Biosynthesis of Glycosaminoglycans by Microsomal Preparations from Cultured Mastocytoma Cells

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Neoplastic mast cells of mice (including long-established and newly derived lines) were grown in large-volume suspension cultures to provide enough cells for preparation of microsomal fractions. Microsomal preparations from P815Y and P815S cells synthesized ^{14}C -labelled glycosaminoglycan when incubated with UDP- ^{14}C glucuronic acid and UDP-*N*-acetylgalactosamine. No significant amount of ^{14}C -labelled glycosaminoglycan was formed when UDP-*N*-acetylglucosamine was substituted for the UDP-*N*-acetylgalactosamine. Microsomal preparations from X163 cells synthesized ^{14}C -labelled glycosaminoglycan when incubated with UDP- ^{14}C glucuronic acid and either UDP-*N*-acetylgalactosamine or UDP-*N*-acetylglucosamine. The ^{14}C -labelled glycosaminoglycan formed in the presence of UDP-*N*-acetylgalactosamine was degradable by testicular hyaluronidase, indicating that it was chondroitin-like. The ^{14}C -labelled glycosaminoglycan formed in the presence of UDP-*N*-acetylglucosamine was not degradable by testicular hyaluronidase. Microsomal preparations from P815S cells were tested for sulphating activity by incubation with adenosine 3'-phosphate 5'-sulphatophosphate, as well as UDP- ^{14}C glucuronic acid, and UDP-*N*-acetylgalactosamine. The resulting newly synthesized polysaccharide was shown by chondroitinase ABC digestion to be 70% chondroitin 4-sulphate and 30% chondroitin. The molecular size of this newly synthesized glycosaminoglycan was determined by gel filtration to be larger than 40000 mol.wt. In general, the glycosaminoglycan-synthesizing ability of the microsomal preparations appeared to reflect glycosaminoglycan synthesis by the intact cells.

Cell-free biosynthesis of the polysaccharide portions of proteoglycans has been obtained by the use of microsomal preparations from several animal tissues, including chick embryo cartilage (Silbert, 1964; Perlman *et al.*, 1964; DeLuca & Silbert, 1968; Silbert & DeLuca, 1969), mouse mastocytoma (Silbert, 1963, 1967*a,b*; Lindahl *et al.*, 1972), rat skin (Schiller *et al.*, 1961), rabbit marrow cells (Olsson, 1972) and horse leucocytes (Olsson & Gardell, 1971). These studies have established that uridine sugar nucleotides are the precursors for the polymerization of the heteropolysaccharide chains and confirmed that adenosine 3'-phosphate 5'-sulphatophosphate is the sulphate donor required for extensive sulphation.

Much less is known about the enzymes and acceptors involved, or about the interrelationships between the various steps in glycosaminoglycan synthesis and regulation. There have been certain difficulties in approaching these questions, owing to the limitations inherent in studies with preparations from whole tissues. In general, whole tissues are heterogeneous, containing not only the desired cells but also extracellular matrix and small amounts of other elements such as blood vessels, fibroblasts etc. Moreover, the ability to control the cellular environment is limited with the use of whole tissues. In con-

trast to whole tissues, cultured cell systems *in vitro* have the advantages of homogeneity, low extracellular matrix content and controllable environment.

In the present study, various lines of mouse mastocytoma-derived cells, previously analysed for their glycosaminoglycan-synthesizing activities (Lewis *et al.*, 1973), were grown *in vitro* in quantities large enough to enable the preparation of microsomal fractions. The glycosaminoglycan-biosynthesizing abilities of these microsomal preparations were determined. The macromolecular products synthesized from the appropriate precursors by these preparations were found to be generally consistent with the glycosaminoglycan products produced by intact cells.

Experimental

Materials

The following materials were obtained from the indicated sources: culture medium, horse serum and antibiotics, Grand Island Biological Co., Grand Island, N.Y., U.S.A.; polyvinylpyrrolidone (Grade 40), Schwarz/Mann, Orangeburg, N.J., U.S.A.; spinner flasks, Bellco Co., Vineland, N.J., U.S.A.; Mes [2-(*N*-morpholino)ethanesulphonic acid] buffer,

Sigma Chemical Co., St. Louis, Mo., U.S.A.; UDP-[^{14}C]glucuronic acid, New England Nuclear Corp., Boston, Mass., U.S.A.; UDP-*N*-acetylglucosamine, Sigma Chemical Co.; UDP-*N*-acetylgalactosamine and adenosine 3'-phosphate 5'-sulphatophosphate prepared as previously described (Silbert, 1964, 1967a). Sources of other materials are given in the preceding paper (Lewis *et al.*, 1973).

Methods

Culture techniques. Mastocytoma cells (as described by Lewis *et al.*, 1973) were grown in large-volume suspension cultures, by using spinner flasks containing Fischer's medium supplemented with 10% (v/v) horse serum, 0.1% (w/v) polyvinylpyrrolidone, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). The medium was equilibrated with CO_2 + air (5:95) throughout the culture period. Each culture was initiated by inoculating a 1-litre spinner flask containing 400 ml of growth medium with enough cells to give an initial culture density of approx. 20000–40000 cells/ml. After 3 days' culture, the contents of this flask were used to seed another spinner flask (9 litre) containing 6 litres of medium. After another 3–4-day culture period, the cells were separated from the medium by centrifugation (400g).

Preparation of microsomal fractions. A 10 ml suspension, containing 3–4 ml of packed cells in 0.25 M-sucrose, was homogenized with a Dounce homogenizer (type B). The degree of cell disruption was checked microscopically, and homogenization was continued until no intact cells were visible. The homogenate was then centrifuged at 10000g for 15 min and the resulting supernatant was centrifuged at 105000g for 30 min. The 105000g pellet was washed by resuspension and re-centrifugation in 0.25 M-sucrose. The washed pellet (approx. 0.2–0.3 ml) was suspended in an additional 0.3–0.8 ml of 0.25 M-sucrose. A sample of this suspension was analysed for protein content (Lowry *et al.*, 1951).

Formation and isolation of glycosaminoglycan. Microsomal preparations (10 μl) were incubated in 0.05 M-Mes buffer (pH 6.5)–0.01 M- MnCl_2 with combinations of the following substrates: UDP-[^{14}C]glucuronic acid, UDP-*N*-acetylgalactosamine, UDP-*N*-acetylglucosamine, and adenosine 3'-phosphate 5'-sulphatophosphate. Unless otherwise noted, the reaction mixtures were in a volume of 25 μl and were incubated at 37°C for 2 h.

Labelled glycosaminoglycan products were isolated by the procedure of DeLuca & Silbert (1968). Reaction mixtures were chromatographed on Whatman no. 1 paper in ethanol–1 M-ammonium acetate, pH 7.5 (5:2, v/v). The chromatographic origins, which contained all the macromolecular material, were cut out and incubated overnight at 37°C with 2 ml of 0.05 M-Tris-HCl buffer (pH 8.5) containing

1% (w/v) pancreatin. Several drops of toluene were added to retard bacterial growth. After incubation, the suspensions were boiled and centrifuged (20000g) for 30 min. The supernatants were retained and the pellets were resuspended in 1 M-NaCl and re-centrifuged. The supernatants were then combined and dialysed against several changes of water and the non-diffusible material was assayed for radioactivity.

Characterization of glycosaminoglycans. The characterization procedures used in this study are described in detail in the preceding paper (Lewis *et al.*, 1973).

Samples of the labelled materials, along with standard chondroitin sulphate, were treated with testicular hyaluronidase. After enzymic treatment, the reaction mixtures were placed on columns (1 cm $\times$ 15 cm) of Sephadex G-50 and eluted with 1 M-NaCl. The amount of material appearing in the included fractions was considered to represent the degraded glycosaminoglycan.

Other samples, together with standard chondroitin sulphates, were incubated with chondroitinase ABC. After degradation, the reaction products were chromatographed overnight on Whatman no. 1 paper in butan-1-ol–acetic acid–1 M-aq. NH_3 (2:3:1, by vol.).

For estimation of molecular weight, samples were placed on columns (1 cm $\times$ 60 cm) of Sepharose 4B with a standard of chondroitin 6-sulphate (mol.wt. 40000) and eluted with 0.1 M-LiCl.

Analytical methods

The methods of analysis used in this paper were described previously (Lewis *et al.*, 1973). Radioactivity was determined with a liquid-scintillation spectrometer or a low-background (0.5 c.p.m.) planchet counter. Culture cell densities were determined with a haemocytometer.

Results

Large-volume culture of mastocytoma-derived cells

All the cell lines previously studied for glycosaminoglycan-synthesizing potential (Lewis *et al.*, 1973) grew well under the suspension culture conditions used in the present study. The average final culture density for five cell lines grown in this fashion was 6.5×10^5 cells/ml, yielding an average of approx. 7.0 ml of packed cells. Therefore, the large quantities of cells necessary for subcellular studies were readily obtainable.

Glycosaminoglycan formation

Microsomal preparations from the cells were tested for heteropolysaccharide-polymerizing activities. Synthesis was measured by the incorporation into macromolecular materials of [^{14}C]glucuronic acid

Table 1. *Incorporation into macromolecular material of [¹⁴C]glucuronic acid from UDP-[¹⁴C]glucuronic acid by microsomal preparations from cultured cells*

Reaction conditions and techniques used for isolating the products are given in the Experimental section. The reaction mixtures contained 10 μ l of microsomal preparation, 0.017 mM-UDP-[¹⁴C]glucuronic acid (80×10^3 c.p.m.), and various combinations of 1.0 mM-UDP-*N*-acetylgalactosamine, 1.0 mM-UDP-*N*-acetylglucosamine and 1.0 mM-adenosine 3'-phosphate 5'-sulphatophosphate. Incorporation values represent the average of two or three determinations.

Source of microsomal preparation	Additions	Incorporation (c.p.m.)	GlcUA incorporated (nmol)
P815Y cells	UDP-GalNAc	1800	0.010
	UDP-GlcNAc	230	<0.002
	None	100	<0.001
X163 cells	UDP-GalNAc	2700	0.014
	UDP-GlcNAc	2100	0.011
	None	70	<0.001
P815S cells	UDP-GalNAc	3600	0.019
	UDP-GalNAc + adenosine 3'-phosphate 5'-sulphatophosphate	4425	0.024
	UDP-GlcNAc	120	<0.001
	None	105	<0.001

Fig. 1. *Time-dependency of glycosaminoglycan formation*

Reaction conditions and glycosaminoglycan isolation techniques are given in the Experimental section. Duplicate reaction mixtures contained 50 μ l of a P815Y microsomal preparation, 1.0 mM-UDP-*N*-acetylgalactosamine, and 1.2 mM-UDP-[¹⁴C]glucuronic acid (4×10^5 c.p.m.) in a volume of 125 μ l. After the indicated reaction time-period, 10 μ l samples of the reaction mixture were spotted on paper and chromatographed. Radioactive material (glycosaminoglycan) remaining at the chromatographic origin was determined directly by removing the origins and counting them in a low-background planchet counter. The points represent the average of the two determinations.

Table 2. *Characterization of [¹⁴C]glycosaminoglycans synthesized by P815S microsomal preparations in the presence of adenosine 3'-phosphate 5'-sulphatophosphate*

Samples were pooled as shown in Fig. 3. After dialysis, total radioactivity of each peak was determined. Portions of the pooled materials were treated with chondroitinase and chondroitinase-degradation products identified. For details see the Experimental section.

Peak	% of total [¹⁴ C]glycosaminoglycan	% degradable by chondroitinase to:	
		Δ Di-OS	Δ Di-4S
I	10	95	<2
II	34	31	67
III	56	<3	96

from UDP-[¹⁴C]glucuronic acid in the presence of necessary co-substrates. Microsomal preparations from P815Y, X163 and P815S cells showed significant polymerizing activity (Table 1), whereas microsomal preparations from HC73 and P815AS cells did not incorporate enough label to allow further study.

When compared relative to microsomal protein content, incorporation of [¹⁴C]glucuronic acid in the presence of UDP-*N*-acetylgalactosamine was of the same order of magnitude for preparations from

P815Y, X163 and P815S cells. For comparison with these microsomal systems from the cultured cells, microsomal fractions from mouse mastocytomas and chick cartilage were prepared by techniques as previously described (Silbert, 1963, 1964). These preparations were tested for incorporation of [^{14}C]-glucuronic acid in the presence of UDP-*N*-acetyl-galactosamine. The mastocytoma preparation, on the basis of microsomal protein content, was two to three times as active as were preparations from the mastocytoma-derived cell cultures. The chick cartilage preparations were approximately ten times as active as the cultured mastocytoma cell microsomal preparations.

Incorporation by a P815Y microsomal prepara-

tion was shown to be time-dependent, with maximal synthesis after 90 min (Fig. 1). At 90 min approx. 70% of the remaining sugar nucleotide substrates were still intact. Incorporation in the presence of 1.2 mM-UDP-glucuronic acid was several times as great on a molar basis as incorporation in the presence of 0.017 mM-UDP-glucuronic acid. However, for identification purposes it was necessary to obtain products of maximal specific radioactivity, and 0.017 mM-UDP- ^{14}C glucuronic acid was used as a routine. This use of less than maximal substrate concentration necessitates certain caution in making quantitative comparisons of the relative activities of the various microsomal preparations.

As in the previous study of glycosaminoglycan

Fig. 2. Chondroitinase degradation of P815S ^{14}C -labelled glycosaminoglycan formed in the presence (b) and in the absence (a) of adenosine 3'-phosphate 5'-sulphatophosphate

Chondroitinase degradation of ^{14}C -labelled glycosaminoglycan and paper chromatography of the resulting products were performed as described in the Experimental section. Materials were eluted from 0.5 cm sections of the chromatograms and radioactivity was determined. The positions of the unsaturated disaccharides were indicated by their u.v. absorbance.

biosynthesis by intact cells (Lewis *et al.*, 1973), the P815S cell system was chosen for more extensive study. Accordingly, adenosine 3'-phosphate 5'-sulphatophosphate was added to the P815S microsomal reaction mixtures to investigate formation of sulphated glycosaminoglycan (Table 2). In the presence of adenosine 3'-phosphate 5'-sulphatophosphate, there was a slight increase in the incorporation of [14 C]glucuronic acid into glycosaminoglycan, but it is questionable whether this increase was significant. Identification of the sulphated product is described in the next section.

Characterization of glycosaminoglycans

All the [14 C]glycosaminoglycan synthesized by the various microsomal preparations in the presence of UDP-glucuronic acid and UDP-*N*-acetylgalactosamine was degradable by testicular hyaluronidase, as expected of chondroitin. The [14 C]glycosaminoglycan obtained after incubation of the X163-cell microsomal preparation with UDP-glucuronic acid and UDP-*N*-acetylglucosamine was resistant to testicular hyaluronidase, as expected of a heparin-like polysaccharide.

Fig. 3. Charge densities of 14 C-labelled glycosaminoglycan synthesized by P815S microsomal preparations in the presence or in the absence of adenosine 3'-phosphate 5'-sulphatophosphate

Macromolecular materials were chromatographed on DEAE-cellulose columns by using a logarithmic [LiCl] gradient (see solid line in *a*). Fractions (2.5 ml) were collected. The reservoir was changed from 1M- to 2M-LiCl after fraction 80. The positions of standard glycosaminoglycans were determined by assay for uronic acid. Fractions were pooled as indicated, dialysed, freeze-dried and characterized further (Table 3). (*a*) Standards; (*b*) no adenosine 3'-phosphate 5'-sulphatophosphate; (*c*) with adenosine 3'-phosphate 5'-sulphatophosphate.

Glycosaminoglycan synthesized by P815S microsomal preparations was also treated with chondroitinase ABC and the resulting degradation products were chromatographed. The [^{14}C]glycosaminoglycan, synthesized in the reaction mixtures that did not contain adenosine 3'-phosphate 5'-sulphatophosphate, was degraded to material co-chromatographing with $\Delta\text{Di-OS}$ (Fig. 2). This further supported the identification of the heteropolysaccharide as chondroitin. Approx. 3–4% of this chondroitinase-degraded material chromatographed in the position of the

saturated disaccharide acetylchondrosine (Di-OS). Since the saturated disaccharide would represent the non-reducing terminal of the chondroitin chain, this suggests that the additional, newly synthesized chains contained an average of 25–33 disaccharide repeating units. When adenosine 3'-phosphate 5'-sulphatophosphate was included in the reaction mixture, the [^{14}C]glycosaminoglycan product was different. Only 30% of products of degradation by chondroitinase co-chromatographed with $\Delta\text{Di-OS}$, whereas 70% co-chromatographed with $\Delta\text{Di-4S}$ (Fig. 2). No

Fig. 4. Sepharose 4B gel chromatography of ^{14}C -labelled glycosaminoglycans synthesized by P815S microsomal preparations

After DEAE-cellulose chromatography (Fig. 3), pooled materials along with standard chondroitin 6-sulphate (mol.wt. 40000) were chromatographed on Sepharose 4B. Fractions (1.0ml) were collected and samples were analysed for radioactivity (●) and uronic acid content E_{530} (Δ). (a) Peak I; (b) peak II; (c) peak III. The void (V_0) and included (V_1) volumes are shown.

radioactivity co-chromatographed with Δ Di-6S. These results indicate the formation of chondroitin 4-sulphate by P815S microsomal preparations.

Labelled products synthesized by microsomal preparations from P815S cells were analysed further by ion-exchange chromatography on DEAE-cellulose (Fig. 3). Glycosaminoglycan synthesized in the presence of adenosine 3'-phosphate 5'-sulphatophosphate had an elution pattern different from the elution pattern of glycosaminoglycan formed in the absence of adenosine 3'-phosphate 5'-sulphatophosphate, demonstrating the change in charge densities owing to the sulphation of chondroitin. The peak III material (Fig. 3) had a charge density greater than that of the chondroitin 4-[35 S]sulphate formed by intact P815S cells (Lewis *et al.*, 1973). Fully sulphated materials formed by microsomal preparations from chick cartilage have also been noted to have the same high charge density (Silbert & DeLuca, 1969).

Pooled, dialysed fractions from the DEAE-cellulose columns were treated with chondroitinase ABC (Table 2). Degradation of peak I material gave Δ Di-0S, indicating that the peak I glycosaminoglycan was chondroitin. Degradation of peak II and III materials yielded 67% and 96% Δ Di-4S respectively, indicating that sulphation of the chondroitin had occurred to form chondroitin 4-sulphate.

The molecular size relative to standard commercial chondroitin 6-sulphate (mol.wt. 40000) of these DEAE-cellulose-separated materials was estimated by Sepharose 4B gel filtration (Fig. 4). Peak I and peak III material chromatographed in a position indicating a larger size than the standard, whereas peak II material appeared to have an average size similar to the standard.

Discussion

Microsomal preparations from cells of three mouse mastocytoma lines (P815Y, X163 and P815S) cultured *in vitro* contained sufficient glycosaminoglycan-synthesizing activity for further study.

Microsomal preparations from both P815Y and P815S cells, when incubated with the appropriate sugar nucleotides, synthesized chondroitin but no heparin-like glycosaminoglycan. In a further study, the microsomal preparations of P815S cells were shown to have sulphating activity; 70% of the newly synthesized chondroitin was sulphated to form chondroitin 4-sulphate when adenosine 3'-phosphate 5'-sulphatophosphate was present. The chondroitin 4-sulphate heteropolysaccharide synthesized by the cell-free system was larger than a chondroitin sulphate standard (of mol.wt. 40000), and larger than the chondroitin 4-sulphate formed by intact P815S cells (Lewis *et al.*, 1973). The significance of these size differences is not known.

Microsomal preparations from X163 cells incorporated sugars into both heparin-like and chond-

roitin-like glycosaminoglycans. In this case, the expression of the glycosaminoglycan-synthesizing potential of X163-cell microsomal preparations appeared to be modified by the loss of the intracellular environment, since intact X163 cells produced predominantly heparin-like material (Lewis *et al.*, 1973).

It is of interest to compare the results of this study with those of earlier experiments, which used microsomal preparations from whole mastocytoma tissue (Silbert, 1963, 1967*a,b*). Previously, microsomal preparations from P815 mouse mastocytomas were shown to synthesize non-sulphated and sulphated heparin-like materials. However, in these earlier experiments, chondroitin sulphate-synthesizing potential was not investigated. Our present studies have shown that microsomal preparations from these mastocytomas now have chondroitin-synthesizing activity and less heparin-synthesizing activity than previously observed. It seems that after years of repeated transplantations there have been changes in the glycosaminoglycan-synthesizing potential of the tumour line.

Cell-free synthesis of highly sulphated chondroitin sulphate *de novo* has been previously demonstrated only with microsomal preparations from chick embryo cartilage (Silbert & DeLuca, 1969). Subsequently, these products have been identified as entirely chondroitin 6-sulphate (S. DeLuca, M. Richmond & J. Silbert, unpublished work). The present work with microsomal preparations from P815S cells has demonstrated the first cell-free synthesis of chondroitin 4-sulphate *de novo*.

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