Isolation and characterization of proteoglycans from human follicular fluid

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Two proteoglycans differing in size and composition were isolated from human follicular fluid. The larger one of high density had a molecular mass of 3.0×10^6 Da, as determined by laser light-scattering, and was substituted with 15–20 chondroitin sulphate (CS) chains (M_r 60000–65000). Half of the CS disaccharides were 6-sulphated, whereas the remaining ones were non-sulphated. Digestion of the CS proteoglycan with chondroitinase ABC lyase, followed by SDS/PAGE, yielded a protein core of 600 to 700 kDa including substituted oligosaccharides, and a band of 70 kDa that was identified as the heavy-chain component of the inter- α -trypsin inhibitor (ITI). Western blotting of the CS proteoglycan showed that this had reactivity with antibodies raised against human versican. Electron microscopy (EM) of the CS proteoglycan also revealed a versican-like structure, with one globular domain at each end of a long extended segment substituted with CS side chains, as well as a structure interpreted as being the heavy chain of ITI attached to CS chains. Laser

light-scattering revealed that the smaller proteoglycan had a molecular mass of 1.1×10^6 Da, and EM demonstrated that it had a globular-protein core structure. The core protein, which showed immunological reactivity with perlecan antibodies, was substituted with approximately seven heparan sulphate (HS) and CS chains of similar size (50–55 kDa), the CS disaccharides being mainly 6-sulphated (68%), with a small proportion being 4-sulphated. The protein core was shown to be heterogeneous, with bands occurring at 215, 330 and 400 kDa after enzymic degradation of the glycosaminoglycan chains followed by SDS/PAGE analysis. The demonstration of intact molecules and fragments obtained after stepwise degradations, as shown by gel chromatography, supported a 'composite' structure of this proteoglycan.

Key words: chondroitin sulphate, heparan sulphate, inter- α -trypsin inhibitor (ITI), proteoglycan, versican-like.

INTRODUCTION

The molecular composition and biological role of follicular fluid (FF) in folliculogenesis, oocyte maturation and fertilization are poorly understood. Follicular development, including antrum formation, involves the creation of a fluid-filled compartment in the extracellular matrix (ECM) surrounding the granulosa cells without increasing intrafollicular pressure, and, theoretically, this process is dependent on the presence of molecules capable of retaining water [1,2]. ECM proteoglycans substituted with glycosaminoglycan (GAG) side chains that carry negatively charged groups could increase the osmoactivity of the ECM, and therefore be implicated in this context [3–5]. The presence of such molecules in the FF has been known for decades [6,7], and a large dermatan sulphate proteoglycan with an estimated molecular mass of $(2\text{--}2.5) \times 10^6$ Da and a protein core of 400 kDa has been shown to be present in porcine ovarian FF, as well as in the medium of both mouse and rat granulosa cell cultures [8,9]. The FF proteoglycans are synthesized by the granulosa cells that are regulated by various systemic hormones and local paracrine factors [10,11]. A variation in the concentration and structure of the proteoglycans at different stages of the follicular maturation process [12] has raised the hypothesis that FF proteoglycans play a physiological role with respect to folliculogenesis and fertilization [13].

Large versican-like proteoglycans are also synthesized by the cumulus cells, which surround the oocyte at the time of ovulation. The ECM of the cumulus complex is organized into a three-dimensional network consisting mainly of hyaluronan, but also the heavy-chain component of inter- α -trypsin inhibitor (ITI),

proteoglycans and at least one protein of 46 kDa [14–18]. A direct interaction between hyaluronan and proteins of the ITI family has been shown to play an important role in stabilizing the ECM following the ovulatory stimulus [16,19–22]. It is also postulated that interaction between hyaluronan and the large proteoglycan enhanced by a 46 kDa link protein contributes to matrix organization and stability [17,23]. As the heavy-chain component of ITI has been shown to bind covalently to CS GAG chains [24], there is the possibility that an interaction between this component and the large proteoglycan might be of importance for cumulus stability.

To elucidate further the functions of the proteoglycans in the FF and the cumulus, proteoglycans were prepared from human FF obtained during *in vitro* fertilization (IVF). Characterization of the proteoglycans showed the presence of a versican-like proteoglycan and a composite perlecan-like heparan sulphate (HS)/CS one. The versican-like proteoglycan improves the motility and viability of human sperm cells *in vitro* [25], and the concentration in the FF is negatively correlated with fertilization and embryo-transfer rates during IVF [13]. A more detailed structural and chemical characterization of these macromolecules is presented in the following report.

EXPERIMENTAL

Materials

Hormones used for ovarian stimulation were clomiphene citrate (Pergotime; Serono, Copenhagen, Denmark), human meno-

Abbreviations used: CS, chondroitin sulphate; DMB, 1,9-dimethyl-Methylene Blue; ECM, extracellular matrix; EM, electron microscopy; FF, follicular fluid; FSH, follicle-stimulating hormone; GAG, glycosaminoglycan; GdmHCl, guanidinium chloride; hCG, human chorionic gonadotropin; HD, high density; HS, heparan sulphate; IVF, *in vitro* fertilization; ITI, inter- α -trypsin inhibitor; LD, low density.

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pausal gonadotropin (Humegon; Organon, Skovlunde, Denmark) and human chorionic gonadotropin (hCG) (Pregnyl; Organon). Sephacryl S-500 HR, Sepharose CL-6B, Superose-12 and Mono-Q HR were obtained from Pharmacia LKB (Uppsala, Sweden); a Lichrosorb-NH₂ column was from Merck (Kebo, Stockholm, Sweden), DEAE-cellulose and Sachs columns were from Whatmann Biosystems Ltd. (Maidstone, Kent, U.K.), and Tris/acetate gels (3–8% acrylamide) and running buffer were from Novex (Frankfurt, Germany). Chondroitinase ABC lyase, chondroitinase AC II lyase and HS lyase were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). SDS/PAGE high-molecular-mass standards and agarose (DNA grade) were from Bio-Rad Laboratories (Sundbybjerg, Sweden) and 1,9-dimethyl-Methylene Blue (DMB) was from Serva (Heidelberg, Germany). [³H]borotritide came from Amersham International (Braunschweig, Germany) and the PVDF nitrocellulose membranes were from Millipore (Eschborn, Germany). Monoclonal mouse anti-perlecan antibodies were supplied by Zymed Laboratories Inc. (San Francisco, CA, U.S.A.) and antibodies raised against human versican were generously given by Professor Dick Heinegård (Lund University, Sweden). Rabbit anti-(human ITI) IgG (code A301) came from Dakopatt A/S (Copenhagen, Denmark), the alkaline-phosphatase-conjugated pig anti-rabbit IgG and rabbit anti-mouse IgG were obtained from Orion Diagnostica (Turku, Finland) and 3,3'-diaminobenzidine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Collection of human FF

Human FF was obtained from infertile women attending the IVF Programme at the Department of Obstetrics and Gynaecology, Aarhus University Hospital, Denmark. The women ($n = 55$) were stimulated by clomiphene citrate and human menopausal gonadotropin. Oocyte maturation was induced by a single injection of 5000 units of hCG when the dominant follicle was larger than 16 mm, and follicular aspirations were performed trans-vaginally 34 h after hCG administration. After removal of the oocyte, FF from multiple follicles was pooled and stored at -20°C . Samples were collected with informed consent, and the study, which was carried out in accordance with the Declaration of Helsinki II, was approved by the Ethical Committee of Aarhus County, Denmark.

Isolation and purification of proteoglycans

The pooled FF was centrifuged at 400 *g* for 60 min at 15°C to remove cells and debris. The supernatant was mixed with 7 M guanidinium chloride (GdmHCl)/0.0875 M sodium acetate, pH 5.8, containing proteinase inhibitors (20 mM 6-aminohexanoic acid/20 mM EDTA/10 mM benzamidinium chloride/10 mM *N*-ethylmaleimide) to a final concentration of 4 M GdmHCl/0.05 M sodium acetate, pH 5.8. CsCl density-gradient centrifugation was performed in a Beckman model L8 60M centrifuge (see the legend to Figure 1). The bottom two fractions, containing high density (HD) material, and fractions 3 to 6, containing lower density (LD) material, were pooled separately and dialysed against 6 M GdmHCl/0.05 M acetate, pH 5.8 (see Figure 1a). The initial density was adjusted to 1.45 g/ml for the HD fractions and 1.30 mg/ml for the LD fractions by adding solid CsCl. The second round of CsCl density-gradient centrifugations were performed under the same conditions as those described above. The bottom six fractions containing proteoglycans were pooled from each of the centrifugations (see Figures 1b and 1c), and subjected to gel chromatography (see the legend to Figure 2). The samples were dialysed against the elution buffer before chromatography.

Laser light scattering

Serial dilutions of the proteoglycans dissolved in 4 M GdmHCl, pH 7.0, were clarified by filtration through a 5 μm Millipore filter before measurement. A Malvern Molecular Analyser System equipped with a 20 mW He/Ne laser and a 64-channel K7027 correlator was used. Data were plotted as described by Zimm [26] and the weight-average relative molecular mass (M_r) and the radius of gyration were determined.

Electron microscopy

Stock solutions of the proteoglycans were dialysed against 0.2 M ammonium hydrogen carbonate, pH 7.9, overnight at 4°C , and prepared for electron microscopy (EM) by glycerol spraying/rotary shadowing [27,28]. The data from the electron micrographs were evaluated as described by Engel and Furthmayr [29].

Degradative methods

Digestions with chondroitinase ABC (0.4 unit/mg proteoglycan), chondroitinase AC II (0.25 unit/mg polysaccharide) and HS lyases (0.2 unit/mg proteoglycan) were carried out as described by Lindblom et al. [30]. Digestion with chondroitinase ABC lyase prior to the HS lyase digestion was carried out in 10 mM Hepes, pH 7.0. Ovalbumin (5 μg) was added as a carrier to all samples, except those analysed by chromatography. All incubations were done at 37°C for 4 or 5 h. Alternatively, HS side chains were cleaved with nitrous acid as described by Shively and Conrad [31].

Deglycosylation by alkali elimination

The proteoglycans were dissolved in distilled water and mild alkali treatment (50 mM NaOH for 5 min at 20°C) was performed as described by Enghild et al. [32]. The GAG chains and oligosaccharides were liberated from the core protein by alkaline borohydride treatment [33], and recovered by gel filtration on Superose-12.

Electrophoresis

SDS/PAGE was performed on 3–12% gradient gels with 3% stacking gels using the buffer system of Laemmli [34], as described by Lindblom et al. [30]. After electrophoresis, the gels were stained with 0.25% (v/v) Coomassie Brilliant Blue R-250, destained and dried. The gels were photographed and subjected to scanning using Adobe PhotoShop 3.0 (Adobe Systems Inc., San José, CA, U.S.A.) and SigmaGel Analysis Software (Jandel Scientific, Erkrath, Germany).

Western immunoblotting

Intact proteoglycans and fragments obtained after chondroitinase ABC lyase digestion were separated by Tris/acetate gels (3–8% acrylamide) and electroblotted on to a PVDF nitrocellulose membrane [35]. Following incubation with affinity-purified rabbit anti-(human ITI) IgG (at a dilution of 1:500), and alkaline-phosphatase-conjugated pig anti-rabbit IgG (at a dilution of 1:1000), the blot was developed with 3',3'-diaminobenzidine, according to the manufacturer's instructions (Sigma). Alternatively, intact proteoglycans were electrophoresed on 2.0% (w/v) agarose gels as described by Björnsson [36], blotted on to the membrane, and digested with chondroitinase ABC lyase and/or treated with nitrous acid [31], before immunostaining with the versican (1:500) or perlecan (1:500) antibodies, as described above.

Chromatography

Ion-exchange HPLC was performed on a Mono-Q HR 5/5 column connected to a HPLC system (Pharmacia LKB), as outlined by Lindblom et al. [30]. Samples were dialysed against buffer containing 6 M urea, 0.1 M NaCl and 0.05 M sodium acetate buffer (starting buffer) before application. Analytical gel chromatography was performed on a column of Sephacryl S-500 HR (1.3 cm × 50 cm). A Superose-12 column (1 cm × 30 cm) was eluted with 0.1 M ammonium acetate, pH 7.0, at room temperature.

Analytical methods

Amino acid analysis was performed with a Beckman 6300 amino acid analyser, after hydrolysis under argon in 6 M HCl at 110 °C for 24 h. Analyses for total hexosamines were carried out using an HPLC system eluted with 0.1 M KH_2PO_4 buffer, pH 7.0, containing 10% (v/v) methanol, as essentially described by Lohmander et al. [37]. Uronic acid and hexose were determined by the carbazole and anthrone methods respectively, both as described by Heinegård [38]. Sialic acid was determined with an automated version of the methods described by Jourdain et al. [39]. GAGs and proteoglycans were detected in microtitre plates, using the reaction of DMB, by modifying the methods described by Farndale et al. [40]. Determination of the molecular mass of the GAG chains liberated by alkaline borotritide was carried out as described by Wasteson [41]. A fraction of the GAG chains liberated from the smaller proteoglycan fraction was subjected to chondroitinase ABC lyase digestion before the determination of the size of the HS side chains. The relative content of non-sulphated, 4- and 6-sulphated disaccharides was estimated in a chondroitinase ABC lyase digest by the method of Hjerpe et al. [42], using a LiChrosorb- NH_2 column eluted with 0.01 M sodium sulphate/0.09 M sodium acetate buffer, pH 5.8, at room temperature.

RESULTS

Isolation of proteoglycans

The density-gradient centrifugation in CsCl/4 M GdmHCl resulted in a separation of material containing sulphated GAG (DMB-reactive material) in the bottom six fractions from proteins and glycoproteins in the upper fractions (Figure 1a). The high-density material (HD; $\rho > 1.44$ g/ml) and the material of lower density (LD; 1.34 g/ml $< \rho < 1.42$ g/ml) were subjected to a second CsCl density-gradient centrifugation step separately. The HD population banding at a density higher than 1.50 g/ml (Figure 1b), and the LD material banding around 1.37 g/ml (Figure 1c), were pooled and subjected to gel chromatography (Figure 2). The proteoglycans were separated into a population of larger (K_{av} of 0 to 0.15) and smaller proteoglycans (K_{av} of 0.17 to 0.31) containing DMB-reactive material, sialic acid and protein, although in different proportions (Figure 2). The proportion of the LD material eluted at the void fractions was subjected to CsCl density-gradient centrifugation at an initial density of 1.45 g/ml, which confirmed its similarity to the HD material (results not shown). The proteoglycans were effectively separated from contaminating proteins and nucleic acid as measured by A_{260} (profile not shown). The purity of the proteoglycans was verified by SDS/PAGE (3–12% gradient gels). The larger and the smaller proteoglycan (Figure 3, lane 1 and Figure 4, lane 1) did not enter the separation gel, but were seen in the stacking gel (not shown). Bands suggesting the presence of contaminating proteins were seen in neither of the preparations.

Figure 1 CsCl density-gradient centrifugation of proteoglycans from human FF

(a) The FF was adjusted to 4 M GdmHCl and an initial density of 1.31 g/ml with CsCl, subjected to centrifugation using a Ti 50.2 rotor (36000 rev./min for 65 h at 14 °C), and fractions (2 ml) collected from the bottom were pooled as indicated. The bottom two fractions (HD) and the fractions designated as LD were pooled separately and adjusted to 6 M GdmHCl and an initial density of 1.45 and 1.30 g/ml respectively, before the second centrifugation step. The HD material is shown in (b), and the material of LD is featured in (c). After the various centrifugations, fractions were collected and analysed for sulphated GAG by the DMB reaction (○), A_{280} (□), sialic acid (Δ) and density (·····). The fractions were pooled as indicated by the horizontal bars.

Chemical characterization

The overall chemical composition of the FF proteoglycans is summarized in Table 1. The approximate protein contents were 22% and 53.9% for the larger and smaller proteoglycans respectively. The predominant amino acids in the larger proteoglycan are glutamic acid, serine, glycine and leucine. In contrast, the smaller proteoglycan has a high content of glycine, arginine and tyrosine, and a lower content of aspartic acid and lysine when compared with the larger one. The large amounts of threonine and serine suggest a relatively high substitution of O-linked carbohydrate units. Whereas the amino acid compositions are relatively similar, differences in the GAG and oligosaccharide substitution were found. Galactosamine was the major hexosamine in the larger proteoglycan (95%), indicating that this proteoglycan only contains galactosaminoglycans. The smaller proteoglycan contained galactosamine and glucosamine in a ratio of approx. 2:1, thus suggesting the substitution of both HS and chondroitin/dermatan sulphate side chains, in addition to oligosaccharides.

Analysis of the GAGs

GAGs and oligosaccharides released after alkali treatment were isolated by gel chromatography (Figures 5a and 5c). Liberated GAG was eluted as a relatively sharp peak in the void volume, whereas almost all of the material containing sialic acid, repre-

Figure 2 Gel chromatography on a Sephacryl S-500 column of (a) the HD, and (b) the LD fractions after the second CsCl density-gradient centrifugation step

The proteoglycans were fractionated by repeated CsCl density-gradient centrifugations into high-density (HD) and low-density (LD) material, as described in the legend to Figure 1. The pooled HD and LD fractions were separated by chromatography on a column of Sephacryl S-500 HR (4.5 cm × 88 cm) eluted with 4 M GdmHCl, pH 7.0, and fractions (14 ml) were collected and analysed for the DMB reaction (○), A₂₈₀ (□) and sialic acid (△). The larger and the smaller proteoglycans were pooled as indicated by the horizontal bars. V₀, void volume; V_t, total volume.

Figure 3 Electrophoresis of the versican-like proteoglycan before and after treatment with chondroitinase ABC lyase or alkali

The CS proteoglycan resolved on (a) SDS/PAGE (3–12% gradient gels) before and after degradation with chondroitinase ABC lyase or alkali elimination, and staining with Coomassie Blue; (b) Tris/acetate (3–8% gels) and Western blotting using anti-ITI IgG; and (c) 2% (w/v) agarose gels and Western blotting using human versican antibodies (for details, see the Experimental section). The migration of standard proteins is indicated by arrows on the left; minor arrows on the right indicate the elution position of enzymes. Versican-like proteoglycan (PGL) and the smaller proteoglycan (PGS) were from bovine sclerae. O, start; F, front.

Figure 4 Electrophoresis of the perlecan-like proteoglycan before and after treatment with chondroitinase ABC lyase and/or HS lyase

The HS/CS proteoglycan resolved on (a) SDS/PAGE (3–12% gradient gels) before and after degradation with chondroitinase ABC lyase (ABC) and/or HS lyase (Hsase) stained with Coomassie Blue and (b) 2% (w/v) agarose gels and Western blotting using human perlecan antibodies (for further details, see the Experimental section). In lanes 1 to 3, most of the material was seen in the stacking gel (not shown). The migration of standard proteins is indicated by arrows on the left; minor arrows on the right indicate the elution position of enzymes. Versican-like proteoglycan (PGL) and smaller proteoglycan (PGS) were from bovine sclerae. O, start; F, front.

senting released oligosaccharides, was eluted at a K_{av} of 0.46 to 0.61. Calculations based on DMB data showed that the sulphated GAG fraction accounted for 30–35% (by mass) of the larger proteoglycan, and 14–18% of the smaller one. The oligosaccharides were eluted as a largish peak containing less sialic acid and a smaller one containing more sialic acid, a finding which suggests the substitution of two or more types of short oligosaccharides (4–8 monosaccharides).

To determine the nature of the GAG, aliquots of the liberated polysaccharides were digested with chondroitinase ABC or ACII lyase, and then subjected to gel chromatography. The polysaccharides from the larger proteoglycan were degraded mainly to disaccharides by chondroitinase ABC lyase (Figure 5b), but small amounts of material were found at a K_{av} of 0.40, probably representing linkage region fragments. Identical results were obtained from chondroitinase ACII lyase digestion (results not shown), indicating that the larger proteoglycan is substituted only with CS side chains. After degradation of the smaller proteoglycan with chondroitinase ABC lyase, part of the GAG was resistant to digestion (Figure 5d), and a similar result was obtained from ACII lyase digestion (results not shown). The ratio between intact and degraded polysaccharides was approx. 1:1, and the treatment of the non-degraded fraction with nitrous acid, followed by chromatography, resulted in a total depolymerization of the GAG (results not shown). These results show that the smaller proteoglycan contains both HS and CS side chains.

The CS side chains of the larger proteoglycan had a molecular mass of 60–65 kDa, and the molecular masses of the HS and CS side chains of the smaller proteoglycan were similar, both being around 50–55 kDa. The degree of sulphation of disaccharides

Table 1 Chemical composition of the CS proteoglycan and HS proteoglycan from human FF

The components of the proteoglycans, and their respective units, are indicated in the furthestmost column on the left; values in parentheses for the amino acid analysis represent the no. of residues per 1000 residues. Δ di-0S, non-sulphated; Δ di-4S, 4-sulphated; Δ di-6S, 6-sulphated.

Composition of proteoglycan	CS proteoglycan	HS/CS proteoglycan
Amino acid (nmol/mg)		
Aspartic acid	130 (97)	339 (76)
Threonine	109 (81)	286 (64)
Serine	139 (103)	451 (101)
Glutamic acid	210 (155)	617 (119)
Proline	Not detected	Not detected
Glycine	116 (86)	535 (120)
Alanine	97 (73)	376 (84)
Cysteine	Not detected	Not detected
Valine	97 (73)	343 (78)
Methionine	22 (16)	50 (11)
Isoleucine	59 (44)	159 (36)
Leucine	114 (85)	423 (95)
Tyrosine	34 (25)	127 (95)
Phenylalanine	58 (43)	140(31)
Histidine	35 (26)	187 (42)
Lysine	67 (50)	99 (22)
Arginine	58 (43)	323 (72)
Tryptophan	Not detected	Not detected
Protein (μ g/mg)*	220	530
GAG (μ g of GAG/mg of proteoglycan)	500†	350‡
Molecular mass (kDa)	60–65	50–55
Hexosamine (nmol/mg)		
Glucosamine	10	208
Galactosamine	820	476
Sulphation of disaccharides of CS side chains		
Δ di-0S (%)	54.2	16.0
Δ di-4S (%)	3.0	15.7
Δ di-6S (%)	42.8	68.2

* Calculated from the amino acid analysis; †calculated from the disaccharides released after chondroitinase ABC lyase digestion and recovery from disaccharide analysis; ‡calculated from the amounts of disaccharides released after chondroitinase ABC lyase digestion and recovery from disaccharide analysis. Results were corrected for a content of 55% HS.

released by the chondroitinase ABC lyase from the intact proteoglycans was estimated (Table 1). The polysaccharides of CS proteoglycan were found to be partly 6-sulphated and partly non-sulphated, whereas the smaller proteoglycan contained primarily 6-sulphated disaccharides (68%) and smaller amounts of non- or 4-sulphated disaccharides. Neither proteoglycan contained significant amounts of disulphated disaccharides.

Molecular size

Relative molecular mass determinations were performed in 4 M GdmHCl, in order to prevent the formation of aggregates. Values of M_r of 3.0×10^6 for the larger proteoglycan and 1.1×10^6 for the smaller proteoglycan were found. The corresponding radii of gyration were 93 nm and 74 nm for the larger and smaller proteoglycans respectively. The apparent translational diffusion coefficients, D_T (water, 20 °C), were 3.2×10^{-8} cm² for the larger and 5.2×10^{-8} cm² for the smaller proteoglycan, which yielded Stokes radii of 66 nm and 41 nm respectively.

Macromolecular assembly of the CS proteoglycan

EM of the CS proteoglycan showed versican-like structures with an extended core protein, from which some 15–20 GAG chains were extended (Figure 6a). The length of the core protein domain is 240 ± 40 nm, and a globule of 12 ± 3 nm is seen at both ends.

The size of the GAG chains were 60 ± 30 nm, which corresponds to the calculated M_r (Table 2). Immunological characterization of the core protein confirmed versican-like properties of the CS proteoglycan, since immunoreactivity with antibodies raised against human versican was demonstrated (Figure 3, lane 6). Particles with a diameter of 20 ± 2 nm were associated with the GAG chains and located at various positions along the side chains (1.2 per CS proteoglycan; Figure 6a). The size of the core protein, including substituted oligosaccharides, as estimated by SDS/PAGE was 600–700 kDa (Figure 3, lane 2). Chondroitinase ABC lyase also released a band at 70 kDa (Figure 3, lane 2). Because the presence of a heavy-chain component of the ITI family with similar M_r has been demonstrated in human FF [23], the reactivity using antibodies raised against ITI was investigated. Western blot analysis showed that this protein is the heavy-chain component of ITI (Figure 3, lane 5). No reactivity was noted when intact CS proteoglycan was subjected to Western blotting (Figure 3, lane 4). The release of the same protein by mild alkali treatment (Figure 3, lane 3) further supported the presence of a GAG–protein linkage involving aspartate/galactosamine, as described for the CS and the heavy-chain components of the ITI complex [19].

Macromolecular assembly of the HS/CS proteoglycan

EM of the smaller HS/CS proteoglycan showed a globular core protein with a diameter of 25 ± 6 nm, to which five to eight GAG

Figure 5 Superose-12 elution profiles of the CS-proteoglycan (a and b) and the HS/CS-proteoglycan (c and d) after alkali treatment (a and c) and followed by chondroitinase ABC lyase digestion (b and d)

An aliquot of the larger and smaller proteoglycan (approx. 1 mg of each) was treated with alkaline borohydride before application on a Superose-12 column connected to an HPLC system. Fractions were analysed for DMB (○), sialic acid (△) and hexose (◆). The DMB-containing peak at V_0 (void volume), representing liberated GAG chains, was pooled in a range of fractions as indicated by the horizontal bars, and re-chromatographed on Superose-12 after enzymic digestion with ABC chondroitinase lyase (b and d). The eluates were analysed for A_{232} (◇) and uronic acid (▼). The GAG resistant to chondroitinase ABC lyase digestion was pooled as indicated in (d), and subjected to nitrous-acid degradation. V_t , total volume.

chains with a length of 50 ± 30 nm were attached (Figure 6b). Immunological characterization by Western blotting showed reactivity with antibodies raised against human perlecan (Figure 4, lane 5). Electron micrographs could not verify a 'beads-on-a-string' appearance of the core protein known from perlecan [43], although the aggregation of core protein particles leading to a cluster formation was occasionally seen. Alkali treatment of the smaller proteoglycan, followed by stepwise degradation of the GAG chains, revealed evidence of substitution of both HS and CS to the perlecan-like core protein (Figure 5d). On the basis of the determination of uronic acid, 55% of the GAG chains are HS, which are resistant to digestion with chondroitinase ABC or ACII lyase.

Separation of the proteoglycan by SDS/PAGE showed the intact HS/CS proteoglycan in the stacking gel, and a small portion of the sample at the top of the gel (Figure 4, lane 1). Digestion of the same amounts of proteoglycan with both chondroitinase ABC and HS lyases resulted in three bands with molecular masses of 215, 330 and 400 kDa respectively (Figure 4, lane 4). This corroborated the results obtained with chromatography on Sephacryl S-500 columns (Figure 7a), as well as on

Figure 6 Structure of the FF proteoglycans as revealed by glycerol spraying/rotary shadowing EM

(a) The CS proteoglycans appear as particles with a single globule (12 ± 3 nm) located at the N- and C-terminals (arrowheads) of the extended core protein (240 ± 40 nm) substituted with GAG chains. In some cases, globular particles of approx. 20 nm can be seen attached to the GAG chains (arrows). (b) The HS/CS proteoglycans appear as particles with one globular core protein (25 ± 6 nm), to which five to eight GAG chains are attached. The bar represents 200 nm.

Table 2 Structural details of FF proteoglycans

The structural features of the proteoglycans were evaluated from a large number of electron micrographs. In total, 100 non-fragmented molecules were assessed, and a mono-modal distribution was found for each population. The data have not been corrected for overestimation (2–3 nm) due to decoration with platinum. The standard errors shown, where applicable, are $\pm$ S.D. of the mean. n.a., non-applicable.

Structural feature	CS proteoglycan	HS/CS proteoglycan
Composition of the length of the extended		
core protein domain (nm)	240 ± 40	—
Diameter of globular domain (nm)	12 ± 3	25 ± 6 nm
Length of GAG chains (nm)	60 ± 30	50 ± 30 nm
No. of GAGs per molecule	15–20	5–8
No. of heavy chains per molecule	1.2	0
Distance between adjacent		
GAG chains (nm)	18 ± 14	n.a.

Mono-Q columns, where it is evident that digestion with both enzymes generates a small protein core with no charged polysaccharide chains (Figure 7b).

Figure 7 Elution profile on (a) Sephacryl S-500 and (b) Mono-Q before and after enzymic degradations of the perlecan-like proteoglycan

Elution profiles of the HS/CS proteoglycan on (a) a Sephacryl S-500 HR column connected to a HPLC system (flow rate of 0.5 ml/min; 1.5 ml fractions), eluted with a 4 M GdmHCl/0.05 M sodium acetate buffer, pH 5.8, and (b) on a Mono-Q 5/5 HR column eluted with a linear gradient of 0.1–1.2 M NaCl in the starting buffer over 60 min (flow rate of 0.5 ml/min; 0.5 ml fractions). The fractions were analysed for A_{280} , and the profiles of the intact HS/CS proteoglycan (—), chondroitinase ABC lyase digestion (---), HS lyase (■ ■ ■) and the HS and chondroitinase ABC lyase digestion (- - -) are shown. The salt concentration is illustrated by a straight line in (b). V_0 , void volume; V_t , total volume.

In order to investigate the possible hybrid structure, the proteoglycans were digested separately with chondroitinase ABC and HS lyase. The digestion with chondroitinase ABC lyase generated material at the top of the separation gel and the same three bands (Figure 4, lane 2) as obtained by digestion with both enzymes (Figure 4, lane 4), in addition to material excluded from the separation gel. This treatment also changed the elution position on the Sephacryl S-500 column from K_{av} 0.24 to 0.29 (Figure 7a). Less than 10% of the protein was identified as non-substituted core protein after chromatography on Mono-Q, whereas most of the proteoglycan was still evident as proteoglycan after chromatography (Figure 7b). The digestion with HS lyase resulted in proteoglycan material in the stacking gel and the same three bands (Figure 4, lane 3). Furthermore, by gel chromatography a change of elution was observed from K_{av} 0.24 to 0.33 (Figure 7a). Ion-exchange chromatography on Mono-Q demonstrated that only 10% of the proteoglycan was substituted solely with HS (Figure 7b).

DISCUSSION

This study demonstrates the existence of a larger versican-like CS proteoglycan and a smaller perlecan-like HS/CS proteoglycan in human FF. The larger, high-density CS proteoglycan has a molecular mass of 3.0×10^6 Da, and a protein core of approx. 600–700 kDa substituted with 15–20 CS side chains, as determined by EM. Approx. 50% of the CS proteoglycan consists

of CS-containing disaccharides, of which about 54% are non-sulphated. This is an unusually high proportion, as other 'large' proteoglycans are normally highly sulphated [3]. According to the physical and chemical data, the number of GAG chains is likely to be approximately 30 per molecule. This discrepancy might be due to an underestimation of the number by EM, as non-sulphated GAG chains have a comparatively lower contrast. Alternatively, adjacent GAG chains might interact and appear as single chains, which, as a general phenomenon, tends to underestimate M_r values calculated by EM [43]. EM revealed a versican-like structure of the CS proteoglycan, comparable with that of versican from the aorta [44]. The reactivity with antibodies directed against human versican, for the first time, confirmed further the versican-like properties of the CS proteoglycan, which have been alluded to by Yanagishita et al. [9]. However, important differences were noted in the type, size and sulphation of the GAG, since the large proteoglycan from pig and rat FF are substituted with mainly 4-sulphated dermatan sulphate GAG chains. These differences could be explained by inter-species variations, or by an altered expression of proteoglycans in the granulosa cell culture. In addition, the hormone treatment might influence the proteoglycan synthetic profile, but human FF obtained from natural cycles contains the identical proteoglycans in the same concentrations and proportions (results not shown) as those found in FF obtained during IVF cycles [13].

At the time of ovulation, both the follicle and the cumulus-oocyte complex are enlarged, owing to a deposition of material in the ECM of these compartments, resulting in expanded three-dimensional structures [14,17,18]. Interactions between FF proteoglycans and hyaluronan might be necessary for matrix formation and stability, as suggested by Camaioni et al. [17]. The existence of a globular structure at the N- and C-terminal ends of the core protein, resembling the G1-hyaluronan-binding domain present in the family of large hyaluronan-binding proteoglycans, suggests that the large FF proteoglycan could interact with hyaluronan through the G1 domain [5,45,46]. Alternatively, the 70 kDa protein attached to the GAG chains might act as the linker protein known to stabilize the interaction between hyaluronan and hyaluronan-binding proteoglycans [17,46]. This is a hypothesis that is further supported by the immunological similarity of the 70 kDa protein with the heavy-chain component of ITI, which is capable of binding hyaluronan [19]. Together, the findings support the existence of interactions between the large CS proteoglycan and hyaluronan in FF and the cumulus-oocyte complex.

A smaller, low-density proteoglycan with an M_r of 1.1×10^6 Da was shown to consist of a globular protein core substituted with five to eight CS and/or HS chains. The application of several separation techniques before and after different enzyme digestions could not distinguish a population of 'pure' CS or HS proteoglycans. This suggests the presence of a composite structure, as described for the syndecan and perlecan group of proteoglycans [47–49]. Only a minor proportion of the proteoglycans was substituted with low amounts of HS or CS side chains, because most of this was co-chromatographed with the non-digested proportion. The core protein appeared to be heterogeneous, as shown by SDS/PAGE, because three bands were found upon enzyme treatment of the proteoglycan. The finding that the bands were apparent after chondroitinase ABC or HS lyase digestion also strongly suggests that the core proteins in most cases are substituted with both types of GAG chains (Figure 4). The size difference could be due to the presence of core proteins with different amino acid sequences, but it is more likely that different glycosylation patterns with N- and O-oligosaccharides generate the observed variation. The presence

of a proteoglycan with a composite structure has never been described in FF, but the medium of rat granulosa cell culture contains a smaller low-density dermatan sulphate proteoglycan and more HS proteoglycans [9].

The physiological significance of the proteoglycans in FF is still uncertain. The established physicochemical properties of the versican-like CS proteoglycan, and the fact that a high concentration of the CS proteoglycan (0.2–0.6 mg/ml) is present in the FF before ovulation [13], highlight the role of the CS proteoglycan in maintaining the structural integrity of the intrafollicular compartment, which might protect the oocyte before ovulation, as suggested by Camaioni et al. [18]. A variation in the concentration of proteoglycans in the FF during follicular maturation has raised the hypothesis that these macromolecules might influence folliculogenesis and oocyte maturation [12, 13, 50]. Furthermore, the FF is taken up by the uterine tubes, together with the oocyte at the time of ovulation, and could therefore influence the fertilization process *in vivo*. Evidence in favour of this is provided by the fact that the incubation of human sperm cells with the CS proteoglycan at physiological concentrations significantly improves sperm motility and viability *in vitro* [25].

The presence of HS side chains on the perlecan-like proteoglycan indicates the role of the proteoglycan as a 'local' anti-coagulation factor in the expanding follicle, since the binding of anti-thrombin III to HS-containing GAG isolated from rat granulosa cells has been demonstrated [51]. In addition, interactions with growth factors, cytokines and various hormones have been described for HS-containing proteoglycans, and a modulating effect of the composite proteoglycan on cell differentiation and growth in the follicular compartment is therefore possible [5]. However, the actual physiological function(s) of the FF proteoglycans with respect to folliculogenesis and fertilization *in vivo* are still hypothetical, and further investigation is needed.

In summary, human FF collected during the IVF procedure contains two distinctly different proteoglycans. One is a larger versican-like CS proteoglycan carrying the heavy-chain component of ITI attached to the GAG chains; the other is a perlecan-like, composite HS/CS proteoglycan, with heterogeneity in the GAG substitution on to a common core structure.

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