Binding of bovine follicular fluid glycosaminoglycans to fibronectin, laminin and low-density lipoproteins

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Summary. Interactions of bovine follicular fluid glycosaminoglycans (GAGs) with extracellular matrix (ECM) components fibronectin and laminin and with low-density lipoproteins (LDL) were examined using affinity chromatography. Glycosaminoglycans from small (diameter <5 mm) and large (diameter 11–20 mm) follicles were isolated from follicular fluid. The dermatan sulphate or heparan sulphate from small or large follicles was applied to Fn-, Lm- or LDL-Sepharose columns. Portions of each fraction of the bound or unbound GAG were then subjected to gel filtration h.p.l.c. for quantification. The binding interaction between dermatan sulphate and fibronectin was significantly greater than between heparan sulphate and fibronectin ($P < 0.05$); the binding interaction between GAGs from small follicles and fibronectin was significantly greater than between GAGs from large follicles ($P < 0.05$). The binding interaction between GAGs from small follicles and laminin was significantly greater than for GAGs from large follicles ($P < 0.05$). Dermatan sulphate from small follicles bound to fibronectin (42%), laminin (36%) and LDL (14%) and that from large follicles bound to fibronectin (14%), laminin (23%) and LDL (14%). Heparan sulphate from small follicles bound to fibronectin (17%), laminin (15%) and that from large follicles bound to fibronectin (13%), laminin (10%) and LDL (6%). These results suggest that dermatan sulphate, but not heparan sulphate, from follicles at different stages of development exhibit a varied ability to interact with components of the ECM. Both substances bound to LDL comparably in small amounts.

Keywords: follicle; glycosaminoglycans; fibronectin; laminin; extracellular matrix; lipoproteins; cow

Introduction

Granulosa cells are hormonally responsive, undergo extensive differentiation, and play an integral role in the development of the ovarian follicle from recruitment through luteinization. At various stages of differentiation, granulosa cells contribute to the growth of the follicle by producing and secreting components of the extracellular matrix such as proteoglycans containing the glycosaminoglycans, heparan sulphate and dermatan sulphate (Ax & Bellin, 1988), and glycoproteins such as fibronectin (Dorrington *et al.*, 1983). In other tissue such as skin and cartilage, glycosaminoglycans (GAGs) associate with the extracellular matrix proteins fibronectin, laminin and collagen (Murata & Yokoyama, 1987). Further, the type of GAG and the degree of sulphation of GAG that is associated with extracellular matrix components is characteristic of the age and physiological state of the tissue in which the biomolecules are located (Murata & Bjelle, 1978, 1980; Hascall, 1981; Fransson *et al.*, 1982; Longas *et al.*, 1986).

At the onset of follicular recruitment, granulosa cells commence proliferation. Before FSH stimulation *in vitro*, granulosa cells produce and secrete fibronectin. Granulosa cell production of

fibronectin *in vitro* is inhibited by addition of FSH (Skinner & Dorrington, 1984). In contrast, production of heparan sulphate and dermatan sulphate and their associated proteoglycan cores is stimulated by FSH (Bellin *et al.*, 1983; Ax *et al.*, 1985). Grimek & Ax (1982) and Grimek *et al.* (1984) reported differences in proteoglycan constitution between small and large follicles of cattle. Proteoglycans from small follicles contained a greater percentage of dermatan sulphate than did large follicles. Additional chemical characterization of the low molecular weight dermatan sulphate proteoglycan indicated that preparations from small follicles contained higher proportions of serine, the amino acid by which polymer side-chains of dermatan sulphate covalently attach to the protein core.

If cell-associated fibronectin functions as a physiological binding site for follicular GAGs, then it is possible that the number of GAG-binding sites on granulosa cells would decrease as the production and secretion of fibronectin by granulosa cells is inhibited. Indeed, as follicles mature, the number of binding sites for the [³H]heparin on the granulosa cells decreases (Ax *et al.*, 1984; Bushmeyer *et al.*, 1985; Bellin *et al.*, 1987a). The ability of follicular GAGs to associate with granulosa cell surfaces may change as granulosa cells differentiate and follicles mature. Dermatan sulphate from large follicles is 10 times more potent at displacing [³H]heparin from cell membranes than is that from small follicles (Bellin *et al.*, 1987b). Heparan sulphate from small follicles will compete for [³H]heparin-binding sites at physiological concentrations, but that from large follicles at doses up to 5 mg/ml is without effect (Bellin *et al.*, 1987b).

Cultured bovine granulosa cells demonstrate preference for extracellular cholesterol as a source of substrate for steroidogenesis (Savion *et al.*, 1981). Commercially available GAGs inhibited steroidogenesis by rat granulosa cells *in vitro* (Campbell & Valiquett, 1982). The GAG chondroitin-4-sulphate alters basal and gonadotrophin-induced secretion of progesterone by pig granulosa cells (Ledwitz-Rigby *et al.*, 1987). Follicular fluid GAGs, at physiological concentrations, inhibit degradation of extracellular low-density lipoproteins and progesterone production by pig granulosa cells (Bellin *et al.*, 1987a).

The purpose of this study was to determine whether follicular fluid GAGs of cattle interacted with extracellular matrix constituents or low-density lipoproteins, and whether the degree of those binding interactions was related to the size of the follicle from which the GAGs were isolated.

Materials and Methods

Isolation of follicular fluid glycosaminoglycans. Bovine ovaries were obtained from Packerland Packing Company, Green Bay, WI, USA, on three separate occasions within 30 min of slaughter. Follicular fluid from small (s, <5 mm) and large (l, 11–20 mm) follicles was aspirated with a 1.0 ml syringe fitted with a 20-gauge needle. Antral fluid was pooled by follicular size and centrifuged (200 g for 10 min) to remove granulosa cells. The follicular fluid was frozen promptly after centrifugation and stored at –20°C until dermatan sulphate and heparan sulphate were isolated by ion exchange chromatography under dissociative conditions as previously described (Bellin & Ax, 1987).

Isolation of low-density lipoproteins. A total of 1 litre of blood was collected from the jugular vein of 3 cows in late lactation and mixed with disodium ethylenediamine tetra-acetate (EDTA) (0.1%), sodium azide (0.05%), gentamicin sulphate (0.005%), epsilon-amino-caproic acid (0.13%) and phenylmethyl-sulphonylfluoride (0.26%) to inhibit coagulation and protease activity. The plasma was adjusted to a density of 1.21 g/ml with potassium bromide. Samples were ultracentrifuged at 112 000 g (Beckman 50.2 Ti rotor) for 20 h at 37°C. The total lipoproteins were concentrated to a volume of 10 ml by ultrafiltration (50 ml Amicon stir cell, Model 8050, Diaflow Membrane: YM10, Amicon, Danvers, MA, USA) and subjected to gel filtration chromatography (Grummer *et al.*, 1983). Absorbance of light by each fraction was determined at 280 nm. Because low- and high-density lipoproteins were not completely separated, the former were further purified by heparin–Sephacrose affinity chromatography (Brantmeier *et al.*, 1988). Purity of lipoprotein fractions was determined by sodium-dodecyl-sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using 5 and 15% acrylamide gels (Brantmeier *et al.*, 1988). Protein concentration of low-density lipoproteins was estimated by Bradford analysis with a gamma-globulin standard (Bradford, 1976).

Coupling of Fn, Lm or LDL ligand to affinity chromatography columns. Plasma fibronectin from cows (Calbiochem, La Jolla, CA, USA; *M_r* 500 000; single band SDS-PAGE), or laminin from mouse tumours (Collaborative Research, Inc., Bedford, MA, USA; 95% pure after reduction, on 7% SDS-PAGE) was coupled to Tressyl-activated Sepharose 4B (Pharmacia, Piscataway, NJ, USA), 0.50 mg dry weight per column. The Sepharose 4B was washed and allowed to

swell in 50 ml 1 M-HCl for 15 min, centrifuged (700 g, 5 min), and the supernatant was discarded. The Sepharose was then washed three times with 10 ml coupling buffer (1 M-NaHCO₃, 0.5 M-NaCl, pH 8.3).

Fibronectin or laminin (5 mg) was dissolved in 10 ml coupling buffer and mixed thoroughly with 1 ml swollen Tresyl-Sepharose for 2 h. After binding, the ligand-conjugated Sepharose was again centrifuged (700 g, 5 min) and the supernatant was saved for protein assay. Excess ligand was washed from beads with coupling buffer, which was saved for protein assay. Unbound active sites on the Sepharose were then blocked by mixing with blocking buffer (0.1 M-Tris-HCl with 0.2 M-glycine, pH 8) for 2 h. The ligand-conjugated Sepharose beads were obtained by centrifugation and washed with three alternating cycles of acetate buffer (0.1 M-NaC₂H₃O₂ · 3H₂O with 0.5 M-NaCl, pH 4) and Tris-washing buffer (0.1 M-Tris buffer with 0.5 M-NaCl, pH 8). The Sepharose bound to the extracellular matrix was washed a final time and equilibrated in Tris buffer (0.05 M-Tris-HCl, 1 mM-MgCl₂, 1 M-CaCl₂, plus 0.01% sodium azide, pH 7.2) and poured into a column consisting of a 1 ml tuberculin syringe. Subsequent use of the term 'Tris buffer' in this paper refers to this equilibration buffer.

The low-density lipoproteins were coupled to a Tresyl-activated Sepharose 4B affinity column with a procedure similar to that described above but the conjugated Sepharose was washed and separated from buffers on a sintered glass filter rather than by centrifugation. Purified low-density lipoproteins (400 mg) were applied to 1 g of swollen Sepharose. The equilibrating buffer was 0.01 M-Tris, 0.01 M-CaCl₂, 0.02% sodium azide, pH 8.0. The gel was poured into a 15 × 35 mm plastic column.

Affinity chromatography of GAGs. [³H]heparin (2 µCi in 100 µl Tris buffer, New England Nuclear, Wilmington, DE, USA; sp. act. 0.34 mCi/mg) was applied to the affinity columns to determine the binding between a commercially available GAG and the extracellular matrix ligand. After allowing a 1 h equilibration time, unbound [³H]heparin was eluted from the column in 10 ml Tris buffer collected in 10 1-ml fractions at a flow rate of 0.33 ml/min. Bound [³H]heparin was eluted from the column with a step-wise 2 M-NaCl gradient in 10 ml Tris buffer collected in 10 1-ml fractions. Each fraction was mixed with 20 ml scintillation cocktail (Ecoscint: National Diagnostics, Manville, NJ, USA) shaken overnight, and monitored for radioactivity for 1 min on a Packard Tri-Carb liquid scintillation spectrophotometer.

Samples of dermatan sulphate and heparan sulphate from large and small follicles (100 µg) were loaded onto each of the fibronectin and laminin columns, and 500 µg samples were loaded onto the low-density lipoproteins column, and equilibrated for 1 h at 4°C. Unbound GAG was eluted from the fibronectin and laminin columns with 10 ml Tris buffer at a flow rate of 0.33 ml/min. Bound GAG was eluted from each column with 2 M-NaCl in 10 ml Tris buffer at the same flow rate. The columns were subsequently equilibrated with 10 ml Tris buffer. The low-density lipoprotein column was eluted initially with 25 ml Tris buffer, followed by 50 ml Tris buffer containing 2 M-NaCl, and then re-equilibrated with 50 ml Tris buffer. Each of those eluates was collected as a single fraction.

Samples of dermatan sulphate and heparan sulphate that did not bind to fibronectin or laminin in previous runs were subsequently applied to the extracellular matrix columns and eluted in a similar manner to confirm that overloading of the columns did not occur. This step was omitted with the low-density lipoprotein column.

Quantitation of the GAGs by h.p.l.c. Samples of each eluted fraction (200 µl) were analysed for the concentration of GAG by gel filtration high performance liquid chromatography (h.p.l.c.) using a Waters Associates I-125 Protein Pak column at a flow rate of 2.0 ml/min and a run time of 12 min. Absorbance was monitored at 205 nm using a Perkin-Elmer LC-75 spectrophotometer (Grimek & Ax, 1982). Glycosaminoglycan concentrations were determined by comparison of integrated areas of known masses (6.25–100 µg) of purified heparan sulphate and dermatan sulphate standards (Bellin & Ax, 1987).

Statistics. Each type of GAG was tested in duplicate or triplicate, with each replication representing heparan sulphate or dermatan sulphate purified from follicles harvested during separate trips to an abattoir. The amount of GAG binding to the extracellular matrix or low-density lipoprotein was expressed as µg adsorbed per 100 µg loaded. A log transformation of the data was performed to normalize variance. The data were analysed using general linear models (GLM) procedures with the Statistical Analysis System (SAS Institute Inc., Box 8000, Cary, NC 27511, USA).

Results

Binding of GAGs to fibronectin or laminin

A portion of the [³H]heparin applied to the fibronectin and laminin columns did not bind (66%) and was eluted with Tris buffer. The remaining portion of [³H]heparin did bind (33%) to the column and was collected in the 2 M-NaCl Tris fraction (Fig. 1).

A larger proportion of GAGs from small follicles bound to the extracellular matrix components than did GAGs from large follicles ($P < 0.05$); however, the significance of this effect was due primarily to the interaction of dermatan sulphate with the extracellular matrix components from small follicles ($P < 0.05$, Fig. 2). A higher percentage of dermatan sulphate from small follicles ($P < 0.05$) bound to fibronectin than did dermatan sulphate from large follicles or heparan sulphate from large and small follicles, which each bound to fibronectin in similar proportions. A

Fig. 1. The interaction of $[^3\text{H}]$ heparin with fibronectin. Unbound $[^3\text{H}]$ heparin was eluted from a fibronectin–Sephacryl affinity chromatography column in 10 fractions. Radioactivity was monitored on a liquid scintillation spectrophotometer. The cumulative GAG from those fractions is depicted in the unbound column. The $[^3\text{H}]$ heparin that did bind to fibronectin was eluted from the column in a step-wise gradient of 10 1-ml fractions of 2 M-NaCl in Tris buffer. The cumulative amount of GAG from those fractions is depicted in the bound column.

greater percentage of GAG from small follicles bound to laminin than did GAG from large follicles ($P < 0.05$, Fig. 2). GAG which initially did not bind to fibronectin or laminin was rechromatographed and did not bind on the subsequent column run, indicating that GAG in the unbound fractions did not represent overloading of the column in the original column runs.

Fig. 2. The percentage of bound portions of follicular fluid glycosaminoglycans (GAG) dermatan sulphate (DS-) or heparan sulphate (HS-) from small (s) or large (l) follicles with fibronectin (Fn) or laminin (Lm). The values represent the mean $\pm$ s.e. for $n = 2$ GAG samples tested with Fn, and $n = 3$ (DS-s and DS-l) or $n = 2$ (HS-s and HS-l) for GAG samples tested with Lm. A greater amount of DS bound to the extracellular matrix proteins than did HS ($P < 0.05$). A greater amount of GAG from small follicles bound to Fn or Lm than did GAG from large follicles ($P < 0.05$).

Binding of GAGs to low-density lipoprotein

Only a small proportion of each follicular fluid GAG interacted with low-density lipoprotein. For each 100 μg of DS-s, DS-l and HS-l applied to LDL, 13.8%, 14.2% and 5.8% bound to the LDL, respectively. Affinity of heparan sulphate from small follicles for low-density lipoprotein was not determined due to lack of available sample. Proportions of the GAGs which bound to low-density lipoprotein were lower than binding observed with extracellular matrix constituents.

Discussion

Glycosaminoglycans isolated from ovarian follicles interacted with components of the extracellular matrix, fibronectin and laminin and with low-density lipoprotein. Furthermore, dermatan sulphate isolated from small follicles exhibited a greater ability to bind to the extracellular matrix components than did dermatan sulphate from large follicles or heparan sulphate from either small or large follicles.

Dermatan sulphate from small follicles bound to fibronectin in higher proportions than that from large follicles. This suggests that dermatan sulphate displays different physicochemical properties at various stages of follicular maturation. That premise is supported by data from our laboratory which indicate that composite follicular GAGs isolated at different times relative to the LH surge in cattle varied in their ability to bind to fibronectin (Vanderboom *et al.*, 1989). Since dermatan sulphate from follicles of different sizes bound fibronectin differently, it is apparent that fibronectin could potentially serve as an affinity ligand to segregate follicular fluid dermatan sulphate isomers. Fibronectin has previously been used for isolating placental proteoglycans (Isemura *et al.*, 1987).

Dermatan sulphate and heparan sulphate account for approximately 90% and 10%, respectively, of the GAGs found in follicular fluid and secreted by granulosa cells *in vitro* (Ax & Bellin, 1988). The dermatan sulphate proteoglycans exist as two forms with different hydrodynamic sizes. Concentrations of the lower molecular weight forms vary with follicular maturation (Grimek & Ax, 1982; Grimek *et al.*, 1984), and production of a similar dermatan sulphate of low molecular weight varies when granulosa cells are exposed to gonadotrophins or cyclic nucleotides (Yanagishita & Hascall, 1981).

The dermatan sulphate chains in the lower molecular weight proteoglycans exhibit a shift in the ratio of galactosamine to glucosamine from approximately 3:1 in small follicles to greater than 8:1 in large follicles (Grimek *et al.*, 1984; Bellin & Ax, 1987). Jensen & Zachariae (1958) reported an increase in the proportions of galactosamine to glucosamine in composite follicular GAG samples. Therefore, the higher binding of dermatan sulphate from small than from large follicles when applied to fibronectin and laminin may be related to a subtle chemical difference in the composition of those GAG chains. Future studies should be designed to compare binding to extracellular matrix constituents of dermatan sulphate preparations with different chemical features. Additionally, dermatan sulphate chains separated from each of the higher and lower molecular weight proteoglycans as well as intact proteoglycans of both hydrodynamic sizes should be applied to fibronectin columns. Current studies are in progress to determine the chemical composition of GAGs which bind or do not bind to extracellular matrix affinity columns to determine chemical characteristics which may be important for those binding interactions.

In contrast with the variance observed in GAG-extracellular matrix interaction, little difference was noted between the interaction of GAGs isolated from follicles of different sizes and low-density lipoprotein in this experiment. Dermatan sulphate from immature pig follicles inhibited low-density lipoprotein degradation and progesterone production by granulosa cells in a dose-dependent manner (Bellin *et al.*, 1987a). Commercial chondroitin-4-sulphate also altered basal and gonadotrophin-induced secretion of progesterone from granulosa cells (Ledwitz-Rigby *et al.*, 1987). Additional studies are needed to determine whether the size of follicle from which GAGs are isolated influences low-density lipoprotein degradation. Two aspects of interaction between GAGs and lipoproteins have been suggested. Glycosaminoglycans may uncouple low-density lipoproteins from cell membranes or may interact directly with them, resulting in steric hindrance or conformational changes in the apolipoprotein that prevent lipoprotein interaction with cell membrane receptors (Strauss *et al.*, 1985). The results of the present study indicate that only a small portion of follicular GAGs interact with low-density lipoproteins with minor differences among GAGs obtained from follicles of different sizes. However, greater binding affinity was observed

between purified follicular fluid GAGs and low-density lipoproteins than between a composite of follicular fluid GAGs and high-density lipoprotein (Brantmeier *et al.*, 1988). In that study, approximately 2% of the GAG bound to high-density lipoprotein.

In conclusion, these studies show that follicular fluid GAG interactions with extracellular matrix proteins are dependent upon the maturity of the follicles from which GAGs are isolated. Further research should focus on specific relationships between extracellular matrix components and isomers of GAG constituents of proteoglycans that are produced in response to various hormonal stimuli. Fibronectin can serve as a potential affinity chromatography ligand to isolate specific sub-populations of GAGs, particularly dermatan sulphate, from follicular fluid for further chemical and functional characterizations.

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