Biosynthesis and secretion of dermatan sulphate proteoglycans in cultures of human skin fibroblasts

Lars CÖSTER, Ingemar CARLSTEDT, Anders MALMSTRÖM and Bengt SÄRNSTRAND
Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund, Sweden

(Received 28 November 1983/Accepted 24 February 1984)

Fibroblasts in culture were incubated with [^3H]leucine and [^{35}S]sulphate for 1–24 h. A large glucuronic acid-rich and a small iduronic acid-rich dermatan sulphate proteoglycan were isolated with the use of isopycnic density-gradient centrifugation, ion-exchange and gel chromatography. After 3 h the accumulation in the cell layer of the small proteoglycan reached a steady state, whereas the large one continued to increase, albeit more slowly. In the medium both proteoglycans accumulated 'linearly', although the large one appeared somewhat later than the small one. The composition of the polysaccharide chains and the size of the protein cores did not vary during the experiment. The two proteoglycans were synthesized at approximately similar rates, but were distributed differently in the culture. The small proteoglycan was mainly confined to the medium, whereas the large one was found in the medium as well as in a cell-associated pool. There was an intracellular accumulation of iduronic acid-rich dermatan sulphate as free polysaccharides.

Fibroblasts in culture synthesize and secrete several different dermatan sulphate and heparan sulphate proteoglycans (Kresse *et al.*, 1975; Cöster *et al.*, 1979; Carlstedt *et al.*, 1981; Vogel & Peterson, 1981). Two different dermatan sulphate proteoglycans, a large one containing glucuronic acid-rich dermatan sulphate and a small one containing iduronic acid-rich dermatan sulphate, were isolated by Cöster *et al.* (1979). The two proteoglycans have different protein cores (Carlstedt *et al.*, 1981); the small proteoglycan has a protein core similar in size to that of the small dermatan sulphate proteoglycan from sclera (Cöster & Fransson, 1981), skin (Damle *et al.*, 1982) and the uterine cervix (Uldbjerg *et al.*, 1983). The cell layer contains, in addition to dermatan sulphate proteoglycans, a heparan sulphate proteoglycan, which is considerably larger than that isolated from the medium (Carlstedt *et al.*, 1981). Fibroblasts from various sources differ with regard to the relative amounts of the large and the small dermatan sulphate proteoglycans. Furthermore, the propor-

tions of these proteoglycans can be manipulated by varying the concentration of serum (Vogel & Sapién, 1982) or by the addition of glucocorticoids (Särnstrand *et al.*, 1982). The present study was undertaken to obtain basic information about the kinetics of synthesis and secretion of the two dermatan sulphate proteoglycans. The size of the protein core and the co-polymeric structure of the dermatan sulphate side chains were followed with time to reveal major modifications of the primary structure.

Experimental

Materials

Guanidinium chloride (technical grade), urea and trypsin (EC 3.4.21.4; type III) were obtained from Sigma Chemical Co. Solutions of guanidinium chloride were purified by chromatography on Celite/activated charcoal. Stock solutions of urea (8M) were passed through a bed of a mixed ion-exchange resin before use. Chondroitin AC lyase (EC 4.2.2.5) and chondroitin ABC lyase (EC 4.2.2.4) were purchased from Miles. L-[4,5- ^3H]Leucine (71 Ci/mmol) and [^{35}S]sulphate (carrier free) were products of Amersham.

Abbreviations used: PG-I, large glucuronic acid-rich dermatan sulphate proteoglycan; PG-II, small iduronic acid-rich dermatan sulphate proteoglycan; PGC, proteoglycans of the cell layer; PGM, proteoglycans of the medium; SDS, sodium dodecyl sulphate.

Isolation of proteoglycans

Human embryonic skin fibroblasts were established and maintained in culture as described previously (Malmström *et al.*, 1975). At 3 days before the addition of the radioactive precursors, the medium was replaced with a sulphate-poor one (Cöster *et al.*, 1979). Cultures (passages 5–15) were used with sulphate- and leucine-poor medium (Cöster *et al.*, 1979) containing 20 μ Ci of [35 S]sulphate/ml and 10 μ Ci of [3 H]leucine/ml. After the specified time periods, the medium was poured off and the cell layer rinsed with cold phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM-Na₂HPO₄/1 mM-KH₂PO₄ buffer, pH 7.4). Proteoglycans were isolated from the medium and the cell layer as described in detail by Carlstedt *et al.* (1981). In brief, guanidinium chloride supplemented with proteinase inhibitors was added to the medium, whereas the cell layer was extracted twice with this solution. After concentration, the extracts of the cell layer and the medium were subjected to isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride (initial density 1.35 g/ml). Proteoglycans were pooled as two major fractions. Materials with a buoyant density higher than 1.41 g/ml and between 1.31 and 1.41 g/ml are referred to below as the high-density and the low-density fractions respectively. After dialysis against 6 M-urea/0.01 M-Na₂EDTA/5 mM-N-ethylmaleimide/0.2 M-sodium acetate buffer, pH 5.8, the fractions were subjected to ion-exchange chromatography on DEAE-cellulose (DE-52). The columns (1 cm $\times$ 2 cm) were eluted stepwise with 6 ml of 6 M-urea/0.01 M-Na₂EDTA/5 mM-N-ethylmaleimide/0.2 M-sodium acetate buffer, pH 5.8, followed by 6 ml of 6 M-urea/0.01 M-Na₂EDTA/0.5 M-sodium acetate buffer, pH 5.8. The proteoglycans were finally recovered by eluting with 6 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8. All buffers used after the dialysis step were supplemented with bovine serum albumin (5 μ g/ml). The proteoglycans were separated into different size classes by gel chromatography on Sepharose CL-2B as described in the legend to Fig. 1, and were finally purified by repeating the ion-exchange-chromatography step as described above. The amount of the various proteoglycans accumulating as a function of time was calculated from data obtained after the final ion-exchange-chromatography step, corrected for differences in recovery during purification. In some experiments the cell layer was digested with trypsin (0.4 g/100 ml) in phosphate-buffered saline for 10 min after rinsing the cells with this buffer. The digestion was terminated by the addition of di-isopropyl phosphorofluoridate (final concn. 5 mM). The cells were collected by

centrifugation at 600g for 10 min, washed with phosphate-buffered saline and extracted with guanidinium chloride, and proteoglycans were isolated by using isopycnic density-gradient centrifugation and gel chromatography as described above. The proteoglycans released from the cell layer by trypsin were subjected to ion-exchange chromatography on DEAE-cellulose (DE-52) after dialysis against 6 M-urea/0.01 M-Na₂EDTA/5 mM-N-ethylmaleimide/0.05 M-sodium acetate buffer, pH 5.8. The proteoglycans were eluted from the column (1 cm $\times$ 2 cm) with a linear gradient of sodium acetate in 6 M-urea (mixing vessel, 30 ml of 6 M-urea/0.2 M-sodium acetate buffer, pH 5.8; reservoir, 30 ml of 6 M-urea/1.5 M-sodium acetate buffer, pH 5.8). The buffers were supplemented with bovine serum albumin (5 μ g/ml). Two peaks were pooled (0.74–0.87 M- and 0.97–1.2 M-sodium acetate). These components were finally chromatographed separately on a column of Sepharose CL-2B.

Analytical and degradative methods

Polyacrylamide-gel electrophoresis in SDS was conducted on gels (T = 8% and C = 2.5% with the notation of Hjertén, 1962) as described by Neville (1971) after digestion of the proteoglycans with chondroitin ABC lyase. Before application the samples were heated at 100°C for 3 min. After electrophoresis the gels were cut into 2 mm slices, which were dissolved in H₂O₂ before addition of the scintillator.

The polysaccharide side chains were isolated after papain digestion and ion-exchange chromatography (Carlstedt *et al.*, 1981). HNO₂ treatment was performed at pH 1.5 as described by Shively & Conrad (1976). Heparan sulphate fragments were separated from dermatan sulphate by chromatography on Sephadex G-50. Periodate oxidation was performed in 0.05 M-sodium formate buffer, pH 3.0, followed by alkaline elimination as described by Fransson & Carlstedt (1974). Digestions of polysaccharides with chondroitin AC lyase and chondroitin ABC lyase were conducted in 0.05 M-sodium acetate buffer, pH 6.0, and in 0.2 M-Tris/acetate buffer, pH 7.4, respectively (Carlstedt *et al.*, 1981). The fragments were separated by chromatography on Sephadex G-50. Digestions of proteoglycans with chondroitin ABC lyase were performed with 0.01 unit of enzyme (1 unit catalyses the formation of 1 μ mol of disaccharide/min) in 0.2 M-Tris/acetate buffer, pH 7.3, containing 0.01 M-Na₂EDTA/5 mM-N-ethylmaleimide/1 mM-di-isopropyl phosphorofluoridate.

Results

After 24h incorporation of [^3H]leucine and [^{35}S]sulphate, radioactivity was found in several dermatan sulphate and heparan sulphate proteoglycan populations (Figs. 1a–1d), the composition of which has been reported previously (Carlstedt *et al.*, 1981). The large dermatan sulphate proteoglycans of the medium (PGM-I; Figs. 1a and 1c) and the large proteoglycans from the cell layer (PGC-I; Fig. 1b) were very similar, and are composed of a relatively large protein core substituted with glucuronic acid-rich dermatan sulphate side chains. The protein core of the small dermatan sulphate proteoglycan of the medium (PGM-II) has apparent M_r 47000 and is substituted with iduronic acid-rich dermatan sulphate. The small dermatan sulphate proteoglycan from the medium (PGM-II; Fig. 1a) of the high-density fraction is contaminated with 5–10% of heparan sulphate proteoglycans, whereas the correspond-

ing fraction from the cell layer (PGC-II; Fig. 1b) contains predominantly free dermatan sulphate chains (see below). The large proteoglycan of the low-density fraction from the cell layer (Fig. 1d) is a heparan sulphate proteoglycan. Less than 5% of radiolabelled proteoglycan remained with the residue after extraction of the cell layer (Cöster *et al.*, 1979).

The accumulation of [^{35}S]sulphate and ^3H into the large (PG-I) and the small dermatan sulphate proteoglycans (PG-II) was monitored in the whole culture (medium and cells together) for 24h (Fig. 2). The proteoglycan PG-I contains more ^{35}S than ^3H radioactivity at all periods of time, whereas the reverse is true for the small one.

The copolymeric structure of the polysaccharide side chains of the proteoglycans PG-I and PG-II obtained from the medium as well as the cell layer at 3 and 24h, respectively, was examined by periodate oxidation–alkaline elimination, followed by gel chromatography on Sephadex G-50. The

Fig. 1. Gel chromatography on Sepharose CL-2B of proteoglycans from the medium (a and c) and the cell layer (b and d). Proteoglycans were isolated from the medium and the cell layer as described in the Experimental section. Proteoglycans with a buoyant density higher than 1.41 g/ml (a and b) and with a buoyant density between 1.3 and 1.4 g/ml (c and d) were pooled from the CsCl density gradient. The column (1.8 cm $\times$ 145 cm) was eluted with 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8, containing 5 μg of bovine serum albumin/ml. Fractions (2.8 ml) were collected and pooled as shown by the bars. —, [^{35}S]Sulphate; ----, ^3H . The data shown are from the 24 h time point. The large less-retarded proteoglycan in panel (d) is a heparan sulphate proteoglycan. V_0 and V_t respectively denote the void volume and the total volume of the column.

Fig. 2. Accumulation of the proteoglycans PG-I and PG-II in the cell culture

Dermatan sulphate proteoglycans were isolated as described in the Experimental section. PG-I represents the sum of such proteoglycans from the low- and high-density fractions of the medium as well as the high-density fraction of the cell layer. PG-II represents the sum of such proteoglycans from the high- and low-density fractions from the medium as well as the cell layer. —, ³⁵S; ----, ³H; ○, PG-I; ●, PG-II.

dermatan sulphate of the proteoglycan PG-I was rich in glucuronic acid at both times, and no difference in copolymeric structure could be detected (Table 1). The proteoglycan PG-II contained iduronic acid-rich dermatan sulphate during the whole experiment (Table 1).

The protein core of the proteoglycan PG-I from the medium and the cell layer and that of the proteoglycan PG-II from the medium were studied after digestion with chondroitin ABC lyase by using polyacrylamide-gel electrophoresis in SDS. The migration of the core from PGC-I and PGM-I was the same, and no change could be detected between 3 and 24 h (Figs. 3a, 3b, 3d and 3e). The proteoglycan PG-II has a considerably smaller core (M_r 47000), and no change in migration could be detected during the experiment (Figs. 3c and 3f). When the fraction PGC-II of the cell layer was studied, only a minute amount of protein core could be detected (see below).

After 3 h the accumulation of PGC-II reached a

Table 1. Copolymeric structure of dermatan sulphate derived from the proteoglycans PG-I and PG-II at different times of incubation

Proteoglycans were isolated after 3 and 24 h of incubation and dermatan sulphate was obtained as described in the Experimental section. The polysaccharides were subjected to periodate oxidation-alkaline elimination, followed by separation of the split products by gel chromatography on Sephadex G-50. The amount of L-iduronic acid was calculated as described by Malmström *et al.* (1975). The numbers represent iduronic acid-containing disaccharides as a percentage of total disaccharides.

Incubation time (h)	Disaccharides (%)	
	3	24
Cell layer		
Large proteoglycan (PG-I)	29	29
Small proteoglycan (PG-II)	40	46
Medium		
Large proteoglycan (PG-I)	22	21
Small proteoglycan (PG-II)	41	44

plateau, whereas PG-I continued to increase, but at a lower rate (Figs. 4a and 4b). In the medium, however, the amounts of the two radioactive proteoglycans increased linearly for 24 h (Figs. 4a and 4b). The proteoglycan PG-II seemed, however, to reach the medium faster than did PG-I (Fig. 4c).

To study the relation between synthesis and secretion, the amount of radioactivity of the proteoglycans PG-I and PG-II in the cell layer and in the medium was compared (Fig. 4). The initial accumulation of the proteoglycan PG-I was higher in the cell layer than in the medium, but was exceeded by that in the medium after 9 h (Fig. 4a). In contrast, the amount of the proteoglycan PG-II in the medium exceeded that of the cell layer already after 3–5 h (Fig. 4b). The most likely explanation is a different distribution of the two proteoglycans. To study this further, the cell layer was digested with trypsin to remove cell-surface-associated proteoglycans. The trypsin-released ³⁵S-labelled material was resolved into two peaks by using ion-exchange chromatography (results not shown). Both components behaved as free chains on Sepharose CL-2B. The major part (60%) of the first peak from the ion-exchange-chromatography step was heparan sulphate, whereas the remainder and the entire second peak contained glucuronic acid-rich dermatan sulphate similar to that obtained from PG-I, determined as described in the legend to Table 1. Chromatography on Sepharose CL-2B of the proteoglycans isolated from the cell residue after the trypsin treatment showed that no large proteoglycan (PG-I type) was present and that most of the ³⁵S radioactivity

Fig. 3. Polyacrylamide-gel electrophoresis in SDS of protein cores from the proteoglycans PG-I and PG-II. Proteoglycans were prepared from the medium and the cell layer after 3 h (a-c) and 24 h (d-f) of incorporation and digested with chondroitin ABC lyase as described in the Experimental section. (a) and (b) The proteoglycan PGC-I from the cell layer; (b) and (e) the proteoglycans PGM-I and (c) and (f) PGM-II from the high-density fractions of the medium. The position of the dye front is indicated by the arrow.

chromatographed as PG-II. This was corroborated by chondroitin AC lyase digestion of the chains, which showed that only 21% of the uronosyl residues were glucuronic acid, a value typical of the proteoglycan PG-II (Carlstedt *et al.*, 1981). The results strongly suggest that the trypsin-released (pericellular) dermatan sulphate proteoglycans are of the PG-I type and that the cell-associated (intracellular) dermatan sulphate proteoglycan is of the PG-II type. The ratios of ^3H to ^{35}S of the proteoglycan PG-II of the cell layer and the medium are very different (Fig. 4b). Furthermore, on Sepharose CL-2B (Figs. 1a and 1d) PGC-II was slightly more retarded than PGM-II, suggesting that the

former may comprise free dermatan sulphate chains rather than proteoglycans. This was confirmed by polyacrylamide-gel electrophoresis in SDS of the proteoglycans from the cell layer and the medium. The two components have different migration distances (Figs. 5a and 5c), and after digestion with chondroitin ABC lyase a protein core with apparent M_r 47000 was found in the proteoglycan from the medium, but was absent from the corresponding fraction from the cell layer (Figs. 5b and 5d). The low-density fraction from the cell layer contains a small amount of the proteoglycan PG-II together with free chains (results not shown).

Fig. 4. Accumulation of the proteoglycans PG-I (a) and PG-II (b) in the cell layer and in the medium

Proteoglycans were prepared from the cell layer and the medium as described in the Experimental section. —, [^{35}S]Sulphate; ---, ^3H . (a): ○, PGC-I from the cell layer; ●, combined PGM-I from high- and low-density fractions of the cell layer; □, combined PGM-II of the high- and low-density fractions of the medium. (b): ■, combined PGC-II from the high- and low-density fractions of the cell layer; □, combined PGM-II of the high- and low-density fractions of the medium. (c): ●, Sum of the radioactivity of PGM-I in high- and low-density fractions from the medium; □, sum of the radioactivity of PGM-II in the high- and low-density fractions of the medium.

Discussion

Fibroblasts in culture synthesize and secrete two different dermatan sulphate proteoglycans. This work shows that the two proteoglycans are synthesized at similar rates and that the composition of the galactosaminoglycan chains and the size of the protein cores were constant for both proteoglycans during the period of time studied. With the use of radioactive precursors it is, however, difficult to determine the actual rates of secretion, as the specific radioactivity of the precursor and product could vary and are not known. Two radioisotopes were used to make interpretations more valid. The $^{35}\text{S}/^3\text{H}$ ratios of the two proteoglycans are different, which may suggest different specific radioactivities. However, the sulphate/leucine ratios of the two scleral dermatan sulphate proteoglycans (Cöster & Fransson, 1981) which correspond to PG-I and PG-II differed to approximately the same extent as the $^{35}\text{S}/^3\text{H}$ ratios of the two radioactive proteoglycans.

Although the apparent amounts of the two proteoglycans in the culture are similar, their distributions are different. The small proteoglycan is mainly confined to the medium and to an intracellular compartment, whereas the larger one is found in the medium and associated with the cell surface. One possible explanation is that there are receptors for the larger proteoglycan on the cell surface. Hyaluronic acid in the pericellular compartment may be a candidate, as the proteoglycan PG-I, in contrast with PG-II, interacts with hyaluronic acid (Norling *et al.*, 1978; Cöster *et al.*, 1979). Hyaluronic acid also interacts with fibronectin (Hedman *et al.*, 1982), and the larger proteoglycan may thus be indirectly associated with fibronectin.

The accumulation of the proteoglycans PG-I and PG-II in the cell layer is complex, owing to the presence of several pools. The precursors are first incorporated into the proteoglycans in the biosynthesis pool. The latter is probably very small, as only minute amounts of the two proteoglycans could be detected intracellularly. The larger ones (PG-I) are subsequently secreted to the cell layer, where they may be retained by specific interactions with other cell-surface components. The proteoglycan PG-II is apparently secreted directly into the medium, as only a small amount was found in the cell-surface-associated pool and because its accumulation in the medium was 'linear' from the start of the experiment. The remainder of the small proteoglycans in the biosynthesis pool might be diverted to a degradation pool, to account for the free dermatan sulphate chains found in the intracellular compartment. Alternatively the intracellular accumulation of dermatan sulphate chains

Fig. 5. Polyacrylamide-gel electrophoresis of the proteoglycan PG-II from the cell layer (a and b) and from the medium (c and d) before and after digestion with chondroitin ABC lyase

Proteoglycans were prepared from the cell layer and the medium after 3 h of label incorporation as described in the Experimental section. The proteoglycan PGC-II from the high-density fraction of the cell layer was subjected to electrophoresis before (a) and after (b) digestion. The proteoglycan PGM-II of the low-density fraction of the medium was subjected to electrophoresis before (c) and after (d) digestion. —, [^{35}S]Sulphate; - - -, ^3H . The dye front is indicated by the arrow.

of the PG-II type intracellularly may be the result of pinocytosis of proteoglycans from the cell surface and/or from the medium. If this is the case, it must be postulated that pinocytosis is specific for the proteoglycan PG-II or that pinocytosis of the proteoglycan PG-I is followed by a very rapid degradation. It is, in our opinion, more likely that the accumulation is a result of a direct transport from

the biosynthesis pool to a degradation pool, as a large amount is detectable already from the start of the experiment. The origin and function of this pool must await further investigations.

This investigation was supported by grants from the Medical Research Council (567, 537), Greta och Johan Kocks Stiftelse, Albert Österlunds Stiftelse, Gustav V

80-års fond and the Medical Faculty of the University of Lund. The expert technical assistance of Lena Åberg and Birgitta Jönsson is acknowledged.

References

- Carlstedt, I., Cöster, L. & Malmström, A. (1981) *Biochem. J.* **197**, 217–225
- Cöster, L. & Fransson, L.-Å. (1981) *Biochem. J.* **193**, 143–153
- Cöster, L., Carlstedt, I. & Malmström, A. (1979) *Biochem. J.* **183**, 669–681
- Damle, S. P., Cöster, L. & Gregory, J. D. (1982) *J. Biol. Chem.* **257**, 5523–5527
- Fransson, L.-Å. & Carlstedt, I. (1974) *Carbohydr. Res.* **36**, 349–358
- Hedman, K., Johansson, S., Vartio, T., Kjellén, L., Vaheri, A. & Höök, M. (1982) *Cell* **28**, 663–671
- Hjertén, S. (1962) *Arch. Biochem. Biophys. Suppl.* **1**, 147–151
- Kresse, N., von Figura, K., Buddecke, E. & Fromme, H. G. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 921–941
- Malmström, A., Carlstedt, I., Åberg, L. & Fransson, L.-Å. (1975) *Biochem. J.* **151**, 477–489
- Neville, D. N. (1971) *J. Biol. Chem.* **246**, 6328–6334
- Norling, B., Glimelius, B. W., Westermark, B. & Wastesson, Å. (1978) *Biochem. Biophys. Res. Commun.* **84**, 914–921
- Särnstrand, B., Brattsand, R. & Malmström, A. (1982) *Connect. Tissue Res.* **10**, 122
- Shively, J. L. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
- Uldbjerg, N., Malmström, A., Ekman, G., Sheehan, J. K., Ulmsten, U. & Wingerup, L. (1983) *Biochem. J.* **209**, 497–503
- Vogel, K. G. & Peterson, D. W. (1981) *J. Biol. Chem.* **256**, 13235–13242
- Vogel, K. G. & Sapién, E. (1982) *Biochem. J.* **207**, 369–379