Synthesis of Proteochondroitin Sulfate by Normal, Nanomelic, and 5-Bromodeoxyuridine-Treated Chondrocytes in Cell Culture
(hyaluronidase/chick embryos/chromatographic heterogeneity)

M. J. PALMOSKI AND P. F. GOETINCK

Department of Animal Genetics, University of Connecticut, Storrs, Conn. 06268

Communicated by Alexander Rich, September 7, 1972

ABSTRACT Chondrocytes grown in cell culture from micromelic chick sterna and 5-bromodeoxyuridine-treated normal cells showed reduced incorporation of sulfate into hyaluronidase-sensitive material as compared to normal chondrocytes. Proteochondroitin sulfate synthesized by normal embryonic chick chondrocytes in cell culture was heterogeneous on chromatography. The major chromatographic component was specifically inhibited in normal chondrocytes treated with 5-bromodeoxyuridine, and this component was absent in nanomelic chondrocytes.

In studies of the metabolism of chondrocytes in vitro, attention has been directed to the metabolism of chondroitin sulfate and to the activity of enzymes involved in the formation of uridine nucleotide sugar precursors of this glycosaminoglycan. Chondroitin sulfate, a repeating unit of uronic acid and sulfated N-acetyl-galactosamine, is part of a much larger molecule, proteochondroitin sulfate, which consists of several chondroitin sulfate molecules linked to a protein backbone by neutral sugar linkage regions. We have studied the synthesis of proteochondroitin sulfate. More specifically, the synthesis of proteochondroitin sulfate in normal chondrocytes was compared to that in normal chondrocytes exposed to 5-bromodeoxyuridine (BrdU) and in chondrocytes from embryos homozygous for the recessive gene, nanomelia (1). The metabolism of chondroitin sulfate in the cartilage of these micromelic embryos is defective (2,3).

In this report, we present evidence for chromatographic heterogeneity of proteochondroitin sulfate in normal embryonic chick chondrocytes in cell culture. In addition we show a specific inhibition of the major chromatographic component in normal chondrocytes treated with BrdU and the absence of this component in nanomelic chondrocytes.

MATERIALS AND METHODS

Chondrocytes from sternal chick cartilage were used in all cultures. Normal and nanomelic sterna were dissected from 14-day embryos that resulted from matings between birds heterozygous for the recessive lethal gene, nm (1). Phenotypically normal siblings are designated as normal. After careful removal of the perichondrial layer, the sterna were disassociated by the method of Cahn et al. (4). The cells were counted in a hemocytometer and plated at an initial density of 2 × 10⁶ cells in 60-mm plastic culture plates. The medium used for all cell cultures consisted of 50% Ham's F-10 with twice the vitamin and 4 times the aminosacid concentration, 38% Hank's balanced salt solution, bovine-serum albumin (5 μg/ml), and 10% fetal-calf serum. A fraction from embryo extract excluded by chromatography on Sephadex G-25 columns was added to the medium for the first 2 days of culture to increase plating efficiency (5). The medium also contained 50 units/ml each of penicillin and streptomycin. When applicable, BrdU was added to a final concentration of 10 μM 4 days after initial plating of the cells. The cells were grown for 12 days in an atmosphere of 5% CO₂-95% air. Medium (4 ml per plate) was changed every other day. Appropriate radioactive precursors were added to the medium on the twelfth day, and the cells were incubated an additional 48 hr in the presence of label. Results expressed in Tables 1 and 2 cannot be compared directly as different batches of medium and isotope were used in each experiment. At the end of the incubation period the medium was decanted and the cells were removed from the plate by incubation in 0.05 M EDTA-0.1 M Tris buffer (pH 7) for 15-30 min. The cells were then centrifuged, resuspended in 0.1 M sodium acetate-0.15 M NaCl (pH 5) and counted.

Glycosaminoglycan synthesis was measured as cpm of hyaluronidase-sensitive material per 10⁻⁶ cells. The cells (after sonication) and media were each divided into two aliquots and boiled for 5 min. Testicular hyaluronidase (EC 3.2.1.35) (1 mg/ml) in hyaluronidase buffer was added to one of the aliquots of the cells and the medium, and the mixture was incubated for 24 hr at 37°. All aliquots were subsequently digested with Pronase (1 mg/ml) in 0.2 M Tris-HCl buffer (pH 8) for 18 hr at 56°. Radioactive glycosaminoglycans were precipitated with 3 volumes of 5% potassium acetate in 80% ethanol for 18 hr at −20° (6) and collected on nitrocellulose filters. The filters were placed in counting vials with 1 ml of 2 N NH₄OH to which 10 ml of Bray's solution (7) was added for counting in a Nuclear Chicago Mark I scintillation spectrometer equipped with external standardization.

Proteoglycans were chromatographed on Bio-Gel P-300 and A-150 columns eluted with 0.5 M NaCl. Media and sera for cell culture work were obtained from Grand Island Biological Co. Bovine-serum albumin and 5-bromo-2-deoxyuridine were purchased from Sigma Chemical Co. Testicular hyaluronidase and Pronase were obtained from Calbiochem. Nitrocellulose filters were purchased from the Matheson-Higgins Co. Bio-Gel resins were obtained from Bio-Rad Laboratories Inc. n-[¹-¹H]glucose, n-[U-¹⁴C]glucose,
and Na\textsuperscript{35}SO\textsubscript{4} were purchased from New England Nuclear Corp.

**RESULTS AND DISCUSSION**

Table 1 shows the relative amounts of glycosaminoglycan synthesized by normal and mutant cultures over a 48-hr period. The synthesis of hyaluronidase-sensitive material by nanomelic chondrocytes is reduced to 9% of that of normal chondrocytes. There is a disproportionate reduction of the material associated with the cell and that found in the medium; the material associated with the cells is reduced to 16% of normal, and that found in the medium is reduced to 9% of normal. The material found in the medium represents 95 and 91% of the total amount synthesized by normal and mutant cultures, respectively. This type of experiment has been repeated four times, and the reduction in total synthesis by nanomelic cultures ranges from 7–13% of normal. Normal and nanomelic cultures in cell culture are indistinguishable morphologically; both show the polygonal morphology characteristic of differentiated chondrocytes.

Genetically, normal chondrocytes cultured in medium containing BrdU displayed characteristic fibroblastic morphology as originally described by Abbott and Holtzer (8). Table 2 shows that the total synthesis of hyaluronidase-sensitive material in BrdU-treated normal cells is reduced to 4% of that of untreated cells. The distribution of chondroitin sulfate between cells and medium is similar for normal cells cultured in the presence or absence of BrdU.

Proteochondroitin sulfate synthesized in the different cultures was examined by gel chromatography. Since 90–95% of the radioactive material was found in the medium, all the characteristics of proteochondroitin sulfate were performed on this material.

In order to separate proteochondroitin sulfate from serum proteins and unincorporated isotope, we chromatographed medium from normal and mutant cultures separately on a Bio-Gel P-300 column (Fig. 1). The normal cells had been incubated in the presence of [\textsuperscript{1}H]glucose and the mutant cells in the presence of [\textsuperscript{14}C]glucose. The included material labeled with glucose seen in Fig. 1 was not observed when similar material was obtained from chondrocytes cultured in the presence of Na\textsuperscript{35}SO\textsubscript{4}. The material excluded from the P-300 column from normal \textsuperscript{3}H-labeled and mutant \textsuperscript{14}C-labeled cultures was mixed and chromatographed on a Bio-Gel A-150 column in order to reduce potential differences between successive chromatographic separations.

The results of chromatography on A-150 are shown in Fig. 2. Normal proteochondroitin sulfate shows two distinct peaks, whereas the mutant material has only one peak, which coincides with peak II of the normal profile. Identical profiles are obtained when Na\textsuperscript{35}SO\textsubscript{4} is used as label and chromatographed separately on A-150 columns. In the normal material, peak I represents 86% of the total radioactivity eluted from the column, while peak II represents 14% of the total. The profile of nanomelic material (Fig. 2) indicates that all of the mutant material chromatographed to a position that corresponds to that of peak II in normal elution profile. The fact that all of the nanomelic material is present in peak II suggests that normal and mutant cells produce equal quantities of peak II material, since the synthesis of hyaluronidase-sensitive material by nanomelic chondrocytes is 7–13% of normal (Table 1).

### Table 1. Incorporation of Na\textsuperscript{35}SO\textsubscript{4} into hyaluronidase-sensitive material by normal and nanomelic chondrocytes*

<table>
<thead>
<tr>
<th>Type of chondrocytes</th>
<th>cpm/10\textsuperscript{-4} cells</th>
<th>% Distribution of total × 100</th>
<th>Mutant Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>692</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>12,624</td>
<td>95</td>
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</tr>
<tr>
<td>Total</td>
<td>13,316</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>113</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Medium</td>
<td>1,147</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>1,260</td>
<td>—</td>
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</tr>
</tbody>
</table>

* Final density in both cultures was about 6 ¥ 10\textsuperscript{6} cells per 60-mm culture dish. Isotope was added to 4 ml of medium at a concentration of 4 μCi/ml.

### Table 2. Incorporation of Na\textsuperscript{35}SO\textsubscript{4} into hyaluronidase-sensitive material by normal cells cultured in the absence or presence of BrdU*

<table>
<thead>
<tr>
<th>Type of chondrocytes</th>
<th>cpm/10\textsuperscript{-4} cells</th>
<th>% Distribution of total × 100</th>
<th>Treated Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
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<tr>
<td>Cells</td>
<td>7,518</td>
<td>10</td>
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<tr>
<td>Medium</td>
<td>70,742</td>
<td>90</td>
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</tr>
<tr>
<td>Total</td>
<td>78,260</td>
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</tr>
<tr>
<td>Normal with BrdU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>224</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Medium</td>
<td>3,225</td>
<td>94</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>3,449</td>
<td>—</td>
<td>4</td>
</tr>
</tbody>
</table>

* Final density in both cultures was about 7 ¥ 10\textsuperscript{6} cells/60-mm culture dish. Isotope was added to 4 ml of medium at a concentration of 5 μCi/ml.
Since normal proteochondroitin sulfate is heterogeneous, the possible effect of BrdU on this heterogeneity was examined. When material excluded from P-300 columns from media of normal cells grown in the presence of BrdU is chromatographed on A-150 columns, only one peak is observed (Fig. 3). This peak corresponds to elution volume to peak II material of normal untreated cells and to the single peak obtained from nanomelic cells.

These peaks were shown to be proteochondroitin sulfate by the reduction in molecular weight of 35S-labeled material after digestion with Pronase and hyaluronidase. Fig. 4 shows such a reduction with peak I material from A-150 columns from normal cells. Peak II from nanomelic cultures and BrdU-treated normal cells shows similar profiles. Peak I material from A-150 columns from normal cells was chromatographed, on a P-300 column (Fig. 4a). Nearly all the radioactivity was in the void volume. These fractions were then pooled and divided equally. Half was dialyzed against 0.2 M Tris·HCl, pH 8, for 24 hr and then digested with Pronase (1 mg/ml) at 56°C for 18 hr. This sample was then chromatographed on the same column (Fig. 4b). A broad peak of radioactivity was retained on the column showing a shift in molecular weight. The other half of the void volume material was dialyzed against 0.1 M sodium acetate·0.15 M NaCl (pH 5) for 24 hr and then digested with hyaluronidase (1 mg/ml) at 37°C for 24 hr. The digest was then chromatographed on a P-300 column of the same dimensions and showed an elution pattern (Fig. 4c) consistent with that of oligosaccharides (9).

The results presented here indicate that embryonic chick chondrocytes in cell culture produce proteochondroitin sulfate that exhibits chromatographic heterogeneity on A-150 columns. A varied population of protein-polysaccharides has also been demonstrated in porcine cartilage of different anatomical origins (10, 11). Simůnek and Muir (12) have recently shown that larger molecular weight protein-polysaccharides of pig articular cartilage predominate during prenatal life, as compared to early postnatal development and old age. Our results suggest that chick embryonic cartilage produces a larger molecule than that of adult cartilage. Shulman and Meyer (9) had shown that proteoglycan extracted from adult chicken xiphoid cartilage was retained in one broad peak on an A-50 column. Our earlier attempts to characterize chick embryonic sternal proteoglycan from chondrocytes in cell culture on an A-50 column did not prove successful. Void volume material from P-300 columns also eluted in the void volume of an A-50 column. Collagenase (1 mg/ml), dithiothreitol (10 mM), and 4 M guanidine hydrochloride extraction, followed by cesium chloride centrifugation (13), were used in an attempt to dissociate the void volume peak; however none of these treatments caused this material to be included in A-50 columns.

The absence of peak I in nanomelic cultures and the specific inhibition of this material in BrdU-treated cultures tempts

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**Fig. 2.** Chromatography of void volume material from P-300 (Fig. 1) on a column (0.7 cm × 120 cm) of Bio-Gel A-150 (1% Agarose) eluted with 0.5 M NaCl at 4°C. (○) 3H-labeled material from normal cells; (●) 14C-labeled material from nanomelic cells.

**Fig. 3.** Chromatography of void volume material from P-300 from culture medium of BrdU-treated normal cells on a column (0.7 cm × 120 cm) of Bio-Gel A-150 eluted with 0.5 M NaCl at 4°C. A-150 columns, only one peak is observed (Fig. 3). This peak corresponds to elution volume to peak II material of normal untreated cells and to the single peak obtained from nanomelic cells.

**Fig. 4.** Chromatography of proteochondroitin sulfate from peak I material from A-150 columns of normal culture medium on Bio-Gel P-300 column (0.9 cm × 55 cm), eluted with 0.5 M NaCl at 4°C. (a) Culture medium; (b) after digestion with Pronase; (c) after digestion with hyaluronidase.
us to conclude that peak I represents cartilage-specific proteo-
chondroitin sulfate while peak II represents nonspecific
proteo-chondroitin sulfate. This conclusion is consistent with
the hypothesis that BrdU inhibits synthesis of cell-specific
products (luxury molecules) in chondrocytes (8, 14). Marzullo
(15) has recently published results that may indicate that two
forms of the enzyme, UDPG-4-epimerase, may be present
in cartilage; one "cartilage-specific" and BrdU-sensitive and
the other ubiquitous and BrdU-resistant.

Scientific Contribution No. 516 of the Agricultural Experiment
Station, The University of Connecticut. P. F. G. is a recipient of a
Career Development Award of the National Institute of General
Medical Sciences (GM16903). This research was supported in
part by Research Grant GB-12880X from the National Science
Foundation.

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