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ISOLATION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS SECRETED BY HUMAN FOETAL LUNG TYPE II PNEUMOCYTES IN CULTURE

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SUMMARY

Hyaluronic acid, but not other glycosaminoglycans are secreted by human foetal Type II pneumocytes in culture.

INTRODUCTION

All major glycosaminoglycans except keratan sulphate are found in animal lung and pleura (Wusteman, 1972). Pleural fluids from patients with several pulmonary diseases contain mostly hyaluronic acid (Friman, Hellstrom, Juvani & Riska, 1977). The high concentration of hyaluronic acid in pleural fluid associated with mesothelioma is considered diagnostic of this disease (Havez, Degand, Boersma & Richet, 1971). We have recently shown that hyaluronic acid is the only glycosaminoglycan detected in the pulmonary secretions of patients with asthma (Sahu & Lynn, 1978) and with pulmonary alveolar proteinosis (Sahu & Lynn, 1979). However, the cellular source of hyaluronic acid in these secretions is not known. A functional role for this secreted hyaluronic acid was recently identified to be directed specifically at alveolar macrophages (Love, Shannon, Myrvik & Lynn, 1977; Lynn & Mukherjee, 1979). Of particular interest are the secretions of the Type II pneumocytes, the metabolically active cells of the alveolar epithelium, which contribute to the production of alveolar secretions. Since we found hyaluronic acid but no other glycosaminoglycans in the alveolar secretions of patients with pulmonary alveolar proteinosis (Sahu & Lynn, 1979), it is possible that the alveolar Type II cells are the source of airway hyaluronic acid. The present report describes the isolation and characterization of glycosaminoglycans secreted by human foetal Type II pneumocytes in culture.

EXPERIMENTAL METHODS

Culture of Type II cells

The cell line (HFLP) used in this study is a clonally isolated Type II pneumocyte line derived from a primary mixed lung cell culture of a 32-week gestation anencephalic fresh

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stillborn infant in a modification of the technique previously described (Tanswell & Smith, 1979*a*). The technique consisted of initially developing a mixed lung cell culture and then a secondary culture derived from a 2-cell inoculum in a 0·5-ml well of a cloning plate. This line has been continuously maintained in a liquid-phase oxygen tension of 55 mm Hg ($6{\cdot}665 \times 10^3$ N m^{-2}) with 1 μM cortisol and thyroxine. Phospholipid analysis of this cell line (Tanswell & Smith, 1979*b*) is very similar to those described for other clonally isolated Type II pneumocyte lines (Mason & Williams, 1977). The cells have a diploid chromosomal configuration and show the characteristic Type II cell lamellar organelles. Cells were grown to confluence in Eagle's minimal essential tissue culture medium with Earle's salts (Grand Island Biological Co. Grand Island, New York), supplemented with 10 % bovine foetal calf serum and 1 μM cortisol and thyroxine in plastic tissue culture flasks with surface area of 75 cm^2 (Corning Glassworks New York). At confluence, the cell monolayer was washed several times with serum-free medium to remove serum and then incubated for 2 consecutive 48-h periods in serum-free medium supplemented with cortisol and thyroxine. The medium collected from several similarly treated culture flasks was pooled and lyophilized. Tho dry powder was stored at −50 °C before use. While 'physiological' concentrations of cortisol and thyroxin have been routinely used in these culture lines, control observations were made on cells cultured in their absence and the results obtained in this study were the same.

Isolation and identification of glycosaminoglycans

The glycosaminoglycans were isolated and identified as previously described (Sahu & Lynn, 1978) from the delipidated (Sahu, DiAugustine & Lynn, 1976) and pronase-digested (Mathews, 1971) sample following the standard methods of isolation and identification (Pearce & Mathieson, 1967; Hardingham & Muir, 1973; Mathieson & Pearce, 1977).

Analytical methods

Hexuronic acid was measured by the carbazole reaction (Dische, 1947) as modified by Bitter & Muir (1962) with glucuronolactone as the standard. Sodium hyaluronate 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 amino acid analyser (Sahu & Lynn, 1977, 1978) after hydrolysis of the sample in 6 M HCl at 100 °C for 3 h (Swann & Caulfield, 1975). Amino acids were determined by amino acid analyser (Sahu & Lynn, 1977, 1978) after hydrolysis of the sample in 6 M HCl at 100 °C for 24 h under vacuum (Swann & Caulfield, 1975). Gel electrophoresis was carried out on 5 % polyacrylamide gels in the presence of sodium dodecyl sulphate as described by Swann & Caulfield (1975) and the gels were stained with Coomassie blue to detect protein.

MATERIALS

The standard hyaluronic acid, chondroitin sulphates, keratan sulphate, dermatan sulphate, heparan sulphate and heparin were kindly supplied by Dr M. B. Mathews and Dr J. A. Cifonelli of the University of Chicago. Pronase (B grade) was obtained from Calbiochem, Los Angeles, Ca. Cetylpyridinium chloride, glucuronolactone and testicular hyaluronidase were purchased from Sigma Chemicals, St Louis, Mo. Bio-Gel P-200 was obtained from Bio-Rad Laboratories, Richmond, Ca. The cellulose acetate (Sepharose III) was obtained from Gelman Instruments, Ann Arbor, Mich.

RESULTS

The cell-free culture medium of human foetal lung Type II pneumocytes, free from any other cell type as evidenced by light- and electron-microscopic studies (Tanswell & Smith, 1979*a*), contained glycosaminoglycans, which could be precipitated as their cetylpyridinium complex and made free of degraded products by gel filtration. Approxi-

Table 1. *Composition of hyaluronic acid secreted by alveolar Type II cells*

Component	% by weight
Hexuronic acid	49·3
Glucosamine	41·6
Galactosamine	0·9
Amino acids	3·1

Values are averages from 3 different preparations of hyaluronic acid, none of the values varied by more than 10·5 %.

Table 2. *Amino acid composition of the protein associated with hyaluronic acid secreted by alveolar Type II cells*

Amino acid	Content (residues/1000 residues)	Amino acid	Content (residues/1000 residues)
Aspartic acid	117	Methionine	13
Threonine	96	Isoleucine	31
Serine	89	Leucine	47
Glutamic acid	98	Tyrosine	34
Proline	64	Phenylalanine	32
Glycine	137	Lysine	18
Alanine	87	Histidine	21
Half-cystine	23	Arginine	29
Valine	61		

Values are averages from 3 different preparations of hyaluronic acid.

mately 1–2 mg of glycosaminoglycans were isolated from 100 mg of dry delipidated powder. Although these cells were routinely cultured in the presence of 'physiological' concentrations of cortisol and thyroxin, results obtained from control studies made on cells cultured in the absence of cortisol and thyroxin, showed no significant differences as far as the glycosaminoglycans are concerned. However, their presence is optimal for the production of dipalmitoylphosphatidylcholine (Tanswell & Smith, 1979*b*).

Electrophoresis on cellulose acetate revealed that hyaluronic acid was the only glycosaminoglycan detected in the cell-free culture medium. The compound migrated with the standard hyaluronic acid on cellulose acetate electrophoresis and disappeared after digestion with testicular hyaluronidase.

The chemical analysis of the isolated hyaluronic acid showed that the ratio of hexuronic acid to hexosamine was approximately 1. Glucosamine constituted over 98% of the total hexosamines (Table 1).

The hyaluronic acid isolated by the methods described in this report was always associated with small amounts of protein which could be detected by amino acid analysis but not by gel electrophoresis with Coomassie blue staining. The protein was relatively rich in aspartic acid, threonine, serine, glutamic acid, glycine and alanine (Table 2). Since hyaluronic acid, when hydrolysed for amino acid analysis, yields a

number of ninhydrin-positive constituents which are eluted together with lysine and histidine (Swann, 1968), these 2 amino acids were calculated using molar ratios of lysine/histidine/aspartic acid of 45:8:94 as suggested by Swann & Caulfield (1975).

DISCUSSION

The role of Type II pneumocytes, the secretory cells of the alveolar epithelium in the synthesis of glycosaminoglycans was not known. The present study is the first report which demonstrates that human foetal lung Type II epithelial cells secrete hyaluronic acid and no other glycosaminoglycan in detectable amounts into their culture medium. This hyaluronic acid was always associated with small amounts of protein, as has been the case with other preparations of hyaluronic acid of mammalian origin even after extensive purification (Swann, 1968; Swann & Caulfield, 1975).

Glycosaminoglycans are most concentrated in the trachea and are present in much lower concentration in the lung parenchyma (Mason, 1971). The types of glycosaminoglycans found in human tracheobronchial cartilage resemble those in costal cartilage (Mason & Wusteman, 1970). Both have an age-dependent increase of sulphated glycosaminoglycans. Hyaluronic acid is present in the highest concentration in foetal lung, and then declines, while the concentration of sulphated glycosaminoglycans rises with age (Horwitz & Crystal, 1975). Keratan sulphate which is unique to cartilage, nucleus pulosis and cornea, has not been found in lung parenchyma or pleura (Wusteman, 1972). Although total glycosaminoglycans constitute approximately 0·5% of the dry weight of adult human lung (Laros, Kuyper & Janssen, 1972), no data are available on the distribution of their types in human lung.

There are over 40 different cell types in the lung. Their contribution to glycosaminoglycans is not known for all cells. Mast cells contain large amounts of heparin (Ringertz, 1965). Lung fibroblasts synthesize chondroitin sulphates, dermatan sulphate, heparan sulphate and hyaluronic acid (Richards & Morris, 1973). Studies of Sampson, Parshley, Mandi & Turino (1975) indicate that endothelial cells derived from mixed cell lines of normal adult rat lung are capable of synthesizing chondroitin sulphates, dermatan sulphate and hyaluronic acid. Also, epithelial cells of embryonic cornea have been shown to synthesize various glycosaminoglycans (Trelstad, Hyashi & Toole, 1974).

Many years ago Macklin (1954) suggested that alveoli are lined by a mucopolysaccharide-rich layer secreted by alveolar Type II cells. In spite of several investigations, the relationship of intra-alveolar carbohydrate to the pulmonary surfactant remains unclear. Although much has been learned about the structure of hyaluronic acid, our knowledge about its possible specific functions is unclear. It does cause the specific aggregation of alveolar macrophages (Love *et al.* 1977; Lynn & Mukherjee, 1979). Other types of cells are not aggregated. Nor are other glycosaminoglycans effective. Since this aggregation is inhibited by treatment of the cells with trypsin, it is likely that these airway cells possess specific receptors for hyaluronic acid. The functional significance of these cell-specific aggregation properties of hyaluronic acid is unclear, but may be involved in granuloma formation by these cells. The nature of

the association of hyaluronic acid with protein also remains an unsettled question. Even after extensive purification, hyaluronic acids of mammalian origin are always associated with small amounts of protein (Swann & Caulfield, 1975; Sahu & Lynn, 1978). It has been suggested that the association of hyaluronic acid with protein is the result of ionic interaction (How, Long & Stanworth, 1969). However, it has also been shown that the protein associated with hyaluronic acid is a part of carbohydrate-protein linkage region (Bader, Ray & Steck, 1972).

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