Biosynthesis of dermatan sulphate proteoglycans

The effect of β -D-xyloside addition on the polymer-modification process in fibroblast cultures

Lars CÖSTER,* Johan HERNNÄS and Anders MALMSTRÖM

Department of Physiological Chemistry, Lund University, P.O. Box 94, S-221 00 Lund, Sweden

Incubation of cultured fibroblasts with *p*-nitrophenyl β -D-xyloside resulted in a concentration-dependent increase in galactosaminoglycan synthesis. At low concentration of added xyloside large and small radiolabelled proteoglycans and xyloside-bound polysaccharides were recovered from the medium, whereas at high concentrations only xyloside-bound polysaccharides were found. In the cell layer proteoglycans and xyloside-bound polysaccharides were found at all concentrations tested. Only galactosaminoglycan chains were polymerized on the xyloside primer. At low concentrations of added xyloside the structure of the galactosaminoglycans formed on the xyloside was similar to that of the small dermatan sulphate proteoglycan, i.e. mainly composed of L-iduronic acid-containing 4-sulphated disaccharides. With increasing concentration of added xyloside the co-polymeric structure of the small dermatan sulphate proteoglycan and the xyloside-bound polysaccharide was changed to contain a larger proportion of D-glucuronosyl residues with only slight changes in the sulphation pattern. No structural change in the polysaccharide chains of the large glucuronic acid-rich proteoglycans occurred. At 1 mM-xyloside, where no proteoglycans were formed, the polysaccharide was shorter and composed mainly of D-glucuronosyl-containing disaccharides with a ratio of 4-sulphate to 6-sulphate substituents of 1:2. This is similar to the structure of the large glucuronic acid-rich proteoglycan synthesized by these cells. Thus the main difference induced by the xyloside treatment was changed polymer modification at high xyloside concentrations. The specific activities of the polymer-modifying enzymes, uronosyl C-5-epimerase and 4-sulphotransferase, were therefore measured and found to be decreased by 30–50 % in fibroblasts treated with high xyloside concentrations. It is suggested that the protein core is of importance for regulating the activity of the polymer-modifying enzymes.

INTRODUCTION

In cultures of human embryonic skin fibroblasts large and small dermatan sulphate proteoglycans (DSPG) are synthesized [1]. The large proteoglycan has a large core protein, M_r 220 000, substituted with five to 30 glucuronic acid-rich dermatan sulphate chains [2–5]. The core protein has been sequenced and shows large similarities to but not identity with the core protein of the large cartilage proteoglycan [5]. Two small proteoglycans, PGS1 and PGS2, both having a core protein of M_r 40 000, have been demonstrated in fibrous tissues and cells thereof [1,2,6]. A 75 % degree of identity of the two core proteins has been shown [7,8]. The two small proteoglycans have been demonstrated in several tissues in various proportions [6,9]. PGS1 is substituted with two polysaccharide chains whereas PGS2 has one. One of these polysaccharide chains is located on a serine residue close to the *N*-terminus [6,10]. The structure of the polysaccharide chains varies considerably between different tissues. In bone and tracheal cartilage the polysaccharide backbone contains only D-glucuronosyl residues [11,12], in articular cartilage 28 % of the uronosyl residues are L-uronosyl residues, and in sclera, skin and cervix 60–80 % of the uronosyl residues are L-iduronosyl residues [1,2,6,13].

In the tissue the various types of proteoglycans have been ascribed different functions. The small iduronic acid-rich dermatan sulphate proteoglycan of the PGS2 type is found located in close contact with the collagen fibrils [14] and implicated to be of importance for fibril growth [15]. PGS1 does not appear to influence fibril formation [16]. The large glucuronic acid-rich dermatan sulphate proteoglycan is present in the interfibrillar space, where it forms large aggregates with hyaluronan, thereby keeping the fibrils apart and endowing the

tissue with resilience [4,9,17,18]. Their different functions are largely dependent on the physicochemical properties of their galactosaminoglycan (GAG) side chains, which have different amounts of iduronic acid. The factors responsible for the determination of the co-polymeric structure of the galactosaminoglycan chains are thus of importance for the properties of the tissue. It is not known what determines the iduronic acid content of a given proteoglycan. In short, the synthesis of galactosaminoglycans is accomplished by alternating addition of glucuronic acid and galactosamine from their respective UDP-sugars on to a Gal-Gal-Xyl-core protein in the Golgi apparatus by the appropriate transferases. By action of the uronosyl C-5-epimerase some of the D-glucuronic acid moieties are converted into L-iduronic acid in the polymer [19,20]. To study whether the core protein influences the co-polymeric structure of the final product it is convenient to use primers other than the cores for the synthesis of galactosaminoglycans. Specific exogenous primers for galactosaminoglycan formation in cell cultures are β -D-xylosides. The xyloside competes with the endogenous xylose-substituted core protein as substrate for the galactosyl-transferases, which results in synthesis of GAG chains on the exogenous xyloside [21,22].

The present study was undertaken to investigate how addition of xylosides to fibroblast cultures influences the polymer-modification processes of the galactosaminoglycan chains.

EXPERIMENTAL

Materials

Guanidinium chloride, urea (both technical grade) and *p*-nitrophenyl β -D-xyloside were obtained from Sigma Chemical

Abbreviations used: DSPG, dermatan sulphate proteoglycans; PGS, small proteoglycans; GAG, galactosaminoglycans; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; HSPG, heparan sulphate proteoglycans; PGL, large proteoglycan; Xyl-GAG, xylose-bound galactosaminoglycans.

* To whom correspondence should be addressed.

Co. Stock solutions of guanidinium chloride were purified by treatment with activated charcoal. Stock solutions of urea (8 M) were passed through a bed of mixed ion-exchange resin before use. Chondroitin AC-II lyase (EC 4.2.2.5), chondroitin ABC lyase (EC 4.2.2.4) and unsaturated disaccharide standards were purchased from Miles Laboratories. [^3H]Leucine, [^3H]glucosamine hydrochloride (5.8 Ci/mmol) and [^{35}S]sulphate (SJS 1) were products of Amersham International. Disulphated disaccharide was prepared from pig skin dermatan sulphate [23]. Mono Q HR 5/5 and Superose 6 HR 10/30 f.p.l.c. columns were obtained from Pharmacia Fine Chemicals.

Isolation of radioactivity labelled proteoglycans and glycosaminoglycans

Human skin fibroblasts were established and maintained as described previously [24]. At 3 days before addition of radioactive precursors to confluent cell cultures the medium was replaced with a sulphate-poor medium [1]. Cultures (passages 5–15) were used with sulphate-poor medium [1] containing 50 μCi of [^{35}S]sulphate/ml and 25 μCi of [^3H]glucosamine/ml. To some cultures *p*-nitrophenyl β -D-xyloside was added at concentrations ranging from 2 to 3000 μM at the same time as radioactive precursors and incubated for 24 h. In other experiments cultures were preincubated with 1 mM-*p*-nitrophenyl β -D-xyloside for various times followed by labelling with radioactive precursors. In these experiments 4 h of preincubation was followed by 4 h of labelling, 20 h of preincubation was followed by 4 h of labelling, or 24 h of preincubation was followed by 24 h of labelling. After incubation the medium was poured off and the cell layer was extracted twice with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, supplemented with 10 mM- Na_2EDTA , 5 mM-*N*-ethylmaleimide and 1 mM-di-isopropyl phosphorofluoridate. The solutions were centrifuged at 10000 *g* for 30 min to remove cell debris. The medium and cell extracts were then dialysed against 6 M-urea/0.05 M-sodium acetate buffer, pH 5.8, containing 10 mM- Na_2EDTA and 5 mM-*N*-ethylmaleimide (buffer 1). The dialysed extracts were subjected to ion-exchange chromatography on columns (0.5 cm $\times$ 2.5 cm) of DEAE-cellulose (DE-52), which were eluted stepwise with 3 bed vol. respectively of buffer 1, 6 M-urea/0.05 M-sodium acetate buffer, pH 5.8, and 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8. Ovalbumin (1 $\mu\text{g}/\text{ml}$) was used as carrier. Proteoglycans and xyloside-GAG were obtained in the last fraction, dialysed against 0.05 M-sodium acetate buffer, pH 5.8, containing 0.1 M-NaCl and 6 M-urea and subjected to ion-exchange chromatography on a Mono Q column connected to an h.p.l.c. system (LKB). This column was eluted with a linear gradient from 0.1 M-NaCl to 1.2 M-NaCl in 6 M-urea/0.05 M-sodium acetate buffer, pH 5.8. Proteoglycan material was recovered and subjected to gel-filtration chromatography in 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8, on a Superose 6 column in an h.p.l.c. system (LKB).

Isopycnic density-gradient centrifugation in $\text{CsCl}/4$ M-guanidinium chloride with an initial density of 1.54 g/ml was performed in a 70.1-Ti rotor at 36000 rev./min for 72 h in a Beckman ultracentrifuge. Each tube was divided into 13 fractions, each 1 ml.

Analytical and degradative methods

Polysaccharide side chains were isolated after papain digestion and ion-exchange chromatography [25]. Digestions of polysaccharides with chondroitin AC-II lyase and chondroitin ABC lyase were performed in 0.05 M-sodium acetate buffer, pH 6.0, and in 0.2 M-Tris/acetate buffer, pH 7.3, respectively [25]. The amount of iduronic acid in the galactosaminoglycans was de-

termined after chondroitin AC-II lyase treatment and separation on Sephadex G-50 [1]. The proportions of L-iduronosyl and D-glucuronosyl residues were calculated by using the formula described by Malmström *et al.* [24].

The amounts of 4-sulphated and 6-sulphated residues in the galactosaminoglycans were determined after simultaneous degradation with chondroitin AC-II lyase and chondroitin ABC lyase followed by separation of the degradation products on a Lichrosorb- NH_2 column (4 mm $\times$ 250 mm) (Merck) connected to an h.p.l.c. system (LKB) [26,27]. After digestion, 4 vol. of ethanol was added to the digest. After 4 h these were centrifuged for 10 min at 10000 rev./min in a Sigma 101 M centrifuge. The supernatants were evaporated to dryness in a Speed Vac Concentrator (Savant). The residues were dissolved in the starting buffer and applied to the column.

Protein was determined by the method of Lowry *et al.* [28], with BSA as standard.

Protein synthesis was determined after incubating cell cultures with *p*-nitrophenyl β -D-xyloside (0, 5 or 1000 μM) for 4 and 20 h respectively. The medium was changed to fresh medium containing xyloside and [^3H]leucine (5 $\mu\text{Ci}/\text{ml}$) and incubated for 4 h. The labelled protein material was recovered and quantified as described previously [29].

Assay of polymer-modifying enzyme activity

Substrate preparations. Chondroitin and dermatan were prepared as described previously [20].

Enzyme preparations. Crude preparations of uronosyl C-5-epimerase and sulphotransferases were obtained from control and β -D-xyloside-treated human skin fibroblast cultures [30]. Briefly, cells were detached from the substrate, then homogenized, and material sedimenting on centrifugation at 10000 *g* was used as enzyme sources.

Uronosyl C-5-epimerase assay. Reaction mixtures contained the following components in a final volume of 100 μl : ^3H -labelled dermatan (5600 d.p.m., specific radioactivity 1450000 d.p.m./ μmol of uronic acid), 2.5 μmol of Mes, pH 6.5, 1 μmol of MnCl_2 , 0.5% Nonidet-P40 and enzyme (5 μg of protein). They were incubated for 1 h [31]. The amount of liberated $^3\text{H}_2\text{O}$ was determined after distillation.

Sulphotransferase assay. Reaction mixtures contained the following components in a final volume of 50 μl : 0.2 M-Mes buffer, pH 6.5, 10 mM- MnCl_2 , 10 mM- NaF , 1% Triton X-100, 12.5 μg of substrate (chondroitin or dermatan), 0.2 mM ^{35}S -labelled PAPS (1 μCi) and enzyme (4 μg of protein). The mixtures were incubated for 20 min at 37 $^\circ\text{C}$. The reactions were interrupted by transferring the mixture on to Whatman 3MM paper discs, which were immersed in isobutyric acid/0.5 M-ammonia (5:3, v/v) [32]. The discs were then subjected to scintillation counting of radioactivity.

RESULTS

After 24 h of incorporation of [^3H]glucosamine and [^{35}S]sulphate in the presence or in the absence of *p*-nitrophenyl β -D-xyloside, the labelled products were isolated by ion-exchange chromatography as outlined in the Experimental section. The sulphated products were isolated as a broad peak free from hyaluronan (Fig. 1a). At 12.5 μM -xyloside the main component was eluted at lower ionic strength than the proteoglycan material (Fig. 1c). At 6 μM -xyloside an intermediate pattern was obtained (Fig. 1b).

The proteoglycans and xyloside-bound polysaccharide formed were further separated by gel chromatography on Superose 6 (Fig. 2). The proteoglycan fraction from the control experiment

Fig. 1. Ion-exchange chromatography on Mono Q of radioactively labelled products recovered from culture medium

Fibroblasts in culture were treated with β -D-xyloside together with radioactive precursors. The medium was then subjected to ion-exchange chromatography on Mono Q HR 5/5 as described in the Experimental section. (a) Control experiment (no β -D-xyloside added); (b) β -D-xyloside was added to the culture medium to a final concentration of $6 \mu\text{M}$; (c) β -D-xyloside was added to a final concentration of $12.5 \mu\text{M}$. —, $[^3\text{H}]$ Glucosamine; ----, $[^{35}\text{S}]$ sulphate; - - - - -, concn. of NaCl. The second component was recovered.

was resolved into a large component (PGL) and a small component (PGS) (Fig. 2a). Most of the HSPG chromatographed in the excluded volume of the Superose column and could be separated by subsequent chromatography on Mono-Q (Fig. 2a inset and Table 1). With increasing xyloside concentration the chromatographic pattern gradually changed to a more included elution position with a K_{av} corresponding to free polysaccharide chains (Fig. 2). At $60 \mu\text{M}$ -xyloside no proteoglycans were secreted into the medium (Fig. 2d and Table 1). With increasing concentration of xyloside the size of the xyloside-bound polysaccharide chains formed decreased (Figs. 2c, 2d and 2e).

At low xyloside concentration ($6 \mu\text{M}$ and $12.5 \mu\text{M}$) it was not feasible to get a clear-cut separation of intact PGS and xyloside-bound GAG by gel filtration on Superose 6 (Figs. 2b and 2c). The final separation was achieved by density-gradient centrifugation, which was performed after the gel-chromatography step. Under the conditions used 95% of PGS will be recovered at densities lower than 1.5 g/ml and the majority of free

Fig. 2. Gel chromatography on Superose 6 of radioactivity labelled proteoglycan material from the medium of fibroblast cultures

Proteoglycans were prepared from fibroblast cultures treated with β -D-xyloside in the presence of radioactive precursors. The proteoglycans were subjected to ion-exchange chromatography followed by gel chromatography on a Superose 6 HR 10/30 column eluted with 4 M -guanidinium chloride/ 0.05 M -sodium acetate buffer, pH 5.8. The individual peaks were pooled as indicated by the bars in the Figures. V_0 and V_t denote the void and total volumes of the column. (a) Proteoglycan fraction from control cultures. The inset is an analytical chromatogram on Mono Q of the peak in the void volume to estimate the amount of heparan sulphate proteoglycan (first peak). The chromatogram was analysed with a Radiomatic Flo-One-Beta flow detector. Radioactive components obtained from cultures incubated with a p -nitrophenyl β -D-xyloside concentration of (b) $6 \mu\text{M}$, (c) $12.5 \mu\text{M}$, (d) $60 \mu\text{M}$ and (e) $1000 \mu\text{M}$. —, $[^3\text{H}]$ Glucosamine; ----, $[^{35}\text{S}]$ sulphate.

polysaccharide of normal chain length will be recovered at densities higher than 1.6 g/ml [33]. In fractions chromatographing as PGS on the Superose column (Fig. 2b) equal amounts

Table 1. Distribution of sulphated glycosaminoglycans in proteoglycan and on β -D-xyloside at various concentrations of *p*-nitrophenyl β -D-xyloside

Proteoglycans and Xyl-GAG were prepared from the medium as described in the Experimental section. The identity of the various side chains was determined after treatment with chondroitin ABC lyase and HNO_2 . The data are given as percentages of total ^{35}S -labelled glycosaminoglycans formed. Abbreviation: N.D., not determined.

Concn. of β -D-xyloside (μM)	GAG synthesis		Fraction (%)			
	Percentage of control	Percentage of total in culture				
			HSPG	PGL	PGS	Xyl-GAG
Medium						
0 (control)	100	60	17	33	50	0
2	126	N.D.	13	26	26	35
6	152	83	7	16	22	55
12.5	292	N.D.	1	2	3	94
60	472	93	0	0	0	100
1000	434	93	0	0	0	100
3000	229	N.D.	0	0	0	100
Cell layer						
0	100	40	22	48	32	0
6	80	17	23	16	24	32
60	84	7	12	12	20	56
1000	79	7	11	11	19	59

of xyloside-GAG and PGS were noted (Fig. 3a). The material chromatographing as free chains contained mainly (> 90%) xyloside-GAG (Fig. 3b). In Table 1 the relative production of different proteoglycans and xyloside-GAG at different xyloside concentrations is summarized. The amount of glycosaminoglycans recovered in the cell layer when xyloside was present represented only a few per cent of total glycosaminoglycans in the cell culture compared with 40% in control cultures (Table 1). Some proteoglycan material was present in the cell layer at all xyloside concentrations. With increasing xyloside added to the medium the amount of intact PGL, PGS and HSPG gradually decreased to zero but that of xyloside-bound galactosaminoglycan increased. In the medium at low concentration of xyloside (2–6 μM) almost equal amounts of proteoglycan and xyloside-GAG were produced. At concentrations higher than 12.5 μM the formation of polysaccharide on the core protein was inhibited and only xyloside-bound galactosaminoglycans were formed. At a xyloside concentration of 1000 μM a maximum of net synthesis of GAG of 4 times over the control was recorded.

Only galactosaminoglycans were formed on the xyloside primer. This was confirmed by the observation that no depolymerization was obtained by nitrous acid treatment (results not shown). The co-polymeric structure of the galactosaminoglycan chains of the large and the small proteoglycans and the polysaccharide formed on the β -D-xyloside polymer was investigated by using chondroitin AC-II lyase digestion followed by separation of the degradation products on Sephadex G-50. The resulting oligosaccharide pattern (Fig. 4) was used to calculate the content of D-glucuronosyl residues and the results are summarized in Table 2. With increasing xyloside concentration the relative glucuronic acid content of the galactosaminoglycan chains of PGS and xyloside-GAG increased, whereas that of PGL remained constant. In the control experiment (no xyloside present) the relative glucuronic acid content in PGL was 91% and in PGS 35% (Figs. 4a and 4b and Table 2). At low xyloside concentrations (6 μM), when PGL, PGS and xyloside-GAG were

Fig. 3. Density-gradient centrifugation of small proteoglycans and Xyl-GAG

Proteoglycans and Xyl-GAG was prepared from a cell culture, which had been incubated with 6 μM *p*-nitrophenyl β -D-xyloside, by using ion-exchange and gel chromatography. Two fractions, (a) PGS and (b) Xyl-GAG, were pooled as indicated in Fig. 2(b) and subjected to density-gradient centrifugation with a starting density of 1.54 g/ml as described in the Experimental section. Fractions corresponding to PGS and Xyl-GAG were pooled as indicated by the bars. ---, [^{35}S]sulphate; ●—●, density.

formed, the relative glucuronic acid content of PGS increased from 35 to 53%, that of PGL was not changed and xyloside-GAG contained 62% glucuronic acid (Figs. 4a, 4c and 4d and Table 2), which is intermediate between that of PGL and PGS. This degradation pattern after chondroitin AC-II lyase treatment of xyloside-GAG produced at low xyloside concentrations (6 μM) was compared with a computer-simulated pattern obtained for a mixture of chondroitin AC-II lyase-digested polysaccharide chains from PGL and PGS obtained from the same xyloside concentration to give the same glucuronic acid content (62%) (Fig. 4c). This pattern was different, the glycosaminoglycan synthesized on the xyloside containing substantially more intermediate-sized fragment containing L-iduronosyl residues. When PGS from control cultures was used for the calculation the difference is even greater. This excludes the possibility that the high proportion of D-glucuronosyl residues of the xyloside-bound polysaccharide could be the result only of a changed ratio of ordinary PGL and PGS side chains being synthesized on the xyloside primer. The presence of some ordinary PGL or PGS side chains on the xyloside cannot, however, be excluded. At intermediate xyloside concentration (60 μM), only xyloside-GAG was formed with an intermediate content of glucuronic acid (68%; Fig. 4e and Table 2). At high xyloside concentrations (1000 and 3000 μM), when no proteoglycans are formed, the

Fig. 4. Gel chromatography on Sephadex G-50 of galactosaminoglycan fractions after treatment with chondroitin AC-II lyase

Proteoglycans and Xyl-GAG were prepared by ion-exchange chromatography, gel chromatography and, at xyloside concentrations lower than $60 \mu\text{M}$, density-gradient centrifugation. The proteoglycan fractions were then digested with papain and the chains recovered by ion-exchange chromatography. Free chains and Xyl-GAG were finally treated with HNO_2 and the degraded heparan sulphate fragments were removed by gel chromatography. The resulting galactosaminoglycans were digested with chondroitin AC-II lyase and subjected to gel chromatography on a column ($1.0 \text{ cm} \times 120 \text{ cm}$) of Sephadex G-50. The column was eluted with $0.5 \text{ M}-(\text{NH}_4)_2\text{CO}_3$ at a rate of 6 ml/h . The elution positions of standard oligosaccharides are indicated. Galactosaminoglycans were from: (a) PGL from control cultures; (b) PGS from control cultures; (c) Xyl-GAG from cultures incubated with $6 \mu\text{M}$ -xyloside (—) and a computer calculation performed by using the PGL and PGS oligosaccharide patterns obtained at $6 \mu\text{M}$ -xyloside to give the same relative glucuronic acid content as that of the Xyl-GAG obtained from the same experiment (●—●); (d) PGS from cultures incubated with $6 \mu\text{M}$ -xyloside; (e) Xyl-GAG from cultures incubated with $60 \mu\text{M}$ -xyloside; (f) Xyl-GAG from cultures incubated with $1000 \mu\text{M}$ -xyloside.

glucuronic acid content of the xyloside-GAG was approx. 80% and similar to that of PGL (Figs. 4a and 4f and Table 2). Few if any fragments of sizes larger than octasaccharides were found after chondroitin AC-II lyase digestion (Fig. 4f), thus excluding the possibility of significant amounts of chains containing high amounts of iduronic acid.

To study whether the observed effect on the co-polymeric structure changed with time, a subset of experiments was designed in which the effect of varying the time of preincubation with

β -D-xyloside and the labelling periods (4 h preincubation/4 h labelling, 20 h preincubation/4 h labelling and 24 h preincubation/24 h labelling) was studied. A concentration of 1 M - β -D-xyloside was chosen since the effect on the glucuronic acid content was most marked at this concentration (see above). The effect on the GAG synthesis was the same at all combinations tested, that is no proteoglycan was formed and the glucuronic acid content was high and similar to that of PGL. This was almost identical with the result obtained without preincubation.

Table 2. Glucuronic acid content of the polysaccharide chains of proteoglycans and β -D-xyloside in the medium formed at different concentrations of *p*-nitrophenyl β -D-xyloside

Proteoglycans and Xyl-GAG were prepared from the medium as indicated in the Experimental section. The amount of D-glucuronosyl residues was calculated as ^{35}S -labelled products after degradation with chondroitin AC-II lyase followed by gel chromatography as described in the Experimental section. Abbreviation: N.A., not analysed. Very similar data were obtained when ^3H label of the oligosaccharides was used for calculation.

Concn. of β -D-xyloside (μM)	Glucuronic acid content (%)		
	PGL	PGS	Xyl-GAG
0 (control)	91	35	0
6	86	53	62
12.5	N.A.	N.A.	63
60	N.A.	N.A.	68
1000	N.A.	N.A.	84
3000	N.A.	N.A.	82

The sulphation of the galactosaminoglycans was determined by separation on a Lichrosorb- NH_2 column after a combined chondroitin AC-II lyase and chondroitin ABC lyase digestion. With increasing xyloside concentrations the degree of 4-sulphation of the xyloside-GAG polymer decreased and non- and 6-sulphation increased (Table 3). The xyloside-bound GAG synthesized at high xyloside concentration (3000 μM) had a relatively large proportion of non-sulphated disaccharides. Furthermore the relative amount of 6-sulphated disaccharides is similar to that of the large proteoglycan but different from the chains of the small proteoglycans, which predominantly have 4-sulphated disaccharide moieties. At a concentration of 60 μM - β -D-xyloside and lower all polysaccharides formed on the β -D-xyloside had a high content of 4-sulphate and low amounts of non- and 6-sulphated residues, which is rather like the content of PGS.

The change in structure of the xyloside-bound polysaccharide and of the dermatan sulphate chains of the PGS proteoglycan with increasing xyloside concentration indicates that the addition of xyloside affects the modification process. Therefore the specific activities (activity/mg of cell protein) of uronosyl C-5-epimerase and sulphotransferases were studied in microsomal fractions from control cultures and cultures treated with *p*-nitrophenyl β -D-xyloside with exogenously added substrates. No decrease in specific activity of C-5-epimerase was noted in cultures treated with a low concentration of xyloside, whereas on treatment with higher concentrations the specific activity decreased by 40–50 % (Table 4). The sulphotransferase activity with chondroitin as substrate was only diminished in cultures treated with a very high xyloside concentration (3000 μM), which may reflect a derangement of the glycosaminoglycan synthesis system. With dermatan as substrate, which accepts sulphate in position 4 of the *N*-acetylgalactosamine residue, a 40–50 % decrease in the specific activity after treatment of the culture with β -D-xyloside concentrations of 60 μM and higher was noted. Thus xyloside addition resulted in a decrease in the specific activity of the enzymes that produce the co-polymeric structure of the small proteoglycans (PGS), especially that of the 4-sulphotransferase. In a control experiment, in which *p*-nitrophenyl β -D-xyloside was added in corresponding amounts to control microsomal fractions, no effect on any of the polymer-modifying enzymes was recorded. Furthermore no effect on overall protein synthesis was seen in the presence of up to 1 mM-xyloside.

Table 3. Sulphation pattern of galactosaminoglycan of PGL, PGS and β -D-xyloside formed at different concentrations of β -D-xyloside

The various components were isolated from the culture medium as described in the Experimental section. They were then subjected to degradation with chondroitin ABC lyase and chondroitin AC-II lyase followed by separation of the resulting disaccharides on a Lichrosorb column as described in the Experimental section.

Concn. of β -D-xyloside (μM)	Fraction	Disaccharide (%)			
		Di-0S	Di-4S	Di-6S	Di-Di-S
0 (control)	PGL	4	29	67	0
	PGS	3	87	8	2
6	Xyl-GAG	5	79	15	0
60	Xyl-GAG	6	77	16	0
1000	Xyl-GAG	9	41	50	0
3000	Xyl-GAG	15	33	52	0

Table 4. Change in the specific activity of polymer-modifying enzymes by treatment of fibroblast cultures with *p*-nitrophenyl β -D-xyloside

Microsomal fractions were prepared from untreated and xyloside-treated fibroblasts and assayed for uronosyl C-5-epimerase and sulphotransferase activities as described in the Experimental section. The activity of the sulphotransferase was determined with chondroitin, which accepts sulphate on C-4 and C-6, and dermatan, which accepts sulphate mainly on C-4 of the *N*-acetylgalactosamine moiety. The data are presented as percentages of control as several different preparations were used with different enzyme activity. Addition of 1000 μM - β -D-xyloside to the incubation with microsomal fraction did not affect the enzyme activity of the control preparation.

Concn. of β -D-xyloside in the culture (μM)	Enzyme (% of control)		
	Uronosyl C-5-epimerase	Sulphotransferase	
		Chondroitin	Dermatan
60	100	100	52
1000	50	100	59
3000	61	79	56

DISCUSSION

Our results confirm earlier studies [21,22] that addition of xyloside increases the net synthesis of glycosaminoglycan and inhibits the formation of proteoglycans. It is interesting to note that at all concentrations some proteoglycan material is still present in the cell layer, thus reflecting the necessity of these compounds for the integrity of the cell. The stimulation was restricted to an increased synthesis of galactosaminoglycans. The maximum increase was 4–5 times that of control. At high concentrations of xyloside a smaller stimulation was noted as well as formation of shorter chains, which indicates that the system is deranged. The enhancement of synthesis is probably an effect of the molar excess of the xyloside primer, which effectively competes with the core proteins for the galactosyltransferase.

No heparan sulphate was formed on the xyloside primer. This probably reflects the fact that the galactosyltransferases involved in the formation of the heparan sulphate linkage region have a much higher affinity for their natural substrate [34,35] than those involved in the galactosaminoglycan synthesis. *p*-Nitrophenyl β -D-xyloside can only serve as a primer for heparan sulphate when synthesis of its core protein is inhibited [36]. The decreased secretion of HSPG into the medium at high xyloside con-

centrations probably reflects diminished synthesis due to depletion of its precursor pool, which is instead used for the increased synthesis of xyloside-GAG.

Not only the synthesis but also the structure of galactosaminoglycans formed both on the β -D-xyloside and on the 43000- M_r core of the small proteoglycans were affected by incubation in the presence of *p*-nitrophenyl β -D-xyloside. In particular a decrease in the content of L-iduronic acid and 4-sulphated *N*-acetylgalactosamine residues was noted. At high xyloside concentration (1000 and 3000 μ M), when only xyloside-GAG was produced, the proportion of D-glucuronic acid and 6-sulphate content was high and similar to that of ordinary PGL chains. A distinct increase in non-sulphated residues was also noted (Table 3). At low xyloside concentration the structure of the glycosaminoglycans on the xyloside and PGS differed from those of ordinary PGL and PGS by having a glucuronic acid content higher than that of PGS but lower than PGL. However, the sulphation pattern of the xyloside-GAG at low xyloside concentrations was similar to that of ordinary PGS side chains (Table 3). Different possibilities to achieve these differences can be envisaged. The synthesis of the various proteoglycans may occur in different compartments and the xyloside primer may be an initiator of polysaccharide chains in both the PGL and PGS compartments. The observed continuous change in structure, especially of the relative L-iduronic acid content, may be a result of different proportions of polysaccharides of the PGL- and PGS-type on the xyloside. This is, however, unlikely, since the computer-simulated chondroitin AC-II lyase degradation pattern of a mixture of PGL and PGS chains was different from the actual pattern, which has more intermediate-sized L-iduronosyl-containing regions (Fig. 4c). Furthermore the sulphation pattern (Table 3) was similar to that of PGS. It thus seems likely that the xyloside primer mainly initiates chains in the PGS compartment, since the iduronic acid content and the degree of 4-sulphation were high. The continuous decrease in L-iduronic acid and 4-sulphate content with increasing xyloside concentration may be achieved by a xyloside-induced decrease in the amount or activity of the polymer-modifying enzymes. A decrease in enzyme amount is unlikely, since no change in overall protein synthesis was found with increasing amounts of xyloside and the presence of xyloside did not produce a gradual change of glucuronic acid content with varied time of preincubation with xyloside. In control microsomal preparations the presence of xyloside did not change the measured enzyme activity of C-5-epimerase or 4-sulphotransferase. The enzyme activity must be modulated by some other mechanism. We propose that the PGS core protein, besides being important for sorting the growing proteoglycan to the right enzyme complex, may have a role as a modulator of the activity of these enzymes. The observed decrease in epimerase and in the 4-sulphotransferase activity would then be the result of a lack of stimulation due to a decrease in or absence of the PGS core protein in this region of the Golgi apparatus.

Further work has, however, to be performed before it can be decided if regulation of enzyme activity by the protein core is the major mechanism by which the co-polymeric structure of the proteoglycan polysaccharide chains is achieved.

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