Glycosaminoglycans of Cultured Human Fetal Uveal Melanocytes and Comparison with Those Produced by Cultured Human Melanoma Cells†

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ABSTRACT: The glycosaminoglycans produced by human fetal uveal melanocytes and by human melanoma cells were examined. The cells were grown in the presence of [^{3}H]glucosamine and [^{35}S]sulfate, and the labeled glycosaminoglycans were isolated from the cells, spent medium, and intracellular material. The distribution of the glycosaminoglycans was similar in both cells and spent media, which together accounted for 95% of the total. Of the total ^{3}H-labeled glycosaminoglycans produced by the melanocyte culture, 42% was in chondroitin 4-sulfate, 25% in heparan sulfate, 16% in chondroitin 6-sulfate, and 17% in hyaluronic acid. In contrast, HM7 human melanoma cultures produced no chondroitin 6-sulfate, increased quantities of heparan sulfate, and less hyaluronic acid. A heparan sulfate fraction obtained from melanocytes required both heparitinase and heparinase for complete degradation, indicating the presence of heparin-like molecules in this fraction. The corresponding fraction from melanoma cells was totally degraded by heparitinase alone.

Several investigators have reported alterations in the glycosaminoglycans (Dietrich & Armelin, 1978; Dunham & Hynes, 1978; Hopwood & Dorfman, 1977; Glimelius et al., 1978; Keller et al., 1980; Klagsbrun, 1976; Muto et al., 1977; Vannucchi et al., 1978; Winterbourne & Mora, 1978) produced by malignant cells compared to normal cells. We have previously described the partial characterization of the glycosaminoglycans produced by cultured HM7[1] human melanoma cells and by a primary culture of human embryonic iris (Banks et al., 1976).

The subject of the present investigation is the study of the glycosaminoglycans produced by two established cell lines of human fetal melanocytes (Giovanella et al., 1976). In contrast to the primary explant of human embryonic iris, this system is a better control for the rapidly growing human melanoma cells. Even though the cells under comparison are of adult (melanoma) and fetal (normal melanocytes) origin, this is not undesirable since the detected differences would exclude the fetal antigens which are common to both tumor and fetal cells.

We describe below the characterization of the glycosaminoglycans produced by the cultured normal melanocytes. Additional characterization of the glycosaminoglycans isolated from human melanoma cells and culture media is also described. As in previous studies, the cell cultures were divided into three compartments: (i) the spent medium (medium), (ii) the intracellular material released by EGTA (EGTA supernatant), (iii) the material which remains associated with the viable cells (cell associated). The glycosaminoglycans of the normal fetal cells (iris and melanocytes) are compared to those produced by tumorigenic human melanoma cells. A preliminary report has been presented (Bhavanandan et al., 1979).

Experimental Procedures

Materials. The human melanoma cell line, HM7, was isolated by Dr. John W. Kreider as described (Banks et al., 1976). The normal human uveal melanocyte cell lines FeMel 6 and FeMel 13 derived from embryonic tissues were kindly donated by Drs. McCormick and Giovanella, Cancer Research Laboratory, St. Joseph Hospital, Houston, TX (Giovanella et al., 1976). Components for culture media and fetal calf serum were obtained from Grand Island Biological Co. (Grand Island, NY) and Flow Laboratories (Rockville, NY), respectively. Pronase CB was from Calbiochem. Vitreous humor hyaluronic acid was obtained from Worthington (Freehold, NJ); chondroitin 4-sulfate was isolated from pig rib cartilage, and reference heparan sulfate was a gift from Dr. A. Linker (Salt Lake City, UT). Leech hyaluronidase (EC 3.2.1.36) was from Biotrics, Arlington, MA; bacterial chondroitinases ABC (EC 4.2.2.4), ACII (EC 4.2.2.5), and the unsaturated reference disaccharides ΔDi–4S, ΔDi–6S, and ΔDi–0S were from Miles Laboratories (Elkhart, IN). Heparitinase and heparinase were isolated from a *Flavobacterium heparinum* strain provided by Dr. A. Linker (Linker & Hovingh, 1972). All the above enzymes were free of contaminating mucopolysaccharidases when tested with isotopically labeled glycosaminoglycans as substrates. Glyceryl-controlled pore glass beads (glyceryl-CPG) were obtained from Electronucleonics (Fairfield, NJ). D-[6-^{3}H]Glucosamine hydrochloride (10–13 Ci/mmol) and $Na_2{}^{35}SO_4$ (carrier free; 80–800 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Sources of other materials used in this study are given in the preceding paper in this issue (Bhavanandan et al., 1981).

Methods. Column Chromatography. Glyceryl CPG-240 beads of 80/120 mesh were packed with constant vibration and then equilibrated and eluted with 0.5 M KCl by using a pump to maintain a constant flow rate of 30 mL/h. For fractionation of glycosaminoglycans, DEAE-Sepharose CL-6B generated in the chloride form was used, and the column was eluted with a linear gradient of 0 → 1.5 M LiCl in 50 mM Tris-HCl buffer, pH 8.0.

Enzyme Digestions. Pronase digestion was performed in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM Ca^{2+} at 37 °C for 120 h, in the presence of toluene. Pronase CB dissolved in the buffer was added at 0, 24, 48, and 72 h. The

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[1] Abbreviations used: glyceryl-CPG, glyceryl controlled pore glass beads; CPC, cetylpyridinium chloride; AcNeu, *N*-acetylneuraminic acid; DEAE, diethylaminoethyl; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; FM6 and FM13, normal human uveal melanocyte cell lines FeMel 6 and FeMel 13; HM7, human melanoma cell line; Tris, tris(hydroxymethyl)aminomethane.

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enzyme preparation used was free of contaminating exoglycosidases and endo-*N*-acetylhexosaminidase when assayed with *p*-nitrophenyl glycosides and [^{3}H]acetylovalbumin glycopeptides, respectively (Umemoto et al., 1977).

The labeled samples (10 000–25 000 dpm, ^{3}H) were mixed with 500 μg of carrier glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparan sulfate, or heparin) and digested with the mucopolysaccharidases as described below. The carrier glycosaminoglycans were completely degraded under the conditions employed. Leech hyaluronidase digestion was carried out by dissolving the sample in 100 μL of McIlvaine's buffer (pH 5.6) and incubating at 37 °C for 6 h with addition of 125 μg of enzyme in 25 μL buffer at 0, 1, and 2 h. Chondroitinase digestions and paper chromatography of the products were performed according to the method of Saito et al. (1968). Heparitinase and heparinase digestions were done in 300 μL of 0.1 M sodium acetate buffer, pH 7.0, containing 1 mM calcium acetate for 24 h at 30 (heparinase) and 43 °C (heparitinase) (Linker & Hovingh, 1972). Blanks prepared as above but using heat-inactivated enzymes did not degrade the reference glycosaminoglycans.

Nitrous acid degradation was done by treating the sample in 100 μL of water with 20 μL of 3 M $NaNO_2$ and 20 μL of glacial acetic acid at room temperature (20 °C) for 80 min. Excess nitrite was destroyed by addition of 50 μL of 3 M glycine, and after 60 min at room temperature, the product was lyophilized.

The enzyme and nitrous acid degradation products were assessed by gel filtration on glyceryl-CPG-240 or Bio-Gel P-4 or P-6 columns.

Liquid scintillation counting was performed on an Intertechnique Model SL-36 or Model SL-4000 spectrometer equipped with disintegration per minute calculating modules. Usually 0.3–1.0-mL aqueous samples were mixed with 3–10 mL of counting liquid (ACS obtained from Amersham) in minivials or regular plastic counting vials, respectively. Efficiencies for ^{3}H and ^{35}S were about 25% and 65%, respectively, with a crossover of about 10% ^{35}S into the ^{3}H channel. When the external standard method was used, quenching was not detectable with any of the buffers.

Cellulose acetate electrophoresis was performed in a Beckman R-101 microzone electrophoresis cell by using 0.2 M calcium acetate (pH 7.0) at 5 mA for 3 h or pyridine/0.1 M formic acid bufer (pH 3.0) at 10 mA for 20 min. Reference glycosaminoglycans were detected by staining with Alcian blue (0.1% in 0.5% acetic acid). Radioactivity on cellulose acetate strips was estimated by dissolving cut pieces in 1 mL of glacial acetic acid in glass vials. After incubating at 37 °C for 3–6 h and cooling to room temperature, scintillation counting liquid was added, and the solution was mixed and counted. The recovery of the applied ^{3}H and ^{35}S radioactivities was between 80% and 90%.

Other methods used in this study are described in the preceding paper in this issue (Bhavanandan et al., 1981).

Cell Culture. The cells were propagated in 16-oz glass prescription bottles as described (Banks et al., 1976). When confluent, the cells were subcultured by using about 2×10^6 cells per bottle. The cultures were routinely tested for bacterial and yeast contamination as described previously (Banks et al., 1976). The cells were also tested for the presence of mycoplasma (Russell et al., 1975). No contamination was detected in the cells used in these studies.

Metabolic labeling of the glycoconjugates was accomplished by growing the cells in a medium containing isotopic precursors but no inorganic sulfate and one-third the usual glucose concentration. The FM6 and FM13 cells were labeled at the 17th and 20th passages, respectively. The isotopes used were [^{3}H]glucosamine and $^{35}SO_4^{2-}$ at 5 and 20 μCi/mL, respectively. The isotope-containing medium (25 mL) was added to logarithmically growing cells (4–8 bottles per experiment), and after 48 h, when confluency was reached, the cells were harvested. Exposure to labeled precursor for this time period is sufficient to provide an accurate estimate of mass ratios from incorporation levels. The medium was decanted, and the cell layer was rinsed 3 times with NaCl/P_i buffer and then treated with 0.02% EGTA in NaCl/P_i buffer, pH 7.2, at 37 °C for 10–20 min. The EGTA supernatant was removed after centrifugation, and the cell pellet was washed 3 times with the NaCl/P_i buffer. The combined medium plus washings of the monolayers and the combined EGTA supernatant plus the washing of the cell pellet were again centrifuged to remove residual cells. The supernatants, referred to as spent medium and EGTA supernatant, respectively, and the cells were all separately examined for their labeled glycoconjugates.

The establishment and isotopic labeling of the human fetal iris explant were described previously (Banks et al., 1976). The labeled spent medium and the tissue (iris) were treated separately and analyzed for glycoconjugates. This system did not yield an EGTA supernatant fraction (extracellular material).

Processing of the Cells, Spent Media, and the EGTA Supernatants. The cells were extracted with chloroform/methanol (2:1 and then 1:2) as described by Hakomori & Murakami (1968). The pellet was treated with Pronase. The spent media and the EGTA supernatants were dialyzed at 4 °C against 0.15 M NaCl for 2 days, followed by distilled water for 4 days in the presence of toluene and chloroform. The nondialyzable materials were recovered by lyophilization and digested with Pronase.

Separation of Glycopeptides and Glycosaminoglycans in the Pronase Digests by Treatment with Cetylpyridinium Chloride (Refer to Scheme I). The Pronase digests were subjected to cetylpyridinium chloride precipitation as described (Bhavanandan et al., 1977). The glycosaminoglycans were recovered in the 0.2, 0.4, 0.8, 1.2, and 2.0 M NaCl extracts of cetylpyridinium chloride precipitate. The mixed glycopeptides present were isolated from the cetylpyridinium chloride supernatant by precipitation of the cetylpyridinium with KCNS followed by dialysis and lyophilization of the nondialyzable material. Table I presents the distribution of the radioactivity in the glycosaminoglycan and glycopeptide fractions.

Fetal Calf Serum–[^{3}H]Glucosamine Control Experiment. This experiment was designed to determine the contribution, if any, to the glycoconjugate pool of spent medium, of artifacts arising from the interaction of [^{3}H]glucosamine with fetal calf serum components. Fetal calf serum (100 mL) from the same batch used for metabolic labeling of cells was mixed with 400 μL of [^{3}H]glucosamine containing 400 μCi, and the mixture was incubated at 37 °C for 48 h. Triplicate aliquots were taken for determining radioactivity, and the balance was dialyzed at 4 °C against 0.15 M NaCl followed by water, two changes of 6 L each for a total of 8 days. When no further radioactivity was detectable in the dialysate, the dialysis was stopped. The nondialyzable material contained 0.76 μCi of radioactivity, which is 0.19% of the original. Two 2-mL aliquots were hydrolyzed (6 N HCl, 110 °C, 24 h), and the hydrolysate was purified on a column of Dowex 50 (H^+) (Boas, 1953) and analyzed on an amino acid analyzer by the stream-splitting technique. About 68% of the radioactivity coeluted with standard glucosamine. The balance of non-

Scheme I: Isolation of Glycosaminoglycans Produced by HM7 Human Melanoma Cells

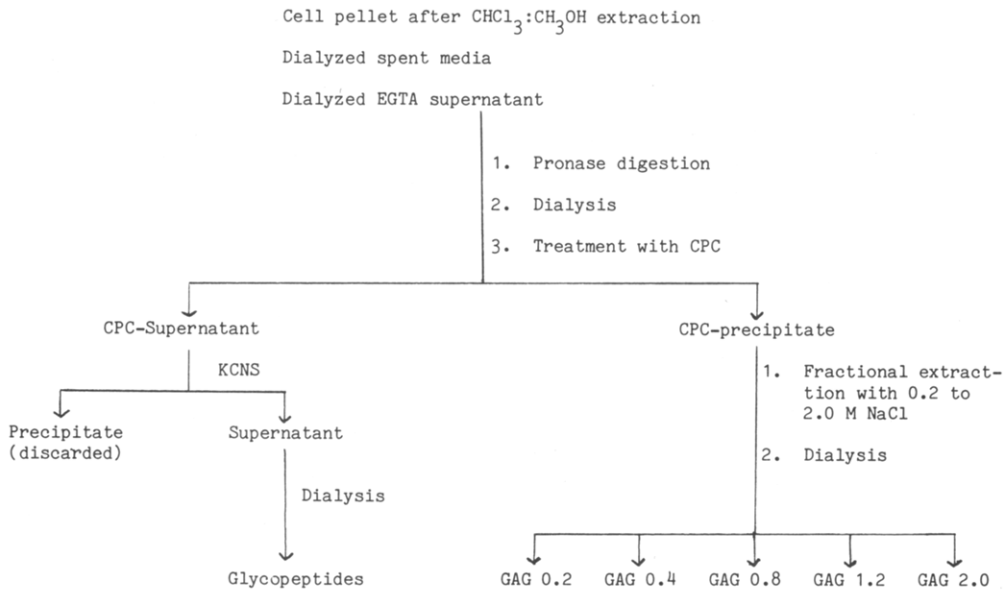

Table I: Distribution of ^3H- and ^{35}S-Labeled Glycoconjugates Produced by Human Fetal Uveal Melanocyte and Melanoma Cell Culture[a]

	human fetal uveal melanocytes			HM7 human melanoma		
	cell	spent medium	EGTA supernatant	cell	spent medium	EGTA supernatant
			glycopeptides			
^3H	26.52 (95.8)	14.63 (56.2)	16.61 (70.5)	54.18 (94.9)	34.78 (85.6)	4.79 (92.2)
^{35}S	0.13 (8.0)	0.84 (4.4)	0.80 (8.7)	0.13 (6.1)	0.44 (5.7)	0.03 (10.0)
			glycosaminoglycans			
^3H	1.17 (4.2)	11.38 (43.7)	6.95 (29.5)	2.91 (5.1)	5.85 (14.4)	0.39 (7.8)
^{35}S	1.49 (92.0)	18.32 (95.6)	8.41 (91.3)	2.04 (93.9)	7.32 (94.3)	0.26 (90.0)

[a] The results are expressed as ^3H and ^{35}S radioactivity (10^{-6} dpm10^7 cells) incorporated into the clycoconjugates. The numbers within parentheses are percentages relative to the total radioactivity which is the sum in the glycopeptides and the glycosaminoglycans in the respective culture compartments. The cells were grown in the presence of [^3H]glucosamine and $Na_2{}^{35}SO_4$ for 48 h during log phase of growth. The culture compartments (cell, medium, and EGTA supernatant) were digested with Pronase and dialyzed. The Pronase digests were treated with CPC to fractionate the glycopeptides and glycosaminoglycans. For details see Methods and Scheme I.

dialyzable material was digested with Pronase, and the digest was dialyzed exactly as for Pronase-digested spent medium. The nondialyzable material after Pronase digestion contained only 10.2% of the radioactivity present before the treatment. The nature of this radioactive material (108 000 dpm), 0.019% of the original, was not further investigated.

Results

Culture Characteristics of the Fetal Melanocytes. The FM6 and FM13 cells grew as nonpiling monolayers of elongated cells compared to the multilayered foci growth of the HM7 human melanoma cells (Figure 1). A brown–black pigmentation was observed in the confluent monolayers of the melanocytes, which is indicative of the production of melanin by these cells. The doubling time of FM6 cells was determined to be 58 h whereas the HM7 cells had a doubling time of 40 h. The doubling times of three other human fetal uveal melanocytes are reported to be 62 (Mel 1B), 52 (Me 7–2A), and 108 (Mel 9) by Giovanella et al. (1976). In contrast to the HM7 cells which have been maintained in culture for over 4 years (>120 passages), both the normal melanocyte cell cultures, FM6 and FM13, failed to grow after about 30 passages.

Tumorigenicity in Athymic (Nude) Mice. Whereas HM7 cells produced large black tumor masses when 10^6 cells were inoculated into nude mice, FM6 cells failed to produce tumors even when 10^7 cells were inoculated. In an earlier work, HM7 cells produced only small nodules in nude mice; the reason for

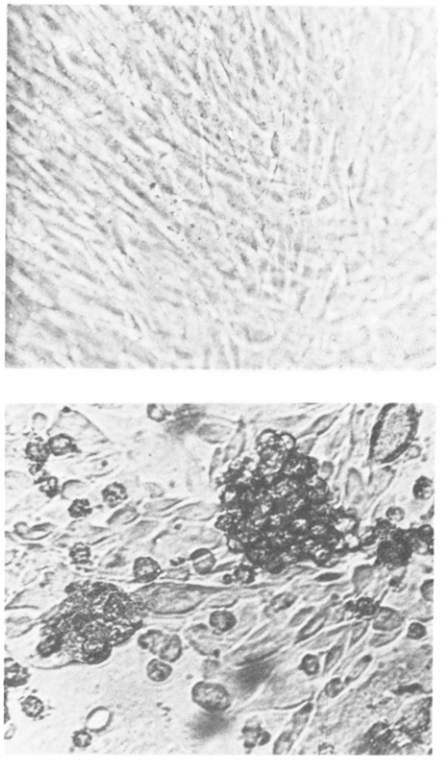

FIGURE 1: Photomicrograph of normal human uveal fetal melanocytes (top) and of HM7 human melanoma cells (bottom). Cultures grown to saturation. Phase contrast 100×.

Table II: Characteristics of GAG 0.8 Fractions[a]

	[^{3}H]GlcNH$_2$ (%)	[^{3}H]GalNH$_2$ (%)	% low mol wt ^{3}H after treatment with: leech hyaluronidase	heparitinase	chondroitinase AC	nitrous acid
cell						
GAG 0.8	100	0	8	83	NT	96
spent medium						
GAG 0.8	88	12	NT	NT	NT	NT
GAG 0.8a	100	0	6	81	4	80
GAG 0.8c	91	9	0	88	10[b]	89
EGTA supernatant						
GAG 0.8	97	3	0	100	NT	88

[a] Percent of radioactivity in the hexosamines was determined by hydrolysis (6 M HCl, 110 °C, 20 h) of the glycosaminoglycans followed by analysis by a stream-splitting technique on an amino acid analyzer as described previously (Bhavanandan & Davidson, 1976). The recovery of radioactivity was between 65 and 75%. The samples were treated as described under Methods with enzymes or nitrous acid, and the mixture was analyzed on Bio-Gel P-4 or P-6 columns. The percentage of radioactivity included in the column is given; the untreated samples were excluded from these columns in all cases. NT = not tested. [b] Paper chromatography showed both ΔDi-4S (65%) and ΔDi-6S (35%).

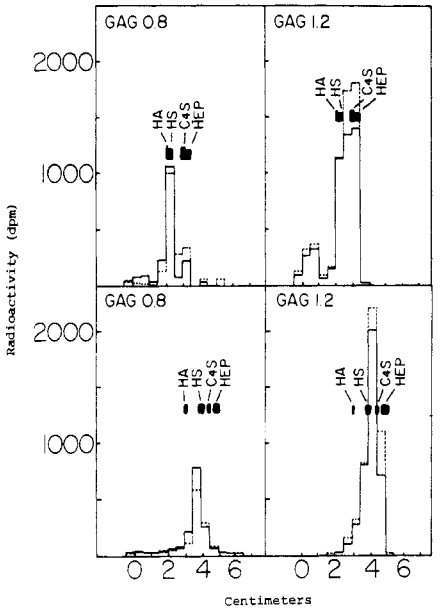

FIGURE 2: Cellulose acetate electrophoresis of human melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2. The human fetal melanocyte glycosaminoglycan fractions GAG 0.8 and 1.2 isolated from the spent medium of cultures labeled with [^{3}H]glucosamine and $Na_2{}^{35}SO_4$ were subjected to electrophoresis on cellulose acetate strips. Electrophoreses were carried out in 0.2 M calcium acetate, pH 7.0, at 5 mA for 3 h (top panels) and in pyridine/0.1 M formic acid buffer, pH 3.0, at 10 mA for 20 min (bottom panels). The radioactivity [^{3}H (—) and ^{35}S (---)] and the reference glycosaminoglycans (HA, hyaluronic acid; HS, heparan sulfate; C4S, chondroitin-4-sulfate; and Hep, heparin) were detected as described under Methods.

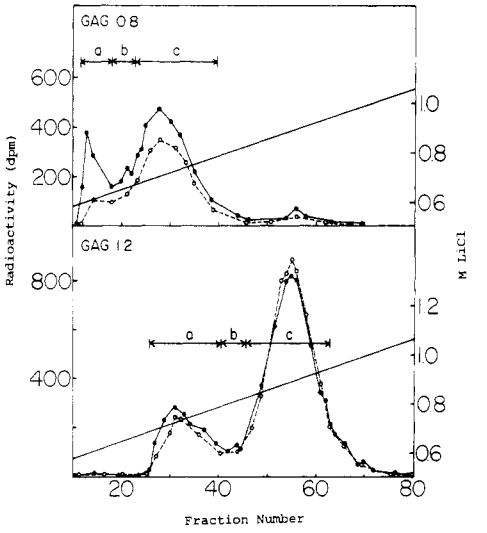

FIGURE 3: Fractionation of human melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2 on DEAE-Sepharose. The human fetal melanocyte glycosaminoglycan fractions GAG 0.8 and GAG 1.2 isolated from the spent medium of cultures labeled with [^{3}H]-glucosamine and $Na_2{}^{35}SO_4$ were chromatographed on a DEAE-Sepharose column (1 × 16 cm). The column was eluted with a linear gradient (total volume 100 mL) of 0.5–1.2 M LiCl. Fractions of 1 mL were collected and aliquots analyzed for radioactivity [^{3}H (●) and ^{35}S (○)]. Material eluting in the area indicated by horizontal arrows marked a, b, and c were recovered by dialysis and lyophilization and designated GAG 0.8a, GAG 0.8b, etc. The GAG 1.2 fraction isolated from the EGTA supernatant was also similarly fractionated.

this is not clear (Banks et al., 1976). FM13 cells were not tested for tumorigenicity. Another fetal melanocyte cell line (Mel 7-2A) is also reported not to have yielded tumors when 10^7 cells were injected subcutaneously and mice were observed throughout their life spans of 1–2 years (Giovanella et al., 1976).

Characterization of the Glycosaminoglycans from Melanocytes: GAG 0.2 and GAG 0.4 Fractions. These fractions from the three culture compartments all contained only [^{3}H]glucosamine and were totally excluded from the CPG column. Treatment with leech hyaluronidase followed by rechromatography on the CPG column indicated that the labeled material in these fractions was completely degraded. Ion-exchange chromatography on DEAE-Sepharose column, using a LiCl gradient for elution, of the GAG 0.2 (medium) and the GAG 0.4 (cell fractions) gave single peaks which coeluted with vitreous humor hyaluronic acid. Heparitinase had no effect on the fractions derived from the medium. Cellulose acetate electrophoresis of the GAG 0.2 (medium), GAG 0.4 (medium), and GAG 0.4 (EGTA) fractions gave single bands with mobilities identical with that of reference hyaluronic acid. Ion-exchange chromatography, heparitinase treatment, and cellulose acetate electrophoresis were not done on the other fractions due to insufficient material. However, on the basis of the results obtained, it was concluded that 0.2 and 0.4 M NaCl fractions from all three compartments contained essentially only hyaluronic acid.

GAG 0.8 Fractions. These fractions contained both ^{3}H and ^{35}S radioactivities. Cellulose acetate electrophoresis of the medium fraction in two buffer systems showed that the major component in this fraction had a mobility similar to that of heparan sulfate (Figure 2). The hexosamine composition (Table II), however, indicated the presence of both glucosamino- and galactosaminoglycans. The GAG 0.8 (medium) fraction was fractionated on a DEAE-Sepharose column (Figure 3), and three subfractions (a, b, and c) were obtained. The hexosamine composition of subfractions 0.8a and 0.8c is given in Table II; the minor intermediate fraction 0.8b was

Table III: Characteristics of GAG 1.2 Fractions[a]

	[^{3}H]GlcNH$_2$ (%)	[^{3}H]GalNH$_2$ (%)	% low mol wt ^{3}H after treatment with: heparitinase	heparitinase and heparinase	chondroitinase AC	nitrous acid
cell						
GAG 1.2	38	62	NT	36	68	NT
spent medium						
GAG 1.2	24	76	NT	NT	NT	NT
GAG 1.2a	100	0	80	100	4	94
GAG 1.2c	0	100	0	NT	100[b,c]	0
EGTA supernatant						
GAG 1.2	21	79	NT	NT	NT	NT
GAG 1.2a	100	0	63	98	NT	100
GAG 1.2c	0	100	NT	8	93[c]	NT

[a] Details are as described in Table II. [b] Paper chromatography showed the presence of both ΔDi-4S (70%) and ΔDi-6S (30%). [c] Chondroitinase ABC digestion gave identical results.

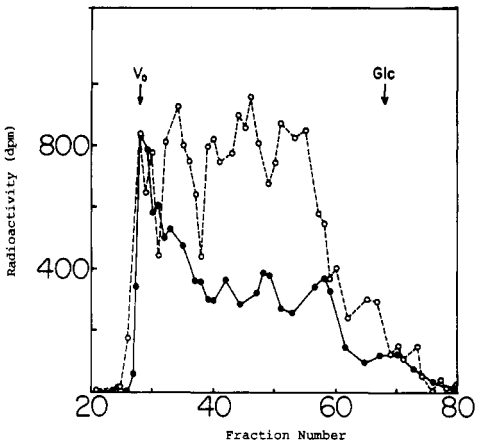

FIGURE 4: Chromatography on Bio-Gel P-6 of ^{3}H-labeled glycosaminoglycan fractions from human fetal melanocytes after treatment with nitrous acid. The ^{3}H-labeled glycosaminoglycan fractions GAG 0.8c (●) and GAG 1.2a (○) from spent medium of human fetal melanocyte cultures were treated with nitrous acid (see Methods for details) and chromatographed on a Bio-Gel P-6 column (0.9 × 105 cm). The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h; 1-mL fractions were collected and analyzed for radioactivity. The void and included volumes of the column determined by the elution of blue dextran (V_0) and glucose (Glc) are indicated. The untreated glycosaminoglycans eluted at the void volume of the column. The results of the treatment of the various melanocyte glycosaminoglycan fractions with nitrous acid are summarized in Tables II and III.

not analyzed. The results of the treatment of GAG 0.8a and 0.8c (medium), GAG 0.8 (cell), and GAG 0.8 (EGTA) with leech hyaluronidase, heparitinase, chondroitinase, and nitrous acid are summarized in Table II. Typical results of the nitrous acid treatment are illustrated in Figure 4.

On the basis of the results, the major component of the GAG 0.8 fractions from all three compartments is characterized as heparan sulfate. The medium fraction, in addition, contained hyaluronic acid (2%) and chondroitin sulfate (12%), and the cell-associated GAG 0.8 contained about 8% hyaluronic acid. The galactosamine component (3%) of GAG 0.8 (EGTA) was not directly identified but is presumed to be chondroitin sulfate.

GAG 1.2 Fractions. These fractions had the largest percentage of ^{35}S radioactivity, and the hexosamine analysis (Table III) suggested that they were mixtures of glucosamino- and galactosaminoglycans. Chromatography on a CPG column indicated components in the molecular weight range 15 000–40 000. Cellulose acetate electrophoresis of GAG 1.2 (cell) showed sulfated material moving as a broad band in the area of heparan sulfate and chondroitin sulfate (Figure 2). The medium and EGTA fractions were fractionated on DEAE-Sepharose to yield two major (a and c) and one minor (b) fraction each (Figure 3). The major fractions were found to have either glucosamine or galactosamine (Table III). The results of the action of nitrous acid, chondroitinase, and heparitinase (or heparitinase followed by heparinase) on these fractions are summarized in Table III. Typical results of chondroitinase treatment and the paper chromatographic pattern of the products are illustrated in Figure 5. Thus the components of the GAG 1.2 fractions from cell, medium, and EGTA supernatant are chrondroitin 4-sulfate (50%, 53%, and 59%), heparan sulfate (38%, 24%, and 21%) and chondroitin 6-sulfate (12%, 23%, 20%), respectively.

GAG 2.0 Fractions. The medium fraction on hydrolysis gave only galactosamine. It gave single peaks on both CPG and DEAE-Sepharose columns. On the basis of its mobility on the CPG column, the molecular weight of the component was estimated to be about 25 000. Chondroitinase AC and ABC completely digested the labeled material. The product was isolated by chromatography on a column of Bio-Gel P-4 and identified as ΔDi-4S by paper chromatography; no ΔDi-6S was detectable in contrast to the GAG 1.2 fraction (Figure 5, bottom). The GAG 2.0 (medium) fraction was not susceptible to either heparitinase or heparinase and was thus identified as chondroitin 4-sulfate. Insufficient material from the cell-associated and EGTA fractions was available for complete characterization. The 100% galactosamine composition of these two fractions, however, suggested that they consisted of chondroitin sulfate(s).

Characterization of the Glycosaminoglycans from Melanoma. Fractions GAG 0.4–2.0 which contain the glycosaminoglycans (hyaluronic acid, chondroitin sulfate, and heparan sulfate) have been partially characterized (Banks et al., 1976). Additional characterization of the melanoma glycosaminoglycans was carried out after further fractionation by chromatography on DEAE-Sepharose CL-6B columns and controlled pore glass as described above. Fraction GAG 0.2 from cell and medium was carefully examined since the corresponding fractions obtained from B16 mouse melanoma consisted mainly of a mucin-type sialoglycopeptide (Bhavanandan & Davidson, 1976; Bhavanandan et al., 1977). However, the human melanoma GAG 0.2 fractions on treatment with neuraminidase or mild acid released insignificant quantities of [^{3}H]sialic acid; further, the labeled material in these fractions did not interact with WGA–Sepharose 4B. Greater than 90% of the radioactivity in this fraction was identified as [^{3}H]glucosamine after acid hydrolysis; on treatment with leech hyalnuronidase, the labeled material was degraded to oligosaccharides. These results as

Table IV: Summary of the ^{3}H-Labeled Glycosaminoglycans Produced by Human Fetal Uveal Melanocytes and Melanoma in Culture[a]

	human fetal uveal melanocytes			human melanoma		
glycan	cell	spent medium (48 h)	EGTA supernatant	cell	spent medium (48 h)	EGTA supernatant
hyaluronic acid	2.78 (35.7)	10.64 (13.8)	8.41 (17.7)	5.00 (15.8)	8.24 (9.0)	1.79 (28.0)
chondroitin 4-sulfate	2.01 (25.8)	35.15 (45.4)	20.15 (42.5)	13.76 (43.5)	51.61 (56.4)	2.53 (39.5)
chondroitin 6-sulfate	0.41 (5.3)	14.13 (18.3)	6.38 (13.5)	0	0	0
heparan sulfate	2.59 (33.2)	17.43 (22.5)	12.46 (26.3)	12.85 (40.7)	31.66 (34.6)	2.08 (32.5)

[a] The results are expressed as ^{3}H radioactivity (10^{-5} dpm) incorporated into the various glycosaminoglycans obtained from the culture compartments cell, spent medium, and EGTA supernatant. The numbers within parentheses are the percentages relative to the total radioactivity present in glycosaminoglycans, in the separate culture compartments (vertical columns). Individual species of glycosaminoglycans were identified and quantitated as described under Methods and Results.

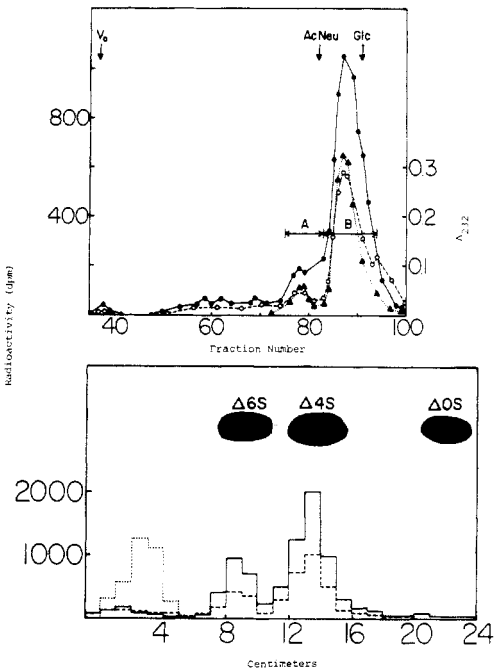

FIGURE 5: Chromatography of ^{3}H- and ^{35}S-labeled glycosaminoglycan fraction GAG 1.2c from human fetal melanocytes after digestion with chondroitinase AC on Bio-Gel P-4 (top) and paper (bottom). (Top panel) The ^{3}H- and ^{35}S-labeled glycosaminoglycan fraction GAG 1.2c isolated from human melanocytes was mixed with carrier chondroitin 4-sulfate treated with chondroitinase AC (see Methods for details) and chromatographed on a Bio-Gel P-4 column (0.9 × 110 cm). The column was eluted with 0.1 M pyridine/0.1 M acetic acid (pH 5.0) at 20 mL/h, and 1-mL fractions were collected. After the fractions were assayed for unsaturated disaccharides by measuring absorbance at 232 nm (▲), aliquots were analyzed for radioactivity [^{3}H, (●) and ^{35}S, (○)]. Untreated GAG 1.2c and chondroitin 4-sulfate or controls prepared by treatment of the glycosaminoglycans with heat-inactivated enzyme eluted at the void volume (V_0). The peak elution positions of *N*-acetylneuraminic acid (AcNeu) and glucose (Glc) are indicated by arrows. The radioactive material eluting in the area marked by horizontal arrows A and B were recovered by lyophilization and examined by paper chromatography. (Bottom panel) The radioactive materials A [^{3}H, (···)] and B [^{3}H (—) and ^{35}S (- - -)] were chromatographed on paper together with standard unsaturated disaccharides (ΔDi-4S, ΔDi-6S, and ΔDi-OS) by using the solvent system 1-butanol/acetic acid/1 M NH_4OH (2:3:1 v/v). The migration positions of the standard unsaturated disaccharides were detected by examining the paper in UV light. Radioactivity on paper strips was estimated by extracting 1-cm cut pieces with 1 mL of water in counting vials followed by scintillation counting. The results of the treatment of the various melanocyte glycosaminoglycan fractions with chondroitinase AC followed by paper chromatography are summarized in Tables II and III.

well as those from cellulose acetate electrophoresis and DEAE-Sepharose chromatography confirmed that the human melanoma GAG 0.2 fractions from cells and spent medium were similar to the GAG 0.4 fractions which comprise hyaluronic acid.

The results of the characterization of the glycosaminoglycans produced by the human fetal uveal melanocyte and human melanoma cultures are summarized in Table IV.

Discussion

The long-term cultures of the uveal melanocytes used in this study were derived from therapeutically aborted, 6–12-week human embryos by Giovanella et al. (1976). The cells were characterized as normal melanocytes on the basis of their ability to produce melanin and by their morphological characteristics (Giovanella et al., 1976). In our laboratory, we confirmed these observations on the FM6 and FM13 cultures. Further, in our studies, the FM6 cells failed to produce tumors in athymic mice under conditions which produced characteristic tumors from HM7 human melanoma cells. This together with the limited culture life (number of passage in culture) of the FM6 and FM13 cells compared to the virtual immortality of the HM7 human melanoma cells under identical culture conditions substantiates the nonmalignant nature of the fetal melanocytes.

[^{3}H]Glucosamine and $Na_2{}^{35}SO_4$ were employed as radioactive precursors to label the newly produced glycoconjugates. The possibility of labeled glucosamine interacting with fetal calf serum components of the culture medium leading to the formation of artifacts (Angello & Hauschka, 1974; Herrmann, 1974) was investigated in a control experiment. After [^{3}H]-glucosamine was mixed and incubated with fetal calf serum, exhaustive dialyses removed 99.81% of the radioactive material. The balance, identified as glucosamine, was apparently trapped or nonspecifically interacting with serum components since 90% of this dialyzed out following Pronase digestions. Thus the artifactual contribution to the pool of glycopeptides and glycosaminoglycans in the Pronase digest of the spent media in our experiments could be shown to be negligible (at the most, 0.019% of the added radioactivity). It should be noted that in the control experiment we used 10 times more glucosamine per milliliter of serum than that employed in the cell labeling experiments.

The total nondialyzable ^{3}H radioactivity in the Pronase digests of the melanocytes and melanoma cells did not differ significantly, but large differences were noted in the extracellular pools (the spent medium and EGTA supernatant). For example, the melanocytes incorporated 3.5 times more sulfate label into the extracellular (medium and EGTA supernatant) glycosaminoglycans than did the melanomas (Table I). A similar difference was noted in the synthesis of sulfated glycosaminoglycans by Balb/c 3T3 and SV40 Balb/c 3T3 cells (Klagsbrun, 1976). Further, the percent of CPC precipitable ^{3}H label (glycosaminoglycans) in the Pronase digests of the spent medium and EGTA supernatant was 14.4% and 7.8% for the melanoma culture and 43.8% and 29.5% in the case of the melanocyte culture (Table I). This indicates an increased secretion (or shedding) of glycosaminoglycans by the

melanocytes into the external environment, most of which was recovered in the GAG 1.2 fractions.

Whereas both melanocyte and melanoma cultures produced hyaluronic acid, chondroitin 4-sulfate, and heparan sulfates, only the melanocyte culture produced chondroitin 6-sulfate (Table IV). The cell, medium, and EGTA supernatant compartments of the melanocyte culture contained 5.3%, 18.3% and 13.5% of glycosaminoglycan-associated ^3H radioactivity in chondroitin 6-sulfate. Dermatan sulfate and keratan sulfate were not produced by either culture. There were quantitative differences in the production of hyaluronic acid, chondroitin 4-sulfate, and heparan sulfate by the two cell cultures. The distribution of ^3H in hyaluronic acid, heparan sulfate, and chondroitin 4-sulfate were 11.6%, 36.0%, and 52.4% for the melanoma culture and 16.5%, 24.5%, and 42.2% for the melanocyte culture (Table IV). An increased production of sulfated glycosaminoglycans, specifically heparan sulfate, with a concomitant decrease in hyaluronic acid production by malignant cells compared to normal counterparts has been previously observed (Goggins et al., 1972; Satoh et al., 1974; Chandrasekaran & Davidson, 1979). The glycosaminoglycans produced by the iris explant were markedly different from those produced by both of the continuous cell cultures (FM6 and HM7). In this case, about 79% of the ^3H radioactivity in the glycosaminoglycans was accounted for by hyaluronic acid, 14% by chondroitin 4-sulfate, 1% by heparan sulfate, and 5% by dermatan sulfate (Banks et al., 1976).

Several forms of heparan sulfate and chondroitin 4-sulfate appear to be present as indicated by the detection of these components in the 0.8, 1.2, and 2.0 M NaCl eluates of the CPC precipitate. Both heparinase and heparitinase were required to digest completely the heparan sulfate in the GAG 1.2 fractions (Table III) whereas heparitinase alone fully degraded the GAG 0.8 heparan sulfate (Table II). In comparison, the heparan sulfates produced by human melanoma culture were always completely degraded by heparitinase alone [Banks et al., (1976) and present results]. These data suggest that the heparan sulfate in the GAG 1.2 fractions of the melanocyte culture may have structural features resembling heparin, for example, a higher degree of N- or O-sulfation. The faster mobility on cellulose acetate electrophoresis of the glycosaminoglycans in GAG 1.2 compared to GAG 0.8 (Figure 2) and the nitrous acid degradation patterns of GAG 0.8c and GAG 1.2a (Figure 4) further supports this possibility. It has been shown that heparan sulfate isolated from normal Swiss 3T3 mouse cells contains 8% more *O*-sulfate than that isolated from SV40 transformed Swiss 3T3 cells (Keller et al., 1980).

Glycosaminoglycans which are major constituents of the cell surface and extracellular matrix are believed to be important in modifying the growth of cells both in vitro and in vivo. These molecules may be involved in the adhesion of cells to each other and to the substratum or membranes (Roblin et al., 1975; Culp et al., 1978; Turley & Roth, 1980).

A role of masking tumor antigens has also been postulated for the cancer cell surface glycosaminoglycans (Takeuchi et al., 1977). However, thus far the studies on the production of glycosaminoglycans by a wide variety of cultured cancer cells have given inconsistent results. For example, whereas increased sulfated glycosaminoglycan production by virus transformed fibroblasts has been reported by Goggins et al. (1972), Satoh et al. (1973), Makita & Shimojo (1973), and Hopwood & Dorfman (1977), others have reported a decrease in the production of these molecules by virus transformed cells (Saito & Uzman, 1971; Terry & Culp, 1974; Roblin et al., 1975). Similarly, both an increase (Temin, 1965; Satoh et al., 1973; Hopwood & Dorfman, 1977) and a decrease (Hamerman et al., 1965; Satoh et al., 1974) in the production of hyaluronic acid by malignant cells have also been recorded.

In the present studies, we have grown the cells in the same medium, labeled them at comparable cell densities and growth phase, and used the same concentration of isotopes in order to minimize the influence of external factors. Accordingly, the observed changes between the malignant and control (fetal) cultures should be considered characteristic of the cell type rather than due to artifactual reasons.

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Neocarzinostatin Chromophore: Purification of the Major Active Form and Characterization of Its Spectral and Biological Properties†

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ABSTRACT: The nonprotein chromophore of neocarzinostatin (NCS-Chrom), which possesses the full biological activity of native NCS, can be separated by high-performance liquid chromatography into two components, A and B, with 90% and 10%, respectively, of the total UV-absorption and in vitro DNA scission activity. Each component possesses in vitro DNA scission activity equal to NCS, as does a third component, C, which is present only when NCS is extracted with nonacidic methanol and appears to be derived from A. NCS-Chrom A possesses in vivo activity equal to NCS; however, B and C have about 5% and 80%, respectively, of the activity of NCS for inhibition of HeLa cell growth. NCS-Chrom A possesses characteristic absorption, fluorescence, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra. The spectral properties of B and C are similar to those of A. These spectral properties cannot be attributed solely to the presence of the known naphthoate component of NCS but are due to the presence of an additional conjugated component (as yet unidentified) in the chromophore. NCS-Chrom D, an inactive minor component of NCS-Chrom preparations, can be generated by hydrolysis of each of the three active components at pH 8. It possesses a very different CD spectrum and, in contrast to NCS-Chrom, possesses no MCD, indicating a major change in or loss of the naphthoic acid residue. NCS-Chrom D is the 490-nm fluorescent product generated when NCS-Chrom spontaneously looses biological activity in aqueoous pH 8 buffers. It is the source of the 490-nm fluorescence in clinical NCS. The optical activity of the biologically active NCS-Chrom accounts for the Cotton effects previously observed for native NCS. Changes in both the absorption and circular dichroism spectra of NCS-Chrom A (and B and C) occur on binding to apo-NCS, generating spectra identical with those of native NCS and providing stoichiometry of the association process. Complex formation between NCS-Chrom and DNA is also detected by CD, with the maximal effect being observed at low molar ratios of NCS-Chrom to DNA. Furthermore, hypochromicity between 290 and 330 nm and a bathochromic shift centered near 365 nm occur upon binding of NCS-Chrom to apo-NCS and to DNA, raising the possibility that similar modifications in the electronic structure of the NCS-Chrom result from both interactions.

Neocarzinostatin (NCS),[1] an antitumor antibiotic, causes single-strand breaks in linear duplex or superhelical DNA in vitro in an oxygen-dependent reaction which is greatly stimulated by mercaptans. NCS also causes DNA strand scission in vivo, and considerable evidence exists indicating that DNA damage is the primary result of its activity in vivo [reviewed in Goldberg et al. (1981)].

We have shown that NCS contains a methanol-soluble, nonprotein chromophoric component (Napier et al., 1979) in addition to the sequenced acidic protein (M_r 10700) (Meienhofer et al., 1972). The isolated chromophore, possessing the full biological activity of NCS (Kappen et al., 1980a; and data reported here), exhibits a characteristic absorption spectrum between 300 and 400 nm and an intense blue

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[1] Abbreviations used: NCS, neocarzinostatin; NCS-Chrom, nonprotein chromophoric component extracted from NCS; apo-NCS, protein component of NCS; HPLC, high-performance liquid chromatography; MCD, magnetic circular dichroism; CD, circular dichroism.

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