an aerosol preparation of HA administered several times a day might provide protection against the endogenous elastases that play a role in pulmonary emphysema.

As a normal constituent of the pulmonary extracellular matrix, HA should be well tolerated by the lung. The current study indicates that repeated exposure to aerosolized HA does not cause pulmonary inflammation. Furthermore, HA has been administered to other tissues without adverse consequences (30–32). In contrast to specific elastase inhibitors, which are now being considered as therapeutic agents for emphysema, HA might provide protection against a variety of harmful agents with fewer potential side effects.

The human macrophage metalloelastase was generously provided by Dr. S. Shapiro of Washington University Medical School.

1. Janoff A. Elastases and emphysema: Current assessment of the protease-antiprotease hypothesis. Am Rev Respir Dis **132**:417–433, 1985.
2. Senior RM, Kuhn C III. The pathogenesis of emphysema. In: Fishman AP, Ed. Pulmonary Diseases and Disorders (2nd ed). New York: McGraw-Hill, pp1209–1218, 1988.
3. Cantor JO, Cerreta JM, Keller S, Turino GM. Modulation of airspace enlargement in elastase-induced emphysema by intratracheal instillment of hyaluronidase and hyaluronic acid. Exp Lung Res **21**:423–436, 1995.
4. Cantor JO, Cerreta JM, Armand G, Turino GM. Further investigation of the use of intratracheally administered hyaluronic acid to ameliorate elastase-induced emphysema. Exp Lung Res **23**:229–244, 1997.
5. Cantor JO, Cerreta JM, Armand G, Turino GM. Aerosolized hyaluronic acid decreases alveolar injury induced by human neutrophil elastase. Proc Soc Exp Biol Med **217**:471–475, 1998.
6. Cleland RL, Wang JL. Ionic polysaccharides. III. Dilute solution properties of hyaluronic acid fractions. Biopolymers **9**:799–810, 1970.
7. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem **4**:330–334, 1962.
8. Augustyniak H, Augustyniak J. A rapid procedure for the estimation of amino sugars on a micro scale. Anal Biochem **15**:167–171, 1966.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
10. Anthony N, Belder DE, Owe Wik K. Preparation and properties of fluorescein-labeled hyaluronate. Carbohydr Res **44**:251–257, 1975.
11. Shapiro SD, Kobayashi DK, Ley TJ. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. J Biol Chem **268**:23,824–23,829, 1993.
12. Dunnill MS. Quantitative methods in the study of pulmonary pathology. Thorax **17**:320–328, 1962.
13. Cantor JO, Willhite M, Bray BA, Keller S, Mandl I, Turino GM. Synthesis of cross-linked elastin by a mesothelial cell culture. Proc Soc Exp Biol Med **181**:387–391, 1986.
14. Zimmerman M, Powers JC. Design and properties of synthetic elastase inhibitors. In: Robert L, Hornebeck W, Eds. Elastin and Elastases. Boca Raton, FL: CRC Press, Vol **2**:pp109–123, 1989.
15. Heinegard D, Paulsson M. Structure and metabolism of proteoglycans. In: Piez KA, Reddi AH, Eds. Extracellular Matrix Biochemistry. New York: Elsevier, pp277–328, 1984.
16. Bhattacharya J, Cruz T, Bhattacharya S, Bray B. Hyaluronan affects extravascular water in lungs of unanesthetized rabbits. J Appl Physiol **66**:2595–2599, 1989.
17. Toole BP, Gross J. The extracellular matrix of regenerating newt limb: Synthesis and removal of hyaluronate prior to differentiation. Dev Biol **25**:57–77, 1971.
18. Toole BP. Glycosaminoglycans and morphogenesis. In: Hay ED, Ed. Cell Biology of Extracellular Matrix. New York: Plenum, pp259–294, 1981.
19. Baccarani-Contri M, Fornieri C, Pasquali-Ronchetti I. Elastin-proteoglycans association revealed by cytochemical methods. Connect Tissue Res **13**:237–249, 1985.
20. Baccarani-Contri M, Vincenzi D, Cicchetti F, Mori G, Pasquali-Ronchetti I. Immunocytochemical localization of proteoglycans within normal elastin fibers. Eur J Cell Biol **53**:305–312, 1990.
21. Bray BA, Hsu W, Turino GM. Lung hyaluronan as assayed with a biotinylated hyaluronan-binding protein. Exp Lung Res **20**:317–330, 1994.
22. Ishibashi M, Nishida T, Murakami H, Shiraishi M, Aritomi T, Yoshida M. The role of interstitial hyaluronan in acute lung injury. Nihon Kyobu Shikkan Gakkai Zasshi **33**(Suppl):225–230, 1995.
23. Konno K, Arai H, Motomiya M, Nagai H, Ito M, Sato H, Satoh K. A biochemical study on glycosaminoglycans (mucopolysaccharides) in emphysematous and in aged lungs. Am Rev Respir Dis **126**:797–801, 1982.
24. Cantor JO, Cerreta JM, Armand G, Keller S, Turino GM. Pulmonary airspace enlargement induced by intratracheal instillment of hyaluronidase and concomitant exposure to 60% oxygen. Exp Lung Res **19**:177–192, 1993.
25. Liou TG, Campbell EJ. Nonisotropic enzyme–inhibitor interactions: A novel nonoxidative mechanism for quantum proteolysis by human neutrophils. Biochemistry **34**:16,171–16,177, 1995.
26. Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, *in vitro* and in tissues. FASEB J **6**:2639–2645, 1992.
27. Shteyngart B, Chaiwiriyakul S, Wong J, Cantor JO. Preferential binding of lysozyme to elastic fibers in pulmonary emphysema. Thorax **53**:193–196, 1998.
28. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke–induced emphysema in mice. Science **277**:2002–2004, 1997.
29. Barrowcliffe MP, Zanelli GD, Ellison D, Jones JG. Clearance of charged and uncharged dextrans from normal and injured lungs. J Appl Physiol **68**:341–347, 1990.
30. Graf J, Neusel E, Schneider E, Niethard FU. Intra-articular treatment with HA in osteoarthritis of the knee joint: A controlled clinical trial versus mucopolysaccharide polysulfuric acid ester. Clin Exp Rheum **11**:367–372, 1993.
31. King SR, Hickerson WL, Proctor KG. Beneficial actions of exogeneous hyaluronic acid on wound healing. Surgery **109**:76–84, 1991.
32. Goldberg EP, Burns JW, Yaacobi Y. Prevention of postoperative adhesion by precoating tissues with dilute sodium hyaluronate solutions. Prog Clin Biol Res **381**:191–204, 1993.