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PRODUCTION OF CHONDROITIN SULFATE IN TISSUE CULTURE OF CARTILAGE

HENRY GROSSFELD

Bureau of Biological Research, Rutgers, The State University, New Brunswick, N. J. (U.S.A.)

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SUMMARY

Production of chondroitin sulfate *in vitro* (a mixture of A and C) by cells growing from explants of beef embryo cartilage has been demonstrated by chemical isolation. In primary cultures of cartilage, fibroblasts retain to a certain extent their physiological differentiation.

INTRODUCTION

In a previous paper[1] it was shown that fibroblasts growing from explants of human and bovine embryo skin, human embryo bone, and rat subcutaneous tissue produce in tissue culture both hyaluronic acid and chondroitin sulfate. The amount of chondroitin sulfate either equaled that of hyaluronate (human bone; rat subcutaneous tissue), or was less than hyaluronic acid (human skin). Where further characterization was carried out, the chondroitin sulfate was found to be C. In cultures of embryonic skin no chondroitin sulfate B, the predominant component of adult bovine and porcine skin was found.

The study of mucopolysaccharide production in tissue culture of cartilage appeared to be of special interest because the mucopolysaccharide pattern of cartilage differs a great deal from that of other tissues. The present experiments were designed to elucidate the question of whether the mucopolysaccharide of hyaline ground substance is produced in tissue culture and to what extent the fibroblasts growing from explants of cartilage retain in tissue culture their specific chemical differentiation.

METHODS AND MATERIALS

Beef-embryo cartilage was used in the present experiments. Fresh, intact uteri, containing beef embryos were obtained from local slaughterhouses. The embryos ranging in size from 5 to 10 in crown-rump were dissected sterile out of the uteri. Limbs were removed and freed of soft tissues, and epiphyseal cartilage was prepared with Parker's blades and cleaned of adherent tissue and blood vessels. The cleaned cartilage was washed several times in sterile physiological salt solution, then cut into small fragments of the size of about 2 mm^3. The cartilage fragments were kept over-

night in a few millilitres of culture medium at 4°. The explantation was carried out the next day. About 2.5 ml of chicken plasma were poured into a Petroff flask (resembles an one-piece petri dish with a large neck and about 10 cm in diameter), and spread over the entire bottom. The cartilage fragments were transferred into the flask and, before coagulation of the plasma, equally distributed in the plasma layer so that a density of about 5 explants/cm^2 was obtained. About 2 ml of culture medium was added and slightly mixed with the plasma to speed up coagulation, and after a few hours, when the plasma was completely clotted, 10 ml of medium was added to each flask. The flasks were tightly closed with rubber cups. The medium consisted of 20% chick-embryo extract, 35% horse serum, 5% placental serum, and 40% Earle's balanced salt solution. 100 units of potassium penicillin and 100 μg streptomycin/ml were added. Renewal of the medium was carried out twice, or rarely three times a week and the cultures were grown for about 8 weeks before harvesting. Since growth of cartilage started only about one to two weeks after explantation, and since production of mucopolysaccharides usually started only some time after good growth of fibroblasts had been established, the culture medium obtained at the first 3–4 changes of medium was discarded and not used for chemical analysis for mucopolysaccharides. In the present work, seven plantings were carried out and the culture fluid and cell mass of a total number of 68 dishes were collected, pooled and submitted to analytical procedures for isolation and determination of acid mucopolysaccharides. It has been found advantageous to keep the cultures growing for longer periods of time before harvesting. The number of culture dishes necessary for obtaining enough material for chemical analysis can be substantially reduced by extending the time of cultivation and production.

After removal of the culture fluid, the cell mass, mixed with clotted plasma, was scraped off the bottom of the flasks and the wet and dry weights were determined.

The total pooled culture fluid amounted to about 11 300 ml, and the wet weight of the cell mass and plasma clot was 55 g. The samples of culture fluid and cell mass which were stored at —15° to —20° until analysis, were precipitated with acetone and the precipitate was washed with acetone and ether and dried. The dried powders were suspended in water and digested with pepsin at pH 1.5 followed by digestion with trypsin at pH 7.5 at 37° in the presence of toluene. The fractions obtained by differential precipitation with ethanol as calcium salts were analyzed for hexosamine, uronic acid, and sulfate, and the optical rotation was determined. The amino sugars of each class of mucopolysaccharides, after acid hydrolysis, were determined by paper chromatography according to STOFFYN AND JEANLOZ[2]. Other analytical methods were used as described by MEYER *et al.*[3].

RESULTS

Growth of fibroblasts in primary cultures of cartilage started only about 1–2 weeks after explantation and was scarce at the beginning. Later, however, it gradually became quite luxuriant and filled in several layers almost all the spaces between the explants. These gradually became smaller and some were no longer distinguishable amidst a thick network of growing cells. At first bipolar, spindle-shaped cells predominated. Later large numbers of polymorph and round cells appeared and frequently occupied large areas of the culture vessels forming dense aggregations of cells

crowded together with indistinct cell boundaries. Occasionally mitotic cell divisions were observed.

In some of the culture dishes, on the second month of cultivation in the neighbourhood of the dense cell aggregations, separated scattered cell groupings consisting of two, four, or more cells were observed. They were separated from other such pairs by blank areas and appeared to be enclosed in separate compartments forming dissected elliptical figures. They resembled familiar pictures of chondrocytes in groups of two, four, or more, enclosed in "lacunae", separated from other groups by areas of hyaline matrix, such as are seen in centers of chondrification in the process of interstitial chondrogenesis. It is possible that chondroitin sulfate was first elaborated by the dense aggregations of round and fibroblast-like cells since formation of chondrogenic cell pairs and islets may have resulted from the presence of cell products separating the cell groupings from each other.

In the culture fluid, a small amount of hyaluronic acid was found. Presumably it derived from the outer layer of perichondrium.

The culture fluid yielded 296.8 g of acetone–ether dried precipitate, which was redissolved, digested with proteases and differentially precipitated with ethanol into three fractions giving the following analysis (expressed in percentage) (Table I).

The first ethanol fraction (188 mg) represents a mixture of chondroitin sulfate and hyaluronic acid, as shown by paper electrophoresis and analysis of the two fractions separated by ion-exchange resin:

145 mg of MVI 42 Aa were placed on Dowex-1 X2 (Cl^- form) column. Two fractions were separated:

Fraction A, 39 mg (0.5–1 N HCl), showed on paper electrophoresis 1 spot with mobility of hyaluronic acid. By paper chromatography galactosamine 1 +, and glucos-

TABLE I

ANALYSIS OF THE ETHANOL FRACTIONS OF USED INCUBATION MEDIA FROM CULTURES OF BEEF-EMBRYO CARTILAGE

Fraction number	*Yield*	*Hexosamine*	*Carbazole*	*Orcinol*	SO_4	$[\alpha]_D$	*Paper chromatography*
MVI 42 Aa	188 mg	30.4	32.6	21.5	3.8	—37	
MVI 42 Ab	326 mg	23.2	29.0	17.7	11.4	—35	Galactosamine 3 + Glucosamine 1 +
MVI 42 Ac	113 mg		9.7	7.9			

TABLE II

ANALYSIS OF THE ETHANOL FRACTIONS OF THE CELL MASS GROWN IN TISSUE CULTURE OF BEEF-EMBRYO CARTILAGE

Fraction number	*Yield*	*Hexosamine*	*Carbazole*	*Orcinol*	SO_4	$[\alpha]_D$	*Paper chromatography*
MVI 43 I	89 mg	26.1	34.0	23.0	13.3	—32	Galactosamine 3 + Glucosamine 1 +
MVI 43 II	62 mg	23.2	29.4	19.4	7.3	—20	Galactosamine 4 +
MVI 43 III	77 mg		17.8	13.1			

amine 3+ were found. The carbazole and the orcinol tests showed 25.6% and 23.8% respectively.

Fraction B, 59 mg (1 N NaCl in 1 N HCl) showed on paper electrophoresis 1 spot with mobility of chondroitin sulfate. By paper chromatography galactosamine 4+ was found. The carbazole and orcinol tests showed 21.7% and 19.7% respectively.

The second ethanol fraction MVI 42 Ab (326 mg) appears to be mainly chondroitin sulfate (Table I).

The plasma clot and cell mass yielded 7.0 g of acetone–ether dried powder. Analysis of the ethanol fractions showed them to be mainly chondroitin sulfate (Table II).

To summarize, the following amounts of mucopolysaccharide have been recovered: 415 mg composed mainly of chondroitin sulfate; 121 mg composed only of chondroitin sulfate; and 39 mg composed mainly of hyaluronic acid. The recovered mucopolysaccharide is thus mainly chondroitin sulfate, with an admixture of hyaluronic acid. Of the mucopolysaccharide recovered, 424 mg were recovered from the used incubation medium and 151 mg from the plasma and cell mass. From alcohol solubility and specific rotation the chondroitin sulfate was assumed to be a mixture of A and C.

In order to clarify whether mucopolysaccharide recovered from the incubation medium was produced in tissue culture, or was only extracted or released from cells in autolysis, cultures were prepared from various tissues of mesenchymal origin which produce mucopolysaccharides *in vitro*. At the same time cultures were prepared from the same tissues, but incubated in a medium of balanced salt solution only. Other control cultures were kept at 4° instead of 37°. Only cultures in a normal incubation medium showed the presence of mucopolysaccharide in the used medium, while in supernatant fluids from cultures in balanced salt solution, or from cultures kept at 4°, mucopolysaccharides could never be found. It is thus evident that the 424 mg mucopolysaccharides recovered from the incubation medium in tissue culture were produced by living metabolizing cells. It is, however, unknown how much of the 151 mg mucopolysaccharide was produced in tissue culture, and how much was originally present in the explants.

DISCUSSION

In previous reports[1,4] it was demonstrated that in primary cultures fibroblasts produce different patterns of acid mucopolysaccharides for long periods of time. Replicate cell cultures of mesenchymal origin, like for instance strain L, do not produce mucopolysaccharides *in vitro*. This is consistent with reports according to which considerably more profound alterations (chromosomal aberrations, giant cell formation) were found to occur in cell strains than in primary cultures[5]. Attempts were made to link gradual loss of differentiation to insufficient respiration and increased anaerobic metabolism[6]. But cartilage shows a deficiency of respiratory enzymes, in particular of cytochrome oxidase and a characteristic anaerobic metabolism[7,8]. Synovial membrane also shows high glycolysis[9]. An association of high glycolysis with enormous increase of mucopolysaccharide production can also be found in Rous sarcoma. It was presumed that this might be a cause and effect relationship[10]. However, other mesenchymal tissues producing acid mucopolysaccharides, like bone

or skin, do not show high glycolysis and, on the other hand, Jensen sarcoma with equally high glycolysis as Rous sarcoma[6] does not produce mucopolysaccharides.

The present results demonstrate that fibroblasts growing in tissue culture from primary explants of embryonic cartilage produce acid mucopolysaccharides which to a certain extent retain the mucopolysaccharide pattern of hyaline matrix of the original cartilage. The small amount of hyaluronic acid evidently derived from the outer layer of perichondrium which had not been completely removed from the original explants. Adult cartilage contains chondroitin sulfate A (analysis of embryonic cartilage are not available). In tissue culture embryonic cartilage produces chondroitin sulfate which is a mixture of A and C. In conclusion, it has been shown that not only the general identification of fibroblasts in tissue culture, but also the identification of physiologically different groups of fibroblasts producing ground substances of different composition can be achieved by chemical analysis.

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