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Sulphated Glycosaminoglycans in Regenerating Rat Liver

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The total weight percentage glycosaminoglycan content of rat liver was found to increase by 50% in the first 30h after partial hepatectomy. The content returned to near normal by the third day, but then increased again to a second maximum at 5–6 days, only to gradually decline to normal by the ninth day, when regeneration was nearly complete. This biphasic pattern was most marked in the chondroitin sulphate A/C component, with a 6-fold increase by the sixth day. Dermatan sulphate showed the same temporal trend, whereas heparan sulphate remained relatively unaltered. No such changes were detected in the livers of rats subjected to sham operation. The possible molecular mechanisms underlying the apparent link between cellular glycosaminoglycan content and proliferative tendency are discussed.

Sulphated polysaccharides have been extracted from a very wide range of multicellular organisms (Cassaro & Dietrich, 1977). Certain kinds have specialized functions within connective tissues, where they are produced in large quantities, but in addition they constitute a major component of the pericellular envelope of probably all tissue-derived animal cells. A conventional view of the possible biological role for these heteropolysaccharides is that they function in intercellular recognition and adhesion, and could thus influence cell movement, growth and differentiation (Chiarugi & Vannucchi, 1976).

The composition of mammalian cell-surface glycosaminoglycans varies depending on the type of tissue from which they are extracted (Dietrich *et al.*, 1977), the extent of growth of cultured cells (Vannucchi *et al.*, 1978) and the progress of differentiation (Augusti-Tocco & Chiarugi, 1976). Differences in glycosaminoglycan composition also occur between tumours and normal counterpart tissues (Takeuchi *et al.*, 1976). Taken as a whole, these data suggest the predominance of highly sulphated 1→4-linked heparan sulphates at the surface of normal cells within well-differentiated slow-growing tissues, and at the surface of stationary-phase cultured cells. Other less-sulphated glycosaminoglycans containing 1→3 glycosidic links occur predominantly in actively-growing normal cells and in neoplastic cells, both in culture and *in vivo*.

The molecular reactions underlying this apparent link between cellular glycosaminoglycans and proliferative tendency remain unclear. Different sulphated polysaccharides characteristically vary, however, in their metal-ion-binding/release properties *in vitro* (Buddecke & Drzenick, 1962), and, as discussed below, differences in such properties *in vivo* may reflect differences in polymer function *in vivo*.

Regenerating liver affords a cell population *in vivo*, the proliferative state of which changes in a determined manner over an experimentally suitable period of time. The present paper reports changes in sulphated glycosaminoglycan composition occurring in rat liver after partial hepatectomy.

Experimental

Materials

Chondroitin 6-sulphate from river sturgeon cranial cartilage, chondroitin 4-sulphate from river sturgeon notochord, chondroitin sulphate from hog mucosal tissue and heparan sulphate from beef lung were given by Professor M. B. Mathews and Professor J. A. Cifonelli, University of Chicago, Chicago, IL, U.S.A. Chrondroitinase AC (EC 4.2.2.5) from *Arthrobacter aurescens*, chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, bacterial α-amylase (EC 3.2.1.1), bovine pancreatic ribonuclease A (EC 3.1.4.22) and bovine pancreatic deoxyribonuclease I (EC 3.1.4.5) were from Sigma Chemical Company, St. Louis, MO, U.S.A. Pronase from *Streptomyces griseus* was from BDH, Poole, Dorset, U.K. Sephrapore III cellulose acetate strips were from Gelman Instrument Company, Ann Arbor, MI, U.S.A. All other reagents were of Analytical grade.

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Methods

Partial hepatectomy and preparation of livers. Female albino rats (180–200g) of the Sprague–Dawley strain were used. Partial hepatectomy (Higgins & Anderson, 1931), with the animals under light diethyl ether anaesthesia, involved removal of two-thirds of the liver (ablation of the main median and left lateral lobes). Sham operations consisted of laparotomy and palpation of the liver. At particular times after operation rats were killed by a blow on the head and cervical dislocation. Livers were perfused with saline (0.15M-NaCl), excised and homogenized in acetone (10vol.) before standing at 0°C for 12h. The homogenate was then centrifuged at 1000*g* for 10min and the pellet was washed twice by resuspension in cold acetone and centrifugation before being dried under vacuum over P_2O_5 and weighed.

Extraction of glycosaminoglycans. For each liver the dried acetone-washed pellet was suspended in Tris/HCl buffer (0.05M, pH7.6) and boiled for 10min. This suspension was then cooled and incubated with Pronase (20mg/g of dry liver) at 50°C under toluene. Further additions of Pronase (10mg) were made at 24 and 48h. At 72h residual Pronase was inactivated by heating at 100°C for 3min. The digest was then cooled, the pH adjusted to 6.0 with HCl and incubated at 37°C with α-amylase (5mg; 695 units/mg) for 24h. Trichloroacetic acid was then added to a final concentration of 10% (w/v) and the tube was left at 0°C for 30min before the resultant precipitate was removed by centrifugation. The supernatant was neutralized with 2M-NaOH, dialysed against several changes of water for 72h and then concentrated by rotary evaporation under reduced pressure before addition of ethanol (3vol.) at 4°C. After standing for 24h at this temperature the precipitate was removed and subjected to further Pronase (20mg) treatment for 24h at 50°C. After inactivation of the Pronase a further incubation was carried out at pH7.6 for 24h at 37°C with a mixture of deoxyribonuclease I (1938 units), ribonuclease A (152 units) and $MgSO_4$ (12mM final concentration). This incubation mixture was then treated with trichloroacetic acid and centrifuged and the supernatant was neutralized, concentrated, dialysed and treated with ethanol. The precipitate was then dried as described above.

Glycosaminoglycans were further purified by ion-exchange column chromatography on a column (0.8cm × 30cm) of Bio-Rad AG 1 X2 (100–200 mesh). After sample application the column was washed with water (40ml) followed by saline (40ml, 0.3M). The adsorbed glycosaminoglycans were then eluted from the column with saline (100ml, 3.0M). Fractions were assayed for total carbohydrate by the method of Dubois *et al.* (1956), and for uronate by the method of Blumenkrantz & Asboe-Hansen (1973). The uronate-containing fractions were pooled, dialysed against water for 16h, concentrated, treated with ethanol and the precipitate was dried as described above.

Characterization and estimation of glycosaminoglycans. Glycosaminoglycans were identified by a combination of electrophoresis, susceptibility to digestion with specific enzymes and reaction with HNO_2.

Flat bed electrophoresis was carried out on cellulose acetate (2.5cm × 17cm) with barium acetate (pH8.0, 0.1M) and with pyridine/formic acid (pH3.0, 0.5M-pyridine) buffers at 12°C with a constant current of 1mA/cm for 4h and 30min respectively. The strips were stained for 30min in Alcian Blue solution (0.2% w/v) containing equal volumes of aqueous sodium acetate (pH5.8, 0.025M) containing $MgCl_2$ (0.1M) and aqueous ethanol (50%, v/v). The same solution was used without Alcian Blue for destaining. Quantitative estimation of individual glycosaminoglycans was by determination of the Alcian Blue complex in each band by the method of Newton *et al.* (1974). Stained bands and control portions from in front of the bands were cut from the wet cellulose acetate strips. The blue polysaccharide complexes were extracted by incubating strips at 37°C in dimethyl sulphoxide containing $MgCl_2$ (0.025M) and sodium acetate (0.025M). A Whirlimixer was used to aid disintegration of the cellulose acetate gel. Maximum colour intensity was achieved 30min after dispersion of the gel particles, when absorbances at 678nm were determined. By using the appropriate standards a separate calibration plot was constructed for each type of glycosaminoglycan. Reproducible estimates were obtained with as little as 0.2μg of polysaccharide.

For testing susceptibility to chondroitinase AC or chondroitinase ABC, samples were dissolved in Tris/acetate buffer (50μl, pH8.0, 0.05M) containing NaCl (0.15M) and bovine serum albumin (0.1%, w/v). After addition of enzyme solution (50μl, 5 units/ml) mixtures were incubated at 37°C for 18h. Reaction was terminated by heating at 100°C for 3min.

Deamination of glycosaminoglycans with HNO_2 was by treatment with 1.8M-acetic acid containing 0.24M-sodium nitrite for 6h at ambient temperature (Lindahl *et al.*, 1973).

Recoveries of individual glycosaminoglycans were estimated as follows: 1mg each of hyaluronate, dermatan sulphate, heparan sulphate, heparin and chondroitin 6-sulphate were dissolved in 2ml of Tris/HCl buffer (0.05M, pH7.6). Of this solution 0.25ml was used for quantitative estimation of the individual components after electrophoretic separation. To the remaining 1.75ml was added

bovine serum albumin (2.5g), DNA (50mg) and RNA (25mg). This mixture was then subjected to the extraction procedure, electrophoresis and finally quantitative estimation of the individual glycosaminoglycans. The percentage recoveries were thus estimated as: heparan sulphate, 85.5; heparin, 82.8; dermatan sulphate, 76.0; chondroitin sulphate, 71.3; hyaluronate, 32.9.

Results

The liver weight returned to near normal by about 9 days after partial hepactectomy (Fig. 1).

Electrophoresis on cellulose acetate strips in barium acetate buffer revealed a changing pattern of sulphated glycosaminoglycans during liver regeneration (Fig. 2).

Two bands, which co-migrated with standards of heparan sulphate and dermatan sulphate, were

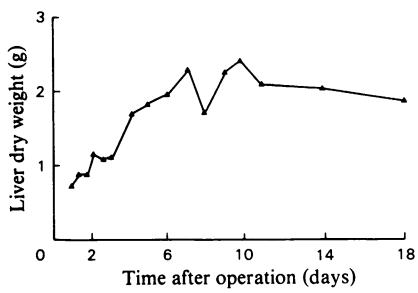

Fig. 1. *Dry weight of rat liver after partial hepatectomy*
Livers were perfused with 0.15M-NaCl, excised and lipid was removed by washing with acetone, before drying under vacuum over P_2O_5. Weight of normal defatted liver from animals not operated on was 2.27g, with a standard deviation of 0.22g (eight animals).

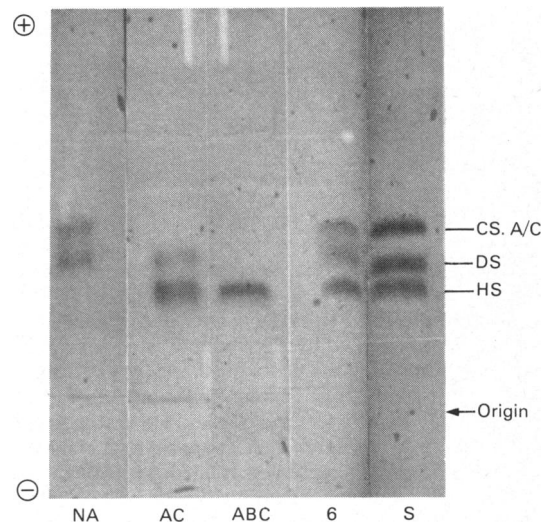

Fig. 3. *Electrophoresis on cellulose acetate strips run in barium acetate buffer (0.1 M, pH 8.0) at 1 mA/cm for 4 h of rat liver extracts subjected to specific degradation*
The glycosaminoglycans extracted from the liver 6 days after partial hepatectomy were incubated with chondroitinase AC (AC), chondroitinase ABC (ABC) and HNO_2 (NA). Untreated day 6 extract (6) and standards (S) of heparan sulphate (HS) dermatan sulphate (DS) and chondroitin sulphate A/C (CS.A/C) are also shown.

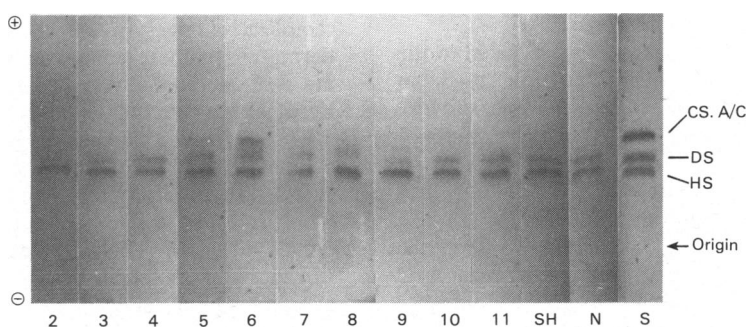

Fig. 2. *Electrophoresis of rat liver extracts on cellulose acetate strips run in barium acetate buffer (0.1 M, pH 8.0) at 1 mA/cm for 4 h*
Glycosaminoglycans were visualized by staining with 0.2% (w/v) Alcian Blue. Strips 2–11 represent extracts prepared from livers taken at 2–11 days after partial hepatectomy. SH refers to the extract from a rat subjected to sham operation and N is the extract from a normal unoperated rat. S is a mixture of standard samples of heparan sulphate (HS), dermatan sulphate (DS) and chondroitin sulphate A/C (CS.A/C).

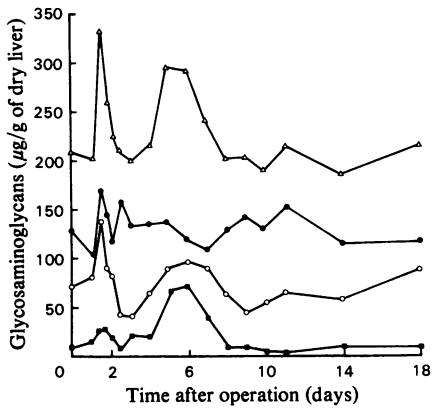

Fig. 4. *Quantitative determination of glycosaminoglycans*

Glycosaminoglycans (△, total; ●, heparan sulphate; O, dermatan sulphate; ■, chondroitin sulphate A/C) isolated from rat liver extracts after partial hepatectomy were separated by electrophoresis on cellulose acetate strips, and stained with 0.2% (w/v) Alcian Blue. The stained bands were cut from the strips, dissolved in dimethyl sulphoxide and the absorbance was measured at 678 nm against appropriate blanks. Calibration graphs were constructed for individual glycosaminoglycans, and corrections were made for losses during extraction. The calculated error limits of the mean values for six normal rats (time zero) were all less than 6% of the mean values.

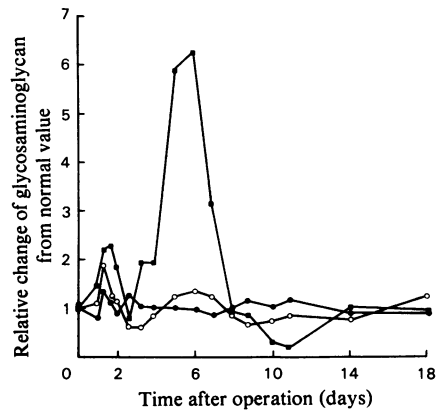

Fig. 5. *Relative changes in content of glycosaminoglycans*

The contents of heparan sulphate (●), dermatan sulphate (O) and chondroitin sulphate A/C (■) in livers of partially hepatectomized rats are expressed as ratios of the contents found in normal unoperated rat livers.

visible in all extracts. Of particular note was the appearance on the fourth day after operation of a third band, which persisted until the ninth day. This band corresponding to chondroitin sulphate A/C and could not be detected in livers of either normal rats or sham-operated rats.

Each of the three bands also corresponded to the appropriate standard when electrophoresed in pyridine/formic acid buffer (results not shown). Positive identification of rat liver glycosaminoglycans was achieved by a combination of specific enzymic and chemical degradation (Fig. 3).

Treatment with chondroitinase ABC resulted in the loss of two bands that corresponded to the dermatan sulphate and chondroitin sulphate A/C standards. Chondroitinase A/C removed only the band corresponding to chondroitin sulphate A/C. The band corresponding to heparan sulphate was degraded by HNO_2, which cleaves the characteristic *N*-unsubstituted or *N*-sulphated glucosamine residues of heparin and heparan sulphate (Lindahl *et al.*, 1973).

These results suggest that the two major bands appearing in all extracts are heparan sulphate and dermatan sulphate, whereas the third band, which appeared only between 4 and 8 days after partial hepatectomy, is chondroitin sulphate A/C.

Quantification of the glycosaminoglycans by spectrophotometric estimation of the Alcian Blue complexes gave two peaks in total glycosaminoglycan content during the 18 day study period (Fig. 4). The increase after operation was maximal at 30 h, decreased to normal amounts by 3 days, peaked again at 5–6 days and returned to normal by 8–9 days.

This biphasic pattern can be accounted for by temporal changes in the chondroitin sulphate and dermatan sulphate constituents. Chondroitin sulphate A/C increased from negligible amounts to 25% by weight of the total glycosaminoglycan content by 5–6 days after partial hepatectomy. Dermatan sulphate increased to its maximum at 30 h, decreased below its normal content to a minimum value at 3 days, returned to normal between 5 and 7 days, decreased again to its low value at day 9 and then slowly returned to normal by day 18. In contrast, heparan sulphate showed little change.

The amounts of individual glycosaminoglycans expressed relative to the amounts present before operation are given in Fig. 5. This shows the marked biphasic increase in chondroitin sulphate A/C, the less dramatic but parallel 'peaking' of dermatan sulphate content and the virtual constancy of heparan sulphate content.

Discussion

The results accord with the report that the chondroitin A/C component of rat liver, which constitutes over 40% of the total liver glycosaminoglycan content at birth, falls to almost zero by 18 days after birth (Sampaio *et al.*, 1977).

A full explanation for the biphasic temporal changes in glycosaminoglycan contents observed in the regenerating liver is not yet available. They are reminiscent of reported biphasic changes in DNA synthesis (Rabes *et al.*, 1965; Rabes & Brändle, 1969), ornithine decarboxylase activity (McGowan & Fausto, 1978), UDP-galactose 4-epimerase activity (Baur *et al.*, 1976) and *N*-acetylglucosaminotransferase activity (Okamoto *et al.*, 1978) in livers after partial hepactectomy, and may reflect the fact that, in intact liver, different populations of hepatocytes replicate asynchronously (Rabes, 1978).

Because some glycosaminoglycans occur predominantly at the surface of cells (Kraemer, 1971), it is likely that their relationship to cell proliferation involves events occuring at the cell surace. The progression of cells through the cell cycle appears to require a transient increase in cytosolic free Ca^{2+} concentration, probably involving an influx of cell-surface-membrane-associated Ca^{2+} into the cytosol (Berridge, 1975). Balk *et al.* (1973) suggested that neoplastic cells proliferate autonomously because of an increased Ca^{2+} uptake, and there is indirect evidence that non-proliferating normal cells have a lower intracellular Ca^{2+} content than corresponding proliferating and transformed cells (Vannucchi *et al.*, 1978).

We have already mentioned the differing metal-ion-binding/release characteristics of different glycosaminoglycans. Indirect evidence suggests that glycosaminoglycans from neoplastic cell surfaces have a lower affinity for Ca^{2+} than do glycosaminoglycans from normal cell surfaces (Yamamoto & Terayama, 1973). It is known that highly sulphated iduronate-rich glycosaminoglycans, which are those predominating in non-proliferating cells, have a higher affinity for Ca^{2+} *in vitro* than do less-sulphated glycosaminoglycans predominating in actively growing cells (Buddecke & Drzenick, 1962; Boyd *et al.*, 1980). Surface glycosaminoglycans may therefore influence the proliferative tendency of cells by limiting the availability of pericellular Ca^{2+}.

An additional possibility involves the interaction of cell-surface glycosaminoglycans with proteinases. Cell-surface proteinases could well be involved in the control of cell proliferation, and different glycosaminoglycans are known to exhibit different capacities for inhibiting serum proteinases *in vitro* (Kindness *et al.*, 1979).

Finally, our data and the results of others provide us with a working hypothesis, which is that the presence of heparan sulphate at the surface of cells decreases their tendency to proliferate and that this effect is diminished in growing cells by an increase in the content of less highly sulphated glycosaminoglycans.

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