The small dermatan sulphate proteoglycans synthesized by fibroblasts derived from skin, synovium and gingiva show tissue-related heterogeneity

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Dermatan sulphate proteoglycans (DSPGs) synthesized in the presence of $^{35}\text{SO}_4$ were characterized in culture media of fibroblast lines obtained from skin, synovium, and gingiva. The molecular mass of DSPG varied from 95–130 kDa as estimated by SDS/polyacrylamide-gel electrophoresis. Gingival fibroblasts constantly produced larger DSPGs than skin fibroblasts. This was due to the larger dermatan sulphate (DS) chains, which also showed tissue-related heterogeneity in the distribution of 4- and 6-sulphated disaccharide units. The *N*-glycosylated cores (44 and 47 kDa) obtained following chondroitinase ABC treatment were of identical size in all tissues. The cores from the different tissues were also of the same size (38 kDa) when addition of the *N*-linked oligosaccharides was inhibited by tunicamycin or when they were removed by *N*-glycanase treatment. No evidence for low-molecular-mass sulphated oligosaccharides was found. All tissues contained two mRNA species (1.6 and 1.9 kb) for the DSPG core protein. These data suggest that the pattern of transferase activities involved in the construction of DS chains differs from one tissue to another. This variation may modulate the functions of DSPG in the extracellular matrix.

INTRODUCTION

The small dermatan sulphate proteoglycans (DSPGs) form a family of 70–130 kDa molecules found in different tissues, such as cartilage, tendon, bone, cornea, and skin (reviewed by Hassell *et al.*, 1986; Poole, 1986). These DSPGs contain one dermatan sulphate (DS) chain covalently linked to a protein core of 40–50 kDa (Hassell *et al.*, 1986; Poole, 1986; Ward *et al.*, 1987). The core also carries *N*-linked oligosaccharides (Hassell *et al.*, 1986; Poole, 1986). The DS chains usually have a high content of iduronic acid. The amino acid sequence of the DSPG core protein has recently been resolved through cDNA cloning of the corresponding mRNA from human fibroblasts (Krusius & Ruoslahti, 1986).

Skin fibroblasts in culture produce both large and small proteoglycans (Cöster *et al.*, 1984). The majority of the radioactive sulphate incorporated by a cell culture appears in the small iduronic acid-rich DSPG (Glössl *et al.*, 1984). It is mainly found in cell-culture medium, but if the cells have been cultured on collagen gels it may be found also in the matrix (Gallagher *et al.*, 1983; Glössl *et al.*, 1984).

DSPG from cultured skin fibroblasts seems to differ from that synthesized by vascular smooth muscle cells (Rauch *et al.*, 1986). The core proteins are thought to be similar, but smooth muscle cells produce larger glycosaminoglycan chains. Less is known about potential tissue-specific or disease-related differences in these molecules. We have found a low-epimerized DSPG in skin fibroblasts of patients with an inherited metabolic disease, aspartylglycosaminuria, and it has been suggested that its presence explains the abnormal distribu-

tion of collagen fibrils in the skin of these patients (Näntö-Salonen *et al.*, 1987). Here we document that the small DSPG shows heterogeneity also within fibroblast cultures derived from skin, synovium, and gingiva. This heterogeneity is based on differences in DS rather than in the core protein.

MATERIALS AND METHODS

Materials

The following materials were purchased as indicated: $\text{Na}_2^{35}\text{SO}_4$ (7–16 Ci/mmol), [α - ^{32}P]dCTP (800 Ci/mmol), and [^{14}C]methylated molecular mass markers from Amersham; cell-culture fluids from Gibco Chemical Company; chondroitinase ABC, tunicamycin, proteinase inhibitors from Sigma Chemical; *O*- and *N*-glycanase from Genzyme, Boston, MA, U.S.A.; protein A–Sephrose and Sephadex 100 SF from Pharmacia Fine Chemicals; peroxidase-conjugated anti-rabbit IgG from Dacopatts; Kodak X-Omat AR from Kodak Eastman Company; Pall Biotryne A nylon membrane from Pall Corporation; and Biogel P2 from BioRad.

Cell culture

Human fibroblasts (four cell lines from each tissue) were cultured from biopsies of skin, gingiva or synovium using the explantation method in each case. Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) foetal-calf serum; 23 mM- Na_2CO_3 /20 mM-Hepes, 50 μg of streptomycin sulphate/ml, 100 i.u. of penicillin/ml, unless otherwise stated. Cells between their fourth and tenth passage were used in the experiments. In the individual

Abbreviations used: DMEM, Dulbecco's modification of Eagle's medium; DS, dermatan sulphate; DSPG, dermatan sulphate proteoglycan.

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experiments the passage numbers were, however, close to each other (± 1 passage). Possible mycoplasma contamination was routinely screened by DNA staining (Chen, 1977). Cultures of skin and gingival fibroblasts resembled each other but were both distinct from synovial fibroblasts under phase-contrast microscopy. The morphology of synovial fibroblasts in culture is more angular than that of skin fibroblasts.

All four cell lines from each tissue type were studied for DSPG-size distribution, however, only one cell line per tissue was used for further analysis of DSPG structure.

Labelling of DSPG

The cells of each line were seeded into cell-culture flasks (175 cm², 10⁶ cells/flask). The media were changed on the following day and the cells were allowed to grow, with a medium change every third day, for 14 days to reach confluency. Then the cultures were preincubated in the presence of 4 μ g of tunicamycin/ml or its vehicle (200 μ g of tunicamycin was solubilized into 1 ml of 0.025 M-NaOH) (Vasilov & Ploegh, 1982). Labelling was performed with carrier-free sulphate (Lee & Huttner, 1983) in DMEM in the presence or absence of tunicamycin. In the first set of experiments, 2% foetal-calf serum was present in the labelling media. After a labelling period of 48 h the media were collected, supplemented with proteinase inhibitors (1:20 of mixture containing 0.5 M EDTA/40 mM-*para*-aminobenzenamidine/0.4 M-*N*-ethylmaleimide/40 mM-phenylmethanesulphonyl fluoride) and dialysed against distilled water at 4 °C for 3 days with six changes. The samples were then lyophilized and stored at 4 °C until used.

Immunoprecipitation of DSPG

Radioactive lyophilizates were dissolved in 500 μ l of 50 mM-Tris/HCl, pH 8.0, containing 200 mM-NaCl, 2% bovine serum albumin, and 0.1% Tween 20. Then 10 μ l of anti-DSPG antiserum was added. The antibodies were raised previously against synthetically-prepared *N*-terminal sequence of fibroblast DSPG in rabbit (Krusius & Ruoslahti, 1986). The samples were shaken at 4 °C overnight and centrifuged (10000 g, 5 min). Protein A-Sepharose CL-4B (4 mg of dry powder) was added to the supernatant and shaken at 4 °C overnight. Over 90% of the precipitable radioactivity was pelleted by this procedure. The pellets were washed three times with 50 mM-Tris/HCl/200 mM-NaCl/0.1% Tween 20, pH 8.0, and then once with the same buffer containing 500 mM-NaCl and 0.1% Tween 20. The final washing was performed with the buffer diluted to 1:10.

Enzyme digestions

The whole medium diffusates or immunoprecipitated samples in 100 μ l of distilled water were digested with chondroitinase ABC in a mixture containing 25 μ l of buffer (50 mM-Tris/HCl/30 mM-sodium acetate, pH 8.0), 5 μ l of proteinase inhibitor mixture (see above), and 10 μ l (0.1 units) of chondroitinase ABC dissolved in water. If subsequent enzyme digestions were performed, the incubation period was 2 h, but otherwise the samples were digested overnight.

In one set of experiments chondroitinase ABC digestions were followed by digestions with neuraminidase (0.1 units per sample; 1 h), endo- α -*N*-acetyl-D-galactosaminidase (6 munits, *O*-glycanase; overnight), or pep-

tide-aspartylglycosylamine aminohydrolase (12 munits, *N*-glycanase; overnight) in phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate), pH 7.0 and pH 6.0, respectively. The enzymes (*O*-glycanase and *N*-glycanase) have been shown to be active under these conditions in our earlier experiments (results not shown).

Electrophoresis and immunoblotting of DSPG

Immunoprecipitated samples bound to protein A-Sepharose were released from the pellet for electrophoresis by boiling the samples in electrophoresis sample buffer containing SDS and mercaptoethanol. To the soluble medium samples a two-fold concentrate of the above buffer was added and it was then boiled for 5 min. The samples were fractionated by SDS/polyacrylamide-gel electrophoresis using 5–22% gradient gels (O'Farrell, 1975). The immunoblotting of proteins from the gels onto nitrocellulose sheets was performed according to the laboratory manual of the manufacturer (LKB). The sheets were incubated in phosphate-buffered saline/2% bovine serum albumin/0.1% Tween 20, pH 7.3, at 37 °C for 1.5 h. Then DSPG antiserum (1:500) in the same buffer was added and incubation continued overnight at room temperature. After several washings peroxidase-conjugated anti-rabbit IgG (1:100) was used as the second antibody. After washings the staining was performed in a substrate solution containing 20 ml of 50 mM-Tris/HCl, pH 7.4, and 200 mM-NaCl, and 1 ml of a mixture containing 3 mg of 4-chloro-1-naphthol/ml solubilized in methanol and 3% H₂O₂. After staining the sheet was allowed to dry and then directly exposed to X-Omat AR film.

Isolation and characterization of DS chains

DS chains were released from immunoprecipitates in overnight incubations with 25 μ g of papain/ml in 5 mM-EDTA and 5 mM-cysteine hydrochloride, pH 6.5. After incubation glycosaminoglycans were precipitated with 1% cetylpyridinium chloride in 0.02 M-NaCl followed by precipitation with ethanol as described in detail elsewhere (Näntö-Salonen *et al.*, 1985). The molecular-mass distribution of the isolated chains from DSPG produced by gingival and skin fibroblasts was studied using gel filtration on a Sephadex 100 SF column (1 cm $\times$ 90 cm). Lyophilized samples were diluted in the gel-filtration buffer (100 mM-NaCl/50 mM-Tris/HCl/1% SDS, pH 8.0) and chromatographed with molecular-mass markers. Radioactivity and absorbance at 278 nm were measured from the 0.5 ml fractions collected.

T.l.c. of chondroitinase ABC-digested glycosaminoglycans was performed as described in detail elsewhere (Säämänen & Tammi, 1984).

Gel filtration of NaBH₄-treated DSPG

Immunoprecipitated DSPGs labelled with ³⁵SO₄ were treated with 0.05 M-NaOH/1 M-NaBH₄ at 37 °C for 16 h and neutralized with acetic acid (Finne & Krusius, 1982). After centrifugation the samples were chromatographed on a Biogel P2 column (0.9 cm $\times$ 56 cm) using 0.1 M-NH₄HCO₃ in 20% (v/v) ethanol as the eluent. Fractions (0.3 ml) were collected and their radioactivity measured.

RNA preparations/hybridizations

Total cellular RNA was extracted from the cell cultures as described earlier (Chirgwin *et al.*, 1979).

Table 1. Molecular mass of dermatan sulphate proteoglycan produced by different fibroblast cell lines

In each experiment different cell lines from the three tissue sources were studied. The experiments were done separately during a period of several months. DSPG was labelled with $^{35}\text{SO}_4$ in sulphate-free medium following dialysis and analysis of the medium by SDS/polyacrylamide-gel electrophoresis. The mean molecular mass of DSPG was estimated from the autoradiograms of the dried gels. DSPG was identified as a chondroitinase ABC-sensitive band in the gels. The other experiments were done using the same cell lines as in experiment 4.

Experiment Number	Source...	Molecular mass (kDa)		
		Skin	Synovium	Gingiva
1		95	120	110
2		95	115	130
3		110	120	125
4		110	115	120

For Northern hybridization each RNA sample was denatured with glyoxal and fractionated electrophoretically on 0.75% agarose gel (Thomas, 1980). Thereafter the samples were transferred to Pall Biotrans A nylon membranes and immobilized to the filters by baking at 80 °C for 2 h. Prehybridization of the filters was carried out as described by Thomas (1980). Hybridizations were performed in the same solution at 42 °C for 20 h using cDNA probes nick-translated with [α - ^{32}P]dCTP (800 Ci/mmol) to specific radioactivities of approximately 2×10^7 c.p.m./ μg . The filter was hybridized with cDNA probes specific for DSPG core protein (PG-40) mRNA (Krusius & Ruoslahti, 1986) and for γ -actin (Gunning *et al.*, 1983). After hybridizations, the washed filters were exposed to Kodak X-Omat AR film at -70 °C using intensifying screens. To remove the probes from the filters between hybridizations, the filters were washed in 50% (v/v) formamide/10 mM-sodium phosphate (pH 6.5) (1 ml/cm 2), at 65 °C for 1 h, followed by washing in $2 \times \text{SSC}/0.1\%$ SDS (1 ml/cm 2) at 25 °C for 15 min.

RESULTS

DSPG

In preliminary experiments four cell lines derived from human gingiva, skin, and synovium were labelled with $^{35}\text{SO}_4$ and the radioactive macromolecules were analysed by SDS/polyacrylamide-gel electrophoresis. Most of the radioactivity incorporated into one major chondroitinase ABC-sensitive 95–130 kDa band and the results suggest a tissue-related variation in molecular mass (Table 1). Gingival and synovial fibroblasts produced consistently larger DSPGs than skin fibroblasts. In all cell lines the 95–130 kDa band showed equal sensitivity to chondroitinase AC (about 80% as estimated from the gels; results not shown). For further experiments one cell line derived from each tissue was selected.

Antibodies directed against DSPG recognized the major $^{35}\text{SO}_4$ -containing molecule produced by all the cell lines studied and also revealed the tissue-related variation in its molecular mass (Fig. 1).

Chondroitinase ABC treatment of immunoprecipi-

Fig. 1. Comparison of DSPGs from three different cell lines by Western blot of electrophoretically separated medium samples

Fibroblast lines were cultured in serum-free medium. The medium samples were collected, dialysed, lyophilized and dissolved in electrophoresis sample buffer. After electrophoresis on SDS/polyacrylamide gels the proteins were transferred onto nitrocellulose membrane and DSPGs were detected with specific antibodies. The samples are: 1, skin fibroblasts; 2, synovial fibroblasts; 3, gingival fibroblasts.

tated DSPG from all cell lines released two core proteins, 47 kDa and 44 kDa in molecular mass (Fig. 2). The core proteins still contained the $^{35}\text{SO}_4$ -label and they were detected by autoradiography. Identity of these bands with DSPG core protein was confirmed by Western blotting (results not shown). When the attachment of the *N*-linked oligosaccharides was inhibited by tunicamycin and DSPG was thereafter treated with chondroitinase ABC, the DSPG from all cell lines released a core of 38 kDa (Fig. 2). A similar decrease was seen in the intact DSPG when tunicamycin was present in the labellings. Treatment of the core protein with *N*-glycanase resulted in degradation products ranging from 38 kDa to 48 kDa but treatment with *O*-glycanase had no effect (results not shown). The incomplete removal of the *N*-glycosidic oligosaccharide chains is consistent with the manufacturer's report that the enzyme mainly acts on denatured proteins and glycopeptides.

Glycosaminoglycan chains of DSPG

The sizes of the glycosaminoglycans released from the protein core with papain were compared by gel filtration. The glycosaminoglycans in DSPGs synthesized by gingival fibroblasts were larger than those produced in skin fibroblasts (Fig. 3).

Fig. 2. The effects of tunicamycin on the size of DSPG core proteins

Fibroblast lines were labelled with $^{35}\text{SO}_4$ in serum-free medium for 48 h in the presence (+) or absence (-) of 4 μg of tunicamycin (TM)/ml. All samples were dialysed, lyophilized, immunoprecipitated with anti-DSPG antibodies and treated with chondroitinase ABC prior to electrophoresis on an SDS/polyacrylamide gel. The samples are: 1, gingival fibroblasts; 2, synovial fibroblasts; 3, skin fibroblasts. Autoradiography was used to detect the radiolabelled bands.

Table 2. Distribution of 4-sulphated and 6-sulphated disaccharides of dermatan sulphate proteoglycan in different fibroblast cell lines

DSPG was immunoprecipitated from cell-culture media, followed by purification of DS chains after papain digestion of the core. DS chains were digested with chondroitinase ABC and the resulting disaccharides were run on t.l.c.

Cell line	Di-4-SO ₄ (1) (%)	Di-6-SO ₄ (2) (%)	Ratio (1/2)
Skin fibroblasts	86.6	13.4	6.4
Synovial fibroblasts	68.9	31.1	2.2
Gingival fibroblasts	81.2	18.8	4.3

The distribution of 4- and 6-sulphated disaccharides of DS-chains synthesized by the different cell lines is shown in Table 2. DS chains of all cell lines were rich in 4-sulphated disaccharides (68.9–86.6%). Synovial fibroblasts synthesized the largest proportion of 6-sulphate disaccharides (31%), whereas skin fibroblast produced DS chains with least 6-sulphate (13.4%) (Table 2).

Fig. 3. Size fractionation of isolated DS chains by gel filtration

Gingival and skin fibroblasts were labelled with $^{35}\text{SO}_4$. DSPGs from medium samples were purified by immunoprecipitation followed by digestion of proteins with papain and fractionation on a Sephadex 100 SF column. Fractions of 0.5 ml were collected and analysed for radioactivity. The elution positions of two protein molecular-mass markers are indicated by arrows: 1, bovine serum albumin, 67 kDa; 2, chymotrypsinogen A, 25 kDa. The samples are: skin fibroblasts (○); gingival fibroblasts (●).

NaBH₄ treatment of DSPG

NaBH₄ treatment releases O-linked oligosaccharides and glycosaminoglycans from the core protein, which undergoes extensive degradation. Following this treatment the DSPG showed a major peak of $^{35}\text{SO}_4$ activity in the exclusion volume of a Biogel P-2 chromatography, corresponding to the DS chains. Less than 8% of the activity appeared in the total volume of the column, and virtually nothing in the intermediate region corresponding to small oligosaccharides. This suggests the absence of sulphated oligosaccharides and is consistent with the lack of effect of O-glycanase on the mobility of the core protein in SDS/polyacrylamide gels (results not shown).

DSPG core protein mRNA

When Northern blots of total RNAs extracted from several fibroblast lines initiated from gingiva, skin or synovial tissue were hybridized with cDNA clone PG-40 for the DSPG core protein mRNA, two transcripts of 1.6 kb and 1.9 kb were observed (Fig. 4). The sizes of the two mRNAs were identical in all the RNA samples studied regardless of origin. Changes were, however, observed in the relative intensities of the two transcripts between the cell lines. This variation was not related to the tissue origin of the fibroblasts.

DISCUSSION

The small DSPGs are found widely distributed in connective tissues (Hassell *et al.*, 1986; Poole, 1986; Voss *et al.*, 1986). It seems that they form a family of molecules with similar size and antigenicity. Here, we have studied fibroblasts cultured from different connective tissues and found relatively small, but consistent, tissue-related heterogeneity in the size of DSPGs. It was

Fig. 4. Northern analysis of mRNAs for DSPG core proteins

Total cellular RNA extracted from fibroblast lines started from gingiva (lanes 1 and 2), skin (lane 3), and synovial tissue (lane 4), were fractionated on an agarose gel, transferred by blotting onto a nylon membrane, and hybridized with cDNA probes specific for human fibroblast DSPG core protein (panel *a*), and for γ -actin, used as a reference probe (panel *b*). To demonstrate equal loading of the gel, the rRNAs are shown on a duplicate gel stained with ethidium bromide (panel *c*).

recently shown that cultured smooth muscle cells synthesize larger glycosaminoglycan chains than fibroblasts on an otherwise similar DSPG (Rauch *et al.*, 1986). Our findings show that differences exist not only between smooth muscle cells and fibroblasts, but also between fibroblasts from different loose connective tissues.

It is likely that only one gene, giving rise to two transcripts with different sizes and 3' ends, codes for the synthesis of DSPG core protein in fibroblasts (Krusius & Ruoslahti, 1986). We found that these two mRNAs (Fig. 4) had sizes similar to those reported before in all fibroblast lines (Krusius & Ruoslahti, 1986). The coding potentials of the two mRNA species are apparently identical since they only differ in their 3' non-coding sequences (Krusius & Ruoslahti, 1986). Also, the core proteins of different cell lines had identical sizes in both the glycosylated and non-glycosylated forms. Further studies are needed to determine the possible biological significance of the two transcripts.

The molecular mass of core proteins was in the same size range as reported earlier (Carlstedt *et al.*, 1981; Glössl *et al.*, 1984; Krusius & Ruoslahti, 1986; Rauch *et al.*, 1986). In previous reports 2–3 *N*-linked oligosaccharides have been reported attached to the DSPG core (Glössl *et al.*, 1984; Rauch *et al.*, 1986), whereas sulphated and *O*-linked oligosaccharides were not found (Glössl *et al.*, 1984), except in cultured gingival fibroblasts (Bartold & Page, 1987). The present results indicate the presence of two *N*-glycosidic oligosaccharides in all cell

lines studied, but we were not able to find any change in the electrophoretic mobility of the DSPG core following *O*-glycanase treatment. Neither did Biogel P2 chromatography of NaBH₄-treated DSPG reveal the small sulphated *O*-linked oligosaccharides previously reported (Bartold & Page, 1987).

In the present study, the ratio of 4- and 6-sulphated disaccharides did not seem to correlate with the chain length, although it should be taken into account that only one cell line from each tissue type was studied here for sulphation. It is not known, how 4- and 6-sulphation is regulated in cultured fibroblasts. Our results agree with a previous study with human skin fibroblasts showing that almost all sulphate is at position 4 (Silbert *et al.*, 1986). Thus a difference in the timing of 4- and 6-sulphation subcompartments of the golgi where sulphation occurs (Poole, 1986), does not explain the size difference, as has been suggested for skin and smooth muscle cell DSPG (Rauch *et al.*, 1986). Epimerization of glucuronic acid to iduronic acid was not specifically studied, but chondroitinase AC treatments revealed similar results between various cell lines, suggesting hardly any difference in epimerizations. The labelling conditions used here (low sulphate) have been shown to produce low-epimerized molecules (Silbert *et al.*, 1986). In general, no major effects on the length of the chains are expected from different epimerization because it occurs at the polymer level (Malmström, 1984; Malmström *et al.*, 1975).

The size and organization of collagen fibres varies in

different connective tissues in spite of the fact that the basic molecular structure of collagen is the same. Thus it is probable that other molecules in extracellular matrix regulate the properties of different connective tissues via their effects on the formation of collagen fibres. The biological function of small DSPG is proposed to be the regulation of collagen fibril formation (Scott & Orford, 1981; Scott *et al.*, 1981, 1983, 1986; Hampson & Gallagher, 1984; Vogel *et al.*, 1984; Longas & Fleischmaer, 1985; Van Kuppevelt *et al.* 1987). Here, we show that fibroblast lines cultured from tissues with different matrix composition produce DSPGs of different sizes. Thus DSPG may be one of the molecules giving specific features to each different type of connective tissue.

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