Hyaluronic Acid in the Pulmonary Secretions of Patients with Asthma

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Hyaluronic acid was the only glycosaminoglycan found in the pulmonary secretions of patients with asthma. The compound had a hexuronate/hexosamine molar ratio of about 1:1. Glucosamine constituted over 98% of the hexosamines, the remaining being galactosamine. The compound moved as a single spot with the mobility of standard hyaluronic acid on cellulose acetate electrophoresis, and this spot disappeared after digestion with testicular hyaluronidase. Even after extensive proteolysis and purification, the compound was associated with small amounts of protein, the major amino acids of which were aspartic acid, threonine, serine, glutamic acid, glycine and valine.

All major glycosaminoglycans except keratan sulphate are found in animal lung parenchyma and pleura (Wusteman, 1972). Pleural fluids from patients with various pulmonary diseases have been found to contain mostly hyaluronic acid (Friman *et al.*, 1977). The high hyaluronic acid concentration of pleural fluid associated with mesothelioma is diagnostic of this disease (Havez *et al.*, 1971). The present paper describes the isolation and characterization of hyaluronic acid from the pulmonary secretions of patients with asthma.

Experimental

Isolation of lavage material

Four patients of either sex, aged 22–34 years with severe asthma, whose asthmatic symptoms could not be controlled with antimicrobial drugs, steroids and bronchodilators, underwent therapeutic broncho-alveolar lavage as previously described (Kylstra *et al.*, 1971) under the direction of Dr. J. A. Kylstra in the Duke University Medical Center. The informed consent of all patients was obtained. Immediately after the lung lavage, the material was processed. The lavage sediment was collected by centrifugation, washed and freeze-dried (Sahu & Lynn, 1977). The supernatant together with the washings were dialysed at 4°C against several changes of water and freeze-dried. Approx. 2–10 g of dry powder from the insoluble sediment and 1–2 g of dry powder from the soluble supernatant were obtained per patient, depending on the disease condition. The dry samples were stored at –40°C.

Delipidation

The dry samples were delipidated as previously described (Sahu & Lynn, 1977) with chloroform/methanol (2:1, v/v).

Pronase digestion

The sample was digested with Pronase (Calbiochem, Los Angeles, CA, U.S.A.; B grade) in the presence of a drop of toluene for 48 h at 60°C in 0.1 M-sodium phosphate buffer, pH 7.0, with a final concentration of 10 µg of Pronase/mg of dry delipidated powder (Mathews, 1971).

Isolation of glycosaminoglycan

To the sample treated with Pronase, solid trichloroacetic acid was added to give a final concentration of 5% (w/v). It was incubated at 4°C for 4 h and was centrifuged at 5000g for 15 min at 4°C. The precipitate was washed with 5% trichloroacetic acid solution. The supernatant and the washings were combined, and the glycosaminoglycans were precipitated by the addition of 3 vol. of ethanol containing 1% (v/v) acetic acid and 1% (w/v) potassium acetate (Mathieson & Pearce, 1977). The mixture was kept at 4°C overnight and centrifuged at 10000g and 4°C for 15 min. The precipitate was washed with ethanol and dried in air.

Alternatively the glycosaminoglycans were precipitated as their cetylpyridinium complex (Pearce & Mathieson, 1967) by the addition of 10% cetylpyridinium chloride in 0.03 M-Na₂SO₄ to a final concentration of 1%. The mixture was kept overnight at 37°C and the precipitate was collected by centrifugation at 10000g for 15 min. The precipitate was washed with water to remove free cetylpyridinium chloride. The complex was dissociated with 2 M-KCl and the glycosaminoglycans were precipitated by addition of 10 vol. of ethanol/water/acetic acid (24:7:1, by vol.) (Pearce & Mathieson, 1967). The mixture was kept at 4°C overnight and the precipitate was collected by centrifugation at 10000g for 15 min. The precipitate was washed with ethanol and dried in air.

Gel filtration

To separate the intact glycosaminoglycans from the degraded products, gel filtration on a column (60cm×2.5cm) of Bio-Gel P-200 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), equilibrated with 0.05M-sodium acetate, pH5.5, containing 0.1M-NaCl was used (Mathieson & Pearce, 1977). The fractions containing intact glycosaminoglycans were pooled, and they were precipitated by either ethanol or cetylpyridinium chloride as described above.

Hyaluronidase digestion

The hydrolysis with testicular hyaluronidase was carried out by the method of Hardingham & Muir (1973). A sample (1mg) was incubated overnight at 37°C in 1 ml of 0.1 M-sodium acetate buffer, pH5.5, with 50 µg of testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO, U.S.A.). Boiled enzyme was used in the control incubation.

Cellulose acetate electrophoresis

Electrophoresis on cellulose acetate (Sephacel III; Gelman Instruments, Ann Arbor, MI, U.S.A.) was carried out in three electrolyte systems: (a) 0.05M-LiCl in 0.01M-HCl, pH2.0 (Breen *et al.*, 1976), (b) 0.3M-cadmium acetate adjusted to pH4.1 with acetic acid (Curwen & Smith, 1977) and (c) 0.1M-calcium acetate solution, pH7.25 (Seno *et al.*, 1970), followed by staining with Alcian Blue (Curwen & Smith, 1977).

Analytical procedures

Hexuronic acid was assayed by the carbazole reaction of Dische (1947) as modified by Bitter & Muir (1962) with glucuronolactone (Sigma) as the standard. Sodium hexuronate concentration was calculated by multiplying the hexuronic acid concentration by 2.06 (Swann & Caulfield, 1975). Hexosamines were determined colorimetrically (Swann & Balazs, 1966) and were identified by using a Beckman amino acid analyser as previously described (Bhattacharya *et al.*, 1976) after hydrolysis of the sample in 8M-HCl at 95°C or 6M-HCl at 100°C for 3h (Swann & Balazs, 1966; Swann & Caulfield, 1975). Amino acids were determined as described by Bhattacharyya *et al.* (1976) after hydrolysis of the sample in 6M-HCl at 100°C for 24h under vacuum (Swann & Caulfield, 1975). Gel electrophoresis was carried out on 5% polyacrylamide gels in the presence of 0.1% of sodium dodecyl sulphate and 4M-urea as described by Swann & Caulfield (1975), and the gels were stained with Coomassie Blue to detect proteins.

Materials

Standard hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate, dermatan sulphate, heparin and heparan sulphate were supplied by Dr. M. B. Mathews and Dr. J. A. Cifonelli of the University of Chicago.

Results

Approx. 2–3mg of glycosaminoglycans were obtained from 100mg of the delipidated pulmonary secretions of the patients with asthma. Most of the glycosaminoglycans were found in the lavage supernatant rather than the insoluble lavage sediment.

Gel filtration with Bio-Gel P-200 was used for the separation of intact macromolecular glycosaminoglycans from the degraded products. Fig. 1 shows the gel-filtration profile of the intact glycosaminoglycan.

Repeated electrophoresis on cellulose acetate in three different electrolyte systems (described in the Experimental section) revealed that hyaluronic acid was the only detectable glycosaminoglycan present. In all the electrolyte systems, the compound migrated with the standard hyaluronic acid. After digestion with testicular hyaluronidase this band disappeared (Fig. 2).

Chemical analysis of the isolated hyaluronic acid showed that it contains 48.8% (by weight) of hexuronic acid, 41.6% of hexosamines and 3.1% of amino acids. Almost 98% of the total hexosamines was glucosamine, the remaining being galactosamine.

The hyaluronic acid isolated even after repeated proteolysis, and gel filtration, was always associated with small amounts of protein as detected by amino acid analysis. It was not possible to detect the protein constituents by gel electrophoresis followed

Fig. 1. Gel filtration of hyaluronic acid isolated from human pulmonary secretions on a Bio-Gel P-200 column. Fractions (2ml) were collected and checked for hexuronic acid by carbazole reaction. Void volume (V_0) was determined by Blue Dextran. For details see the Experimental section.

Fig. 2. Tracing of an electrophoretogram on cellulose acetate paper of hyaluronic acid isolated from human pulmonary secretions together with reference standards

The electrolyte system composition was 0.05 M-LiCl/0.01 M-HCl, pH 2.0, at a constant current of 1.0 mA/cm for 60 min. Staining was by Alcian Blue. Reference standards: heparin (1), heparan sulphate (2), dermatan sulphate (3), keratan sulphate (4), chondroitin 6-sulphate (5), chondroitin 4-sulphate (6), hyaluronic acid (7). The position of isolated pulmonary hyaluronic acid is shown before (8), and after (9), digestion with testicular hyaluronidase.

by Coomassie Blue staining. The amino acid composition of the protein showed relatively high contents of aspartic acid, threonine, serine, glutamic acid, glycine and valine (Table 1). Hyaluronic acid, when hydrolysed for amino acid analysis, yields a number of ninhydrin-positive constituents, that are eluted together with lysine and histidine (Swann, 1968). Although errors introduced by these artifacts are negligibly small for hyaluronic acids containing 5–10% of protein, lysine and histidine cannot be accurately determined in samples containing 1–2% of protein (Swann & Caulfield, 1975). Therefore the lysine and histidine values have been calculated by using molar proportions of lysine/histidine/aspartic acid of 45:8:94 as suggested by Swann & Caulfield (1975).

Discussion

Studies reported in the present paper indicate that hyaluronic acid is the only glycosaminoglycan present in the pulmonary secretions of patients with asthma. Most of the hyaluronic acid is present in the lavage supernatant rather than the insoluble lavage sediment.

Hyaluronic acids of mammalian origin are always associated with small amounts of protein even after extensive purification (Swann & Caulfield, 1975). However, the nature of association between the

Table 1. Amino acid composition of hyaluronic acid isolated from human pulmonary secretions

Values are the average of four different preparations of hyaluronic acid.

Amino acid	Content (residues/1000 residues)
Hydroxyproline	0
Aspartic acid	142
Threonine	101
Serine	113
Glutamic acid	122
Proline	61
Glycine	123
Alanine	66
Half-cystine	28
Valine	75
Methionine	11
Isoleucine	28
Leucine	29
Tyrosine	23
Phenylalanine	19
Lysine	10
Histidine	19
Arginine	29

protein and hyaluronic acid is obscure. It is not clear if this protein is a part of the carbohydrate-protein linkage region. According to Hamerman *et al.* (1966) and Bader *et al.* (1972), such a linkage between protein and hyaluronic acid does exist. However, How *et al.* (1969) consider that the association of protein and hyaluronic acid may be the result of their ionic interaction.

Animal lung tissue contains all the major glycosaminoglycans except keratan sulphate (Wusteman, 1972). Alterations in the type and quantity of these compounds occur in the developing lung and continue with maturation. The proportion of hyaluronic acid synthesized is highest in foetal lung and then declines, whereas that of the sulphated glycosaminoglycans rises with age (Horwitz & Crystal, 1975). Pleural effusions resulting from pathological conditions of the lung are found to contain hyaluronic acid along with chondroitin sulphate and dermatan sulphate (Arai *et al.*, 1975).

Diploid mouse-embryo lung fibroblasts have been shown to synthesize heparan sulphate during their exponential-growth phase (Kraemer, 1971). The release of hyaluronic acid, chondroitin sulphate and dermatan sulphate into the cellular medium of growing rabbit lung fibroblasts has also been demonstrated (Richards & Morris, 1973). Studies by Sampson *et al.* (1975) indicate that endothelial cells derived from mixed cell lines of normal adult rat lung are capable of synthesizing hyaluronic acid, chondroitin sulphate, heparan sulphate and dermatan sulphate.

In the present study, hyaluronic acid was the only

glycosaminoglycan found in the pulmonary secretions of patients suffering from asthma. No other glycosaminoglycan could be detected by the methods described in the present paper. It is noteworthy, however, that these patients had been on a variety of medicaments that could affect the normal pulmonary secretions. It appears, however, that the secretion of only hyaluronic acid and no other glycosaminoglycan may be specific for this particular disease and may prove to be of clinical significance. It would be of interest to know if hyaluronic acid is a secretory product of the 'normal' lung or is a product of the pathological conditions associated with this particular disease. Unfortunately, it is very difficult to obtain enough pulmonary secretory material of 'normal' human lung to answer this question. First, it is hard to find a healthy volunteer willing to undergo lung lavage. Secondly, less than 1 mg of material was obtained from the lung lavage of a healthy volunteer (Sahu *et al.*, 1976). This problem is easily overcome in the case of chronic obstructive lung diseases, where grams of dry pulmonary secretions are obtained from the therapeutic lung lavage of each patient. However, cellular source, mechanism of accumulation and physiological role of hyaluronic acid in the pulmonary secretions as well as in the aetiology of the disease remain to be explored.

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