Glycobiology vol. 11 no. 3 pp. 183–194, 2001

Anticoagulant heparan sulfate proteoglycans expression in the rat ovary peaks in preovulatory granulosa cells

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Received on June 27, 2000; revised on October 3, 2000; accepted on October 4, 2000

Ovarian granulosa cells synthesize anticoagulant heparan sulfate proteoglycans (aHSPGs), which bind and activate antithrombin III. To determine if aHSPGs could contribute to the control of proteolytic activities involved in follicular development and ovulation, we studied the pattern of expression of these proteoglycans during the ovarian cycle. aHSPGs were localized on cells and tissues by ^{125}I-labeled antithrombin III binding followed by microscopic autoradiography. Localization of aHSPGs has shown that cultured granulosa cells, hormonally stimulated by gonadotropins to differentiate *in vitro*, up-regulate their synthesis and release of aHSPGs. *In vivo*, during gonadotropin-stimulated cycle, aHSPGs are present on granulosa cells of antral follicles and are strongly labeled in preovulatory follicles. These data demonstrate that aHSPG expression in the ovarian follicle is hormonally induced to culminate in preovulatory follicles. Moreover, we have shown that five heparan sulfate core proteins mRNA (perlecan; syndecan-1, -2, and -4; and glypican-1) are synthesized by granulosa cells, providing attachment for anticoagulant heparan sulfate chains on the cell surface and in the extracellular matrix. These core proteins are constantly expressed during the cycle, indicating that modulations of aHSPG levels observed in the ovary are likely controlled at the level of the biosynthesis of anticoagulant heparan sulfate glycosaminoglycan chains. This expression pattern enables aHSPGs to focus serine protease inhibitors in the developing follicle to control proteolysis and fibrin formation at ovulation.

Key words: Anticoagulant heparan sulfate/antithrombin III/ heparan sulfate proteoglycan/ovarian granulosa cells/coagulation

Introduction

Heparan sulfate proteoglycans (HSPGs) are synthesized by most cells and are present on cell surfaces and in extracellular matrix, according to the nature of their core protein (David, 1992). The structural diversity of heparan sulfate chains (HS) provides HSPG with numerous biological activities corresponding to precise sulfated oligosaccharide structures (Lyon and Gallagher, 1998). Anticoagulant HS (aHS) contain a pentasaccharide sequence common to heparin that specifically binds and activates antithrombin III (AT) by inducing a conformational change in the inhibitor that stabilizes its active conformer (Carrell *et al.*, 1997; Rosenberg *et al.*, 1997).

Anticoagulant HSPGs (aHSPGs) are synthesized by endothelial cells and endow vessel walls with antithrombotic properties (de Agostini *et al.*, 1990; Rosenberg and Aird, 1999). In addition, a heparin-like activity has been detected in ovarian granulosa cells and follicular fluid (Andrade-Gordon *et al.*, 1992), and we have demonstrated that cultured rat granulosa cells synthesize significant amounts of aHSPGs, similar to that produced by endothelial cells (Hosseini *et al.*, 1996). Granulosa cell HSPG core proteins have been originally described by Yanagishita and Hascall, who reported the synthesis of a membrane-spanning and a glycosyl-phosphatidylinositol (GPI)–anchored HSPGs (Yanagishita and Hascall, 1984, Yanagishita and McQuillan, 1989). Recently, expression of the four members of the syndecan family of HSPGs was reported in the mouse ovary, syndecan-4 being up-regulated in degenerating atretic follicles (Ishiguro *et al.*, 1999). Moreover, human follicular fluid was shown to contain a composite form of HSPG core protein immunologically related to perlecan (Eriksen *et al.*, 1999).

The inner ovarian follicle is formed by granulosa cells surrounding the oocyte and remains avascular until ovulation (Fortune, 1994). The production of aHSPGs requires the assembly of a complex biosynthetic pathway able to generate AT-binding pentasaccharide sequences (Rosenberg *et al.*, 1997). This synthesis in ovarian granulosa cells suggests a previously unsuspected function of aHSPGs outside from the vascular bed. At ovulation, plasma proteins leak from permeabilized vessels surrounding ovulatory follicles and fibrin gets deposited in the outer layers of the follicle (Dvorak *et al.*, 1999).

Granulosa cell aHSPGs could be critically expressed during the development of ovarian follicles, to prevent clotting of follicular fluid and thus contribute to the maintenance of fluidity in the environment of the oocyte. To test this hypothesis, this study evaluated the expression pattern of aHSPGs in granulosa cells in response to hormonal stimulations *in vitro* and *in vivo*. We measured the aHSPGs produced by granulosa cells following gonadotropin stimulations *in vitro* and simultaneously visualized aHSPGs on the cells by microscopic autoradiography. The aHSPGs have then been localized on ovary cryosections taken along gonadotropin-stimulated cycles in

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immature rats to show the modulation of aHSPG expression in follicles from the ovulatory cohort. In parallel, heparan sulfate core proteins expression was assessed at the mRNA level to clarify the identity of the proteoglycans involved in this system.

Results

Modulation of cultured rat granulosa cell aHSPGs by gonadotrophins stimulation

Primary rat granulosa cells were sequentially stimulated in culture by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and cell-bound and soluble aHSPGs were determined using ^{125}I-labeled-AT (^{125}I-AT) cell- and ligand-binding assays, respectively. The cells responded to FSH by increasing their estradiol synthesis and to LH by markedly increasing their progesterone synthesis (data not shown). Stimulation by FSH altered the partitioning of aHSPGs between cell-surface and soluble forms, favoring the release of aHSPGs in culture medium, as previously reported (Hosseini *et al.*, 1996). Further stimulation with LH accentuated this trend, and we observed a sixfold increase of soluble aHSPGs in culture media from LH-stimulated cells as compared with nonstimulated controls (Student paired *t*-test, $p < 0.01$, $n = 7$) whereas cell-bound aHSPGs were not significantly modified (data not shown).

We next examined the partition between cell-bound and soluble aHSPGs using normalized values, and Table I shows that 83% aHSPGs were present on the cell surfaces and 17% in the medium under basal conditions. In FSH-primed granulosa cells, aHSPGs were evenly distributed between the cell layer (59%) and the medium (41%), and after LH stimulation a 36% decrease was observed in the cell-bound aHSPGs, which were released into the medium. Statistical analysis showed highly significant differences between stimulated and nonstimulated conditions for soluble and cell-bound aHSPGs (Student paired *t*-test, control versus FSH, $p < 0.05$; control versus LH, $p < 0.01$). These results demonstrate that granulosa cells respond to FSH and furthermore to LH by increasing their aHSPG output, thereby altering their distribution of aHSPGs between cell surface and culture medium in favor of the liberation of cell-bound aHSPGs.

Visualization of granulosa cell aHSPGs by ^{125}I-AT microscopic autoradiography

Endothelial cell aHSPGs can be visualized by ^{125}I-AT-binding in the extracellular matrix and in subendothelial basement membranes (de Agostini *et al.*, 1990). Because our data suggest that isolated granulosa cells release much of their aHSPGs in soluble form, we have ascertained that aHSPGs could be localized on granulosa cell layers by ^{125}I-AT microscopic autoradiography on cultured granulosa cells stimulated with FSH and LH (in parallel to the experiments described in Table I). A strong labeling of granulosa cells by ^{125}I-AT was observed both after FSH and LH stimulations (Figure 1a,d), demonstrating that aHSPGs are readily detected on gonadotropin-stimulated granulosa cells. The specificity of the autoradiographic signal for ^{125}I-AT-bound aHSPGs was verified by incubating ^{125}I-AT in the presence of polysaccharide competitors and showed that the labeling is abolished in the presence of heparin (Figure 1b,c,e), whereas dextran sulfate

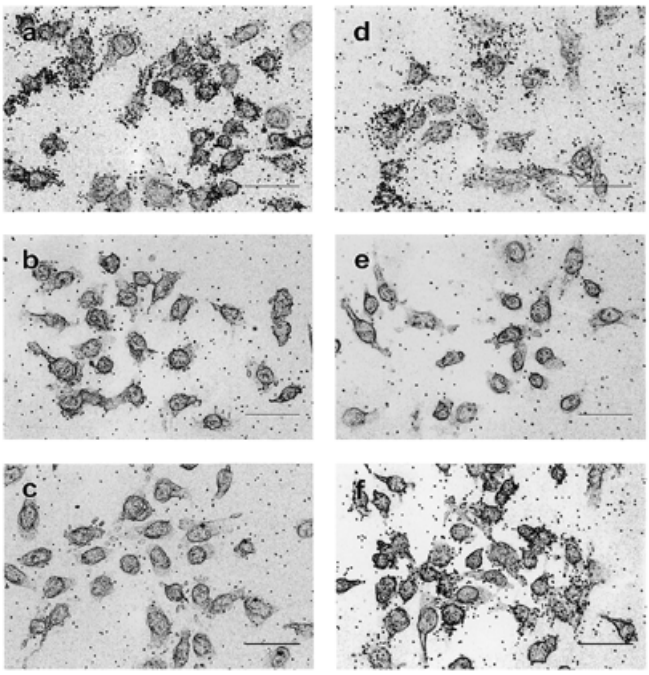

Fig. 1. Microscopic autoradiography of ^{125}I-AT bound to aHSPGs on cultured granulosa cells. DES-primed primary rat granulosa cells were cultured for 48 h in the presence of 50 ng/ml FSH (a–c) or of 50 ng/ml FSH followed by 500 ng/ml LH for an additional 48 h (d–f). Granulosa cell cultures were incubated with ^{125}I-AT alone or in the presence of competitors, fixed in ethanol, and exposed for autoradiography. (a, d) ^{125}I-AT; (f) ^{125}I-AT + heparin 10 µg/ml; (c, e) ^{125}I-AT + heparin 100 µg/ml; (f) ^{125}I-AT + dextran sulfate 100 µg/ml. Scale bar = 50 µm.

Table I. Distribution of cell-bound and soluble aHSPGs in cultured granulosa cells.

	Cell layer			Culture medium		
	Basal	FSH-primed	LH	Basal	FSH-primed	LH
% aHSPG Average	83	59*	47**	17	41*	53**
SD	8	20	14	8	20	14

% of aHSPGs = (c.p.m. in the medium or on the cell layer) / [(c.p.m. on cell layer) + (c.p.m. in the medium)] × 100. Student's paired *t*-test, ** $p < 0.01$, * $p < 0.05$. Data from seven independent experiments, average ± SD.

does not affect the labeling because it does not compete with aHSPGs for ^{125}I-AT binding (Figure 1f).

aHSPG localization in ovary cryosections

To localize aHSPGs in ovarian follicles matured *in vivo*, we labeled aHSPGs in rat ovary cryosections with ^{125}I-AT and revealed the labeling by autoradiography. Figure 2a shows the labeling of aHSPGs on the granulosa cell layers of a large preovulatory follicle. Additional punctuated labeling is present in the outer theca layers of the follicle and in the ovarian stroma, corresponding to aHSPGs synthesized by endothelial cells in blood vessels. The specificity of aHSPG labeling by ^{125}I-AT was shown on serial sections using specific glycosidases and sulfated polysaccharide competitors. Digestion of HS chains by preincubating the ovary section with heparitinase abolished subsequent binding of ^{125}I-AT to the cryosection, on granulosa cells as well as on the ovarian vasculature (Figure 2b). In contrast, digestion of chondroitin sulfate and dermatan sulfate chains by preincubating the section with chondroitinase ABC did not affect subsequent ^{125}I-AT binding to aHSPGs (Figure 2c). In addition, the binding of ^{125}I-AT to ovary cryosections in the presence of polysaccharide competitors shows that heparin competed with ovarian aHSPGs and decreased the signal to background levels (Figure 2d), whereas dextran sulfate did not compete with ovarian aHSPGs (Figure 2e). Moreover, no labeling was observed when ^{125}I-AT was incubated in the presence of excess unlabeled AT (10 µM) or of the heparin AT-binding pentasaccharide (10 µM) (data not shown).

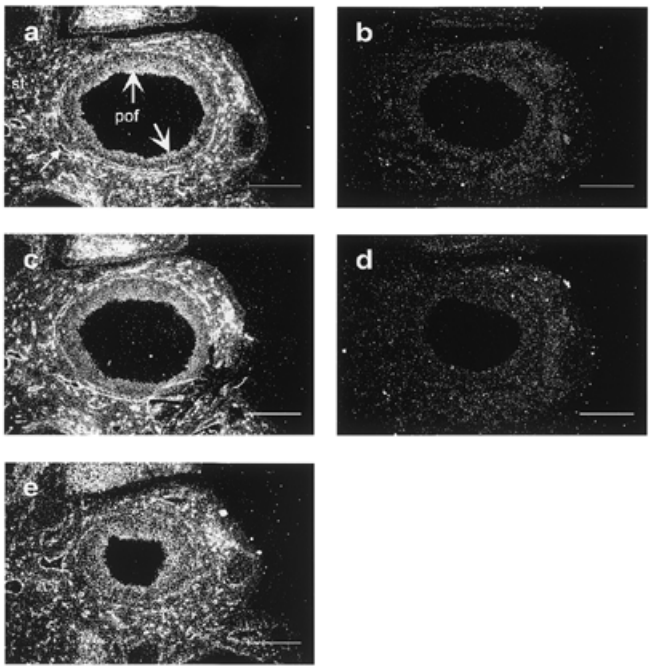

Fig. 2. Specificity of ^{125}I-AT-binding to aHSPGs in ovary cryosections: Autoradiography of ^{125}I-AT-labeled ovary cryosections in dark field exposure, oxidized silver grains appearing in white. (a) Incubation with ^{125}I-AT alone. Preovulatory follicle (pof) ; stroma (st); granulosa cells (bold arrows), blood vessels (arrow); (b, c) preincubation of the cryosection with heparitinase (b) or with chondroitinase ABC (c) and subsequent incubation with ^{125}I-AT; (d, e) incubation of ^{125}I-AT in the presence of 100 µg/ml of the competitors heparin (d) or dextran sulfate (e). Scale bar = 200 µm.

Granulosa cell aHSPGs are independent from vascular wall aHSPGs

Labeling of ovary cryosections with ^{125}I-AT highlights the aHSPGs present in the subendothelial basement membranes of capillaries and vessels. To ascertain that granulosa cell aHSPGs could be distinguished from aHSPGs originating from the vasculature, we compared serial ovary sections, stained for histology (Figure 3a,d), for aHSPGs (Figure 3b,e), and for endothelial cells (Figure 3c,f). The ovaries chosen to illustrate this point were taken during a gonadotropin-stimulated cycle, during the follicular phase (48 h after stimulation by pregnant mare serum gonadotrophin [PMSG], Figure 3a–c), and just before ovulation (6 h after stimulation with human chorionic gonadotrophin [hCG], Figure 3d–f). Histological staining (Figure 3a,d) shows the presence of large and small follicles with distinct inner compartments containing granulosa cells and the oocyte and delimited by a basement membrane. aHSPGs are detected over the whole surface of granulosa cells in large follicles and also, as punctuated labeling, on the capillaries embedded in theca layers (Figure 3b,e). Theca cells themselves, as well as the oocyte, are negative for aHSPG labeling. Specific immunostaining of endothelial cells confirms the presence of numerous capillaries in outer theca layers of the follicles, as well as in the ovarian stroma (Figure 3c,f). In contrast, the inner compartment of follicles is completely devoid of vasculature, as shown by the absence of endothelial cell staining inside the follicular basement membrane. These results demonstrate that the aHSPGs are located on granulosa cell layers of large antral follicles in the absence of vasculature in this compartment. Moreover, aHSPGs are detected on granulosa cells in antral follicles but not in neighboring small secondary follicles (Figure 3b,e), in keeping with *in vitro* observations showing an increase of aHSPG production upon stimulation of granulosa cells by FSH.

aHSPG localization in the rat ovary during the cycle

The pattern of expression of aHSPGs in the ovary during the reproductive cycle was revealed by ^{125}I-AT-binding followed by autoradiography on ovary cryosections. We have used a model of gonadotropin-induced ovulation in immature female rats and analyzed ovaries taken 48 h after PMSG administration, as well as 6 h, 12 h, 24 h, and 72 h after ovulation induction by hCG. We have followed aHSPG labeling in the large cohort of follicles induced by gonadotropins to develop, ovulate, and form corpora lutea (Figure 4).

In untreated 23-day-old immature animals, the rat ovary contains some early antral follicles, many small preantral and primary follicles, and many primordial dormant follicles clustered in the cortical region (Figure 4a,b). aHSPG labeling was present around primordial follicles, associated with the single granulosa cell layers and/or with the follicular basement membrane (Figure 4a). This labeling vanished in small growing primary and secondary follicles, which are not labeled (Figure 4b). A weak aHSPG labeling appears on granulosa cells from follicles in the early antral stage, observed in animals treated with the estrogen analog diethyl-stilbestrol (DES) (Figure 4c). In contrast, 48 h after stimulation with

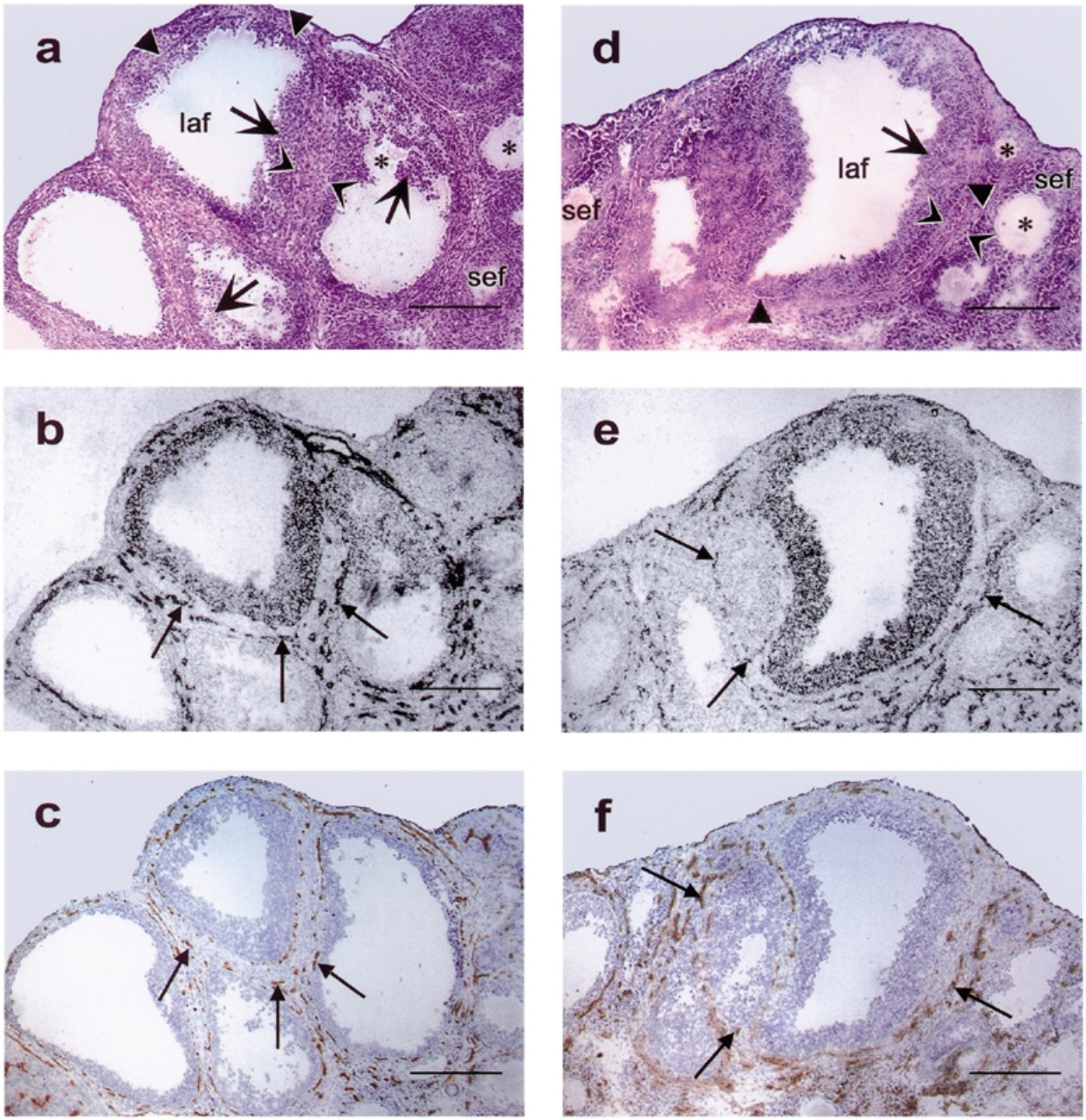

Fig. 3. Granulosa cell aHSPGs are independent from vascular wall aHSPGs : Ovary sections from immature rats stimulated with PMSG for 48 h (a–c) followed by hCG for 6 h (d–f). Serial cryosections showing tissue histology, aHSPGs and endothelial cells. (a, d) hematoxylin/eosin, large antral follicle (laf); secondary follicle (sef); oocyte (*), granulosa cells (bold arrows), blood vessel (arrows), theca cells (partial arrowheads), follicular basement membrane (full arrowheads). (b, e) autoradiogram of section incubated with ^{125}I-AT in light-field exposure, oxidized silver grain appearing in black. (c, f) immunohistological detection of endothelial cells with RECA-1 monoclonal antibody. Scale bar = 200 µm.

FSH, many follicles developed to large antral follicles, and the granulosa cell layer of these follicles is strongly labeled for aHSPGs (Figure 4d). Upon ovulation induction by hCG, aHSPGs remain abundant on the whole granulosa cell layer of follicles at the preovulatory stage (6 h after hCG, Figure 4e), as well as at the time of ovulation, 12 h after hCG induction (Figure 4f). After ovulation, 24 h after hCG injection, the aHSPG signal has largely decreased, and a low diffuse signal is observed on the luteinized cells of the postovulatory follicle (Figure 4g). Seventy-two hours after hCG treatment, the corpus luteum is labeled for aHSPGs in a complex manner. The diffuse labeling observed on luteal cells 24 h after hCG is still present and is most evident in the center of the corpus luteum, which is still devoid of capillaries. In addition, the radial capillary network of the corpus luteum is labeled for aHSPGs (Figure 4h).

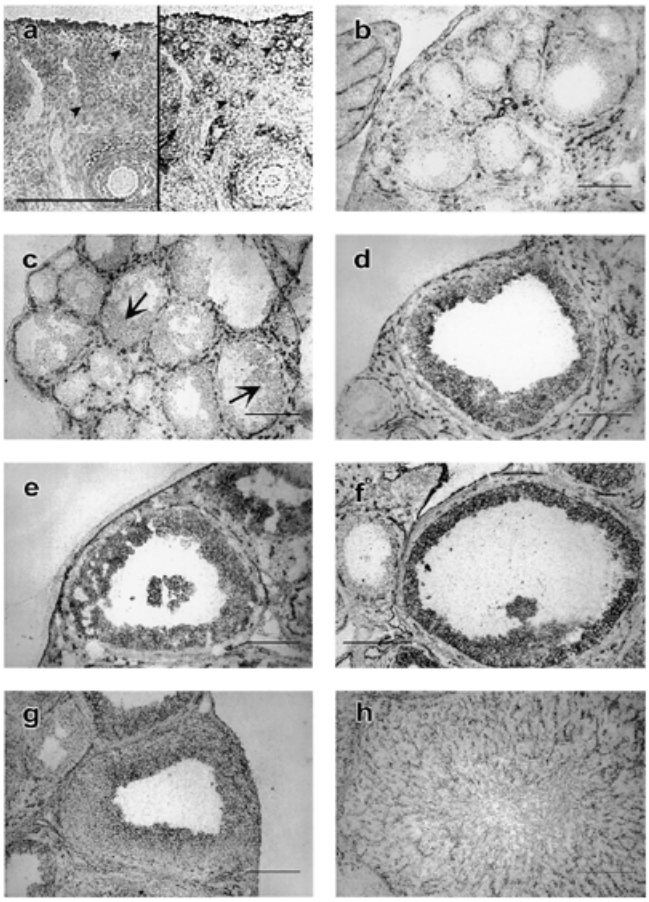

Fig. 4. aHSPG localization in the rat ovary during stimulated cycles: Autoradiographic detection of ^{125}I-AT-labeled aHSPGs on ovary cryosections. (a) immature ovary, serial sections; left: histological staining, right: autoradiography; primordial follicles (arrowheads) are labeled for aHSPGs. (b) immature ovary with unlabeled primary and secondary follicles. (c) ovary from an immature female treated with DES, granulosa cells of early antral follicles are weakly labeled (bold arrows). (d–h) Autoradiography of ovary sections illustrating aHSPG labeling on ovulatory follicles during a gonadotropin-stimulated cycle. (d) PMSG 48 h, (e) hCG 6 h, (f) hCG 12 h, (g) hCG 24 h, (h) hCG 72 h. Scale bar = 200 µm.

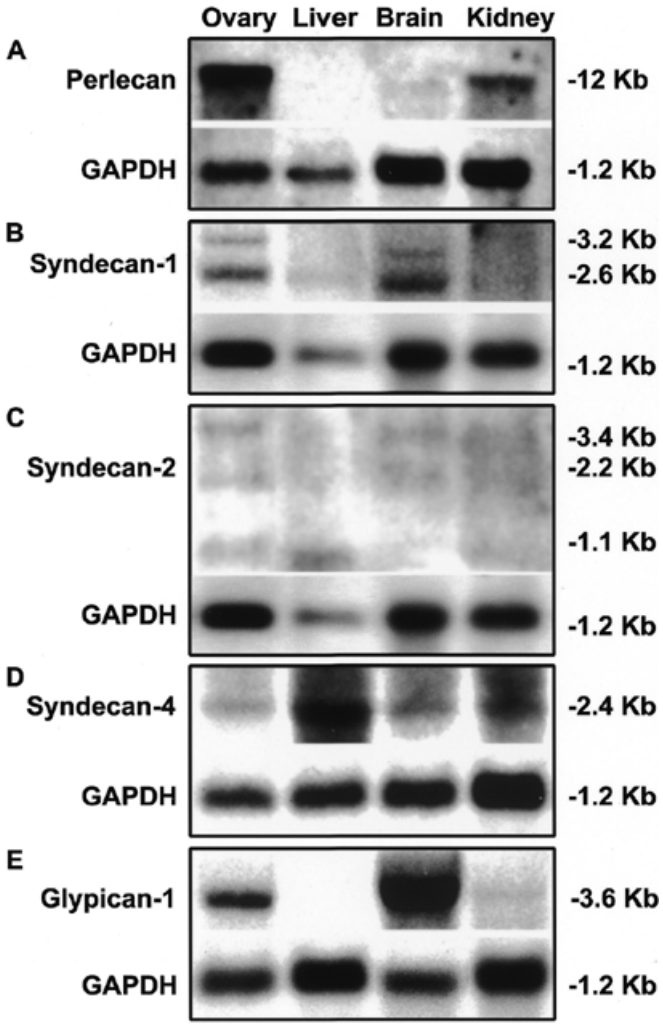

Fig. 5. mRNA expression of HSPG core proteins in the ovary: Northern blot analysis of total RNA (10 µg) from rat ovary, liver, brain, and kidney were hybridized with HSPG core protein probes as outlined in Materials and methods. The upper part of each frame represents the hybridization with the different core protein probes; the lower part, the hybridization with GAPDH control probe. The size of the mRNA (kb) are indicated on the right. Perlecan: 12 kb mRNA; syndecan-1: 3.2 kb and 2.6 kb mRNA; syndecan-2: 3.4 kb, 2.2 kb, and 1.1 kb; syndecan-4: 2.4 kb mRNA; glypican-1: 3.6 kb mRNA.

Expression of HSPG core proteins mRNA in the rat ovary and in isolated granulosa cells

To identify the nature of the HSPG core proteins expressed in the ovary, in particular by granulosa cells, we performed Northern blot analysis on RNA extracted from rat ovaries and from isolated granulosa cells at different stages of the cycle.

We first assessed the expression of HSPG core proteins in ovary extracts and compared their level of mRNA to that in control organs, known for abundant or low expression of these core proteins (Figure 5). The mRNA of five distinct HSPG core proteins were found in the ovary; the basement membrane HSPG perlecan; the membrane-spanning HSPG syndecan-1, syndecan-2, and syndecan-4; and the GPI-anchored HSPG glypican-1. Perlecan, syndecan-1, and glypican-1 mRNA were readily detected; their level, normalized by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, is comparable to that of highly expressing organs. Perlecan mRNA is particularly abundant in the ovary, with a signal stronger than for the kidney, which is a rich source of perlecan. Syndecan-1 mRNA expression in the ovary is comparable with that found in the liver and the brain, with a conserved ratio (3:1) between the two mRNA splicing variants. The signal obtained for glypican-1 mRNA in the ovary is high, representing about 40% of that seen in the brain, which is the major source of glypican-1. Low level of syndecan-2 mRNA was detected in the ovary, similar to the brain and kidney, with comparable expression of the three characteristic splicing variants. Syndecan-4 expression was found at low levels in all organs, consistent with its ubiquitous distribution, with an mRNA content in the ovary comparable to the levels seen in brain and kidney and lower than in the liver.

To determine if the HSPG mRNA detected in the ovary was produced by granulosa cells, we next compared the signal obtained in the ovary and in isolated granulosa cell. In addition, to find out if the variations in aHSPGs observed during the cycle by ^{125}I-AT-binding are reflected on HSPG core protein expression, we analyzed their mRNA levels at different stages of the cycle. The expression levels of HSPG core proteins mRNA were compared after normalization against GAPDH. The membrane-bound HSPG core proteins syndecan -1, -2, and -4, and of glypican-1 mRNA were all detected in isolated granulosa cells, as well as the mRNA of the extracellular matrix HSPG perlecan (Figure 6). These data demonstrate that these HSPG core proteins are expressed in ovarian granulosa cells coordinately with aHSPG synthesis, and hence aHS chains could be associated with both membrane-bound and secreted HSPGs. The level of expression of HSPG core proteins mRNAs was similar in isolated granulosa cells and in total ovary, indicating that these core proteins are also expressed by other cell types in the ovary. However, except in endothelial and granulosa cells, HSPG core proteins do not bear aHS chains, as shown by the pattern of ^{125}I-AT-labeling in ovary cryosections. The levels of expression of perlecan, syndecan-1, -2, and -4, and of glypican-1 mRNA remained relatively constant during the ovarian cycle, both in total ovary extracts and in purified granulosa cells. Signal quantification, done for perlecan, syndecan-1, and glypican-1, showed no consistent trends for variations in HSPG core proteins mRNA expression during the ovarian cycle, in three independent experiments (data not shown). Similarly, the qualitative estimation of the low signals obtained for syndecan -2 and -4 mRNA indicated no consistent differences during the cycle. In particular, the core proteins expression was not decreased after ovulation, at metestrus, or 24 h after hCG treatment, in contrast to the profound variations observed in the levels of aHSPGs present in follicles during the cycle.

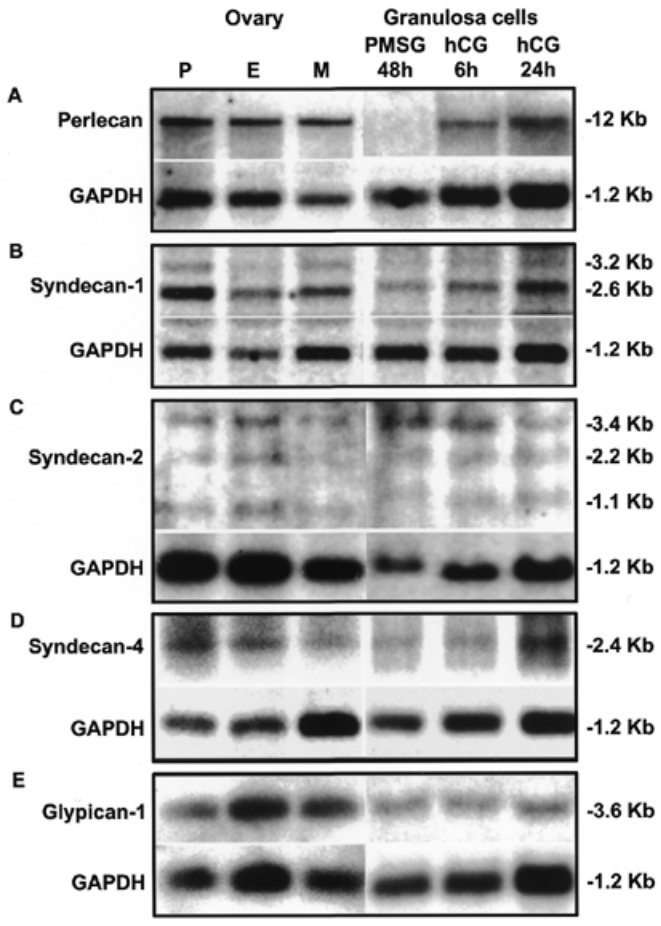

Fig. 6. HSPG mRNA expression in the ovary and in isolated granulosa cells during the cycle: Northern blot analysis of total RNA (10 µg) from rat ovary and from isolated primary granulosa cells were hybridized with HSPG core protein probes and with GAPDH probe as described. The size of the mRNA (kb) are indicated on the right. Ovary mRNA was isolated from naturally cycling animals at proestrus (P), estrus (E), and metestrus (M). Primary granulosa cell mRNA was extracted from immature animals stimulated with PMSG for 48 h, followed by hCG for 6 h and 24 h.

D-glucosaminyl-3-O-sulfotransferase-1 expression in the rat ovary and in granulosa cells

To confirm the local synthesis of aHSPG and possible biosynthetic modulation in granulosa cells, we examined the expression of D-glucosaminyl-3-O-sulfotransfease-1 in rat ovary extract and in isolated granulosa cell mRNA. The enzyme mRNA was detected in both extracts, as seen in Figure 7 by RT-PCR, and these results were confirmed by Northern blot analysis (data not shown). The signals obtained for rat ovary and granulosa cells mRNA were comparable to that of rat microvascular endothelial cells (RFPs), used as positive control. The specificity of the RT-PCR signal was ascertained by the absence of signal in control samples in which the RT enzyme was omitted.

Discussion

The results presented in this study demonstrate the dynamic expression of aHSPGs in ovarian follicles during the reproductive cycle. *In vitro*, granulosa cells increased their aHSPG synthesis in response to gonadotropin stimulations and released increased amounts of soluble aHSPGs. *In vivo*, maximal expression of aHSPGs was detected on granulosa cells of dominant follicles during the periovulatory period of gonadotropin-stimulated cycles. Furthermore, we report that ovarian granulosa cells express mRNA of five HSPG core proteins: perlecan; syndecan-1, -2, and -4; and glypican-1. No major modification of HSPG core proteins mRNA levels was noted at

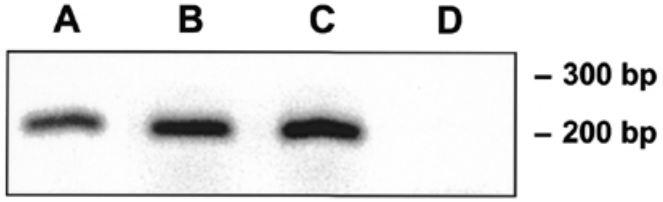

Fig. 7. D-glucosaminyl-3-O-sulfotransferase-1 expression in the rat ovary and in granulosa cells: Resolution of RT-PCR products (219 bp, primers 1S/2A as described in Materials and methods) by 2% agarose gel electrophoresis with SYBR green staining. The positions of relevant DNA size markers are indicated. (A) rat ovary, (B) rat endothelial cells (RFP), (C) isolated rat granulosa cells, (D) negative control, rat ovary control with RT omitted.

different stages of the ovarian cycle, in contrast with the impressive variations in aHSPGs detected during the cycle. These data demonstrate that the modulation of aHSPG production in granulosa cells is most likely not directly governed by the level of HSPG core proteins expression and suggest that it occurs at the level of the biosynthesis of aHS chains.

The growth and differentiation of ovarian follicles is largely dependent on sequential stimulation by FSH and LH; FSH induces follicular development and maturation, and LH triggers ovulation and luteinization of the follicle to develop a corpus luteum. We have previously studied the modulation of aHSPG expression in rat granulosa cells stimulated by FSH (Hosseini *et al.*, 1996). The present study extends these observations to subsequent LH stimulation, thereby mimicking the differentiation stage achieved by granulosa cells *in vivo* at ovulation. We have found that FSH and LH reinforce each other to up-regulate granulosa cell biosynthesis of aHSPGs and to increase the fraction of aHSPGs released in soluble form in the culture medium. The general biosynthesis of HSPG in granulosa cells is known to be increased after gonadotropin stimulations (Adashi *et al.*, 1986; Ax and Ryan, 1979; Salustri *et al.*, 1989; Yanagishita *et al.*, 1981). However, aHS chains represent about 7% of the total HS synthesized by granulosa cells (Hosseini *et al.*, 1996), and their production depends on the coordinated action of several biosynthetic enzymes, directing the construction of the pentasaccharide sequence specifically binding AT. In particular, D-glucosaminyl-3-O-sulfotransferase-1 is a key enzyme in aHS synthesis, and its expression level has been directly correlated to aHS synthesis (Shworak *et al.*, 1997; Zhang *et al.*, 1998). A similar mechanism could up-regulate aHSPG production by ovarian granulosa cells during gonadotropin-induced differentiation, in parallel to the modulation of the general biosynthesis of HSPGs.

We next set out to evaluate the pattern of expression of aHSPGs during the cyclic development of ovarian follicles *in vivo*. We have established a method of detection based on the specific binding of aHSPGs by ^{125}I-AT, revealed by microscopic autoradiography. The aHSPGs could be visualized on isolated cultured granulosa cells and on ovary cryosections, and the specificity of aHSPG labeling was demonstrated using glycosidase digestions and glycosaminoglycan competitors. The aHSPG labeling was seen on granulosa cells of antral follicles and on blood vessels endothelial cells, but not on theca cells. The inner follicular compartment remains avascular until ovulation (Amsterdam and Rotmensch, 1987), hence the intense labeling of granulosa cells from large antral follicles is due to aHSPGs endogenous to the follicle, independently from endothelial cell labeling.

We defined the localization of aHSPGs throughout the ovarian cycle induced by gonadotropin treatment of immature animals. Interestingly, primordial follicles, (diameter 19–28 μm) containing 8 to 12 flattened granulosa cells in cross sections with no distinguishable theca cells (Hirshfield, 1991), are strongly labeled for aHSPGs at their periphery, and, by virtue of their ability to activate serine protease inhibitors, they might serve to protect primordial follicles from the active proteolysis occurring in neighboring developing follicles. The aHSPGs quickly disappear from follicles at the onset of development in primary and secondary preantral follicles. This down-regulation of aHSPGs is reminiscent of the observed decrease in syndecan-1 expression in migrating keratinocytes during wound healing or in cells with high metastatic potential (Matsumoto *et al.*, 1997; Subramanian *et al.*, 1997). The stimulation of animals with DES mimicks the initial stages of follicular development, brought about by the elevated estrogen levels occurring in the preceding cycle. Granulosa cells from DES-treated animals are weakly labeled for aHSPGs, indicating that estrogens alone do not induce aHSPGs. In contrast, after FSH induction, granulosa cells from large antral follicles are heavily labeled for aHSPGs, and this labeling is consistently found throughout the periovulatory period. These observations are in agreement with the increased production of aHSPGs induced by gonadotropins in cultured granulosa cells. Stimulated granulosa cells released increased amounts of aHSPGs in soluble form. In localization experiments, soluble aHSPGs cannot be detected, because the labeling procedure is performed on unfixed material, resulting in the loss of soluble species in washes. In addition, cells in culture are widely exposed to the culture medium and constitutively release increased amounts of soluble material, including proteoglycans, while in tissues the tridimensional architecture provides extensive cell–cell and cell–matrix interactions (Kim *et al.*, 1994). Therefore, the increased labeling of aHSPGs on granulosa cells of preovulatory follicles does not preclude the parallel release of aHSPGs into follicular fluid. Indeed, we have detected aHSPGs in rat follicular fluid, collected during the preparation of granulosa cells from animals stimulated by gonadotropins (data not shown), and large amounts of HSPGs have been detected in human follicular fluid (Eriksen *et al.*, 1999). These data suggest that aHSPGs accumulate in follicular fluid and are released at ovulation. After ovulation, aHSPG labeling is decreased but still present on luteinized granulosa cells. The follicular architecture is rapidly evolving during corpus luteum formation, the antral cavity fills with a fibrin clot, and granulosa and theca luteal cells intermix to invade this space, closely followed by rapidly expanding capillaries (Fortune, 1994; Kamat *et al.*, 1995; Phillips *et al.*, 1990; Reynolds *et al.*, 1992). aHSPG staining of corpus luteum is a superposition of a diffused weak yet positive staining of granulosa luteal cells and of the focused labeling of capillaries.

In summary, aHSPGs are very strongly expressed on granulosa cells of ovulatory follicles until ovulation. Hence, we postulate that the inner follicle displays a strongly anticoagulant surface protecting it from fibrin deposition, which ensures the maintenance of fluidity of follicular fluid until expulsion of the oocyte at ovulation. The widespread localization of aHSPGs on the entire granulosa cells volume, and likely also in follicular fluid, is probably necessary to control the large influx of procoagulant plasma proteins that leak from blood vessels during the inflammatory vascular permeabilization occurring at ovulation (Dvorak *et al.*, 1999). It is noteworthy that fibrin depositions occur in outer theca layers, which are devoid of aHSPGs, underlining their functional importance on granulosa cells in the inner follicle. The rapid decrease in aHSPGs observed on the surface of luteinized granulosa cells after ovulation corresponds to a change to a more procoagulant surface, which is permissive for a fibrin clot to form, filling the antral cavity with a provisional matrix for invading luteal cells. Alternatively, aHSPGs could interact with other heparin-activated serine protease inhibitors present in the follicle, such as protease nexin-1 and plasminogen activator inhibitor-1, and be involved

in the control of the proteolytic breakdown of the follicular wall at ovulation.

To further analyze the nature of aHSPGs in the follicle, we investigated the identity of HSPG core proteins expressed in the ovary by granulosa cells, to which aHS can be attached. We have identified five HSPG core proteins mRNA expressed by ovarian granulosa cells. The basement membrane HSPG perlecan is present in granulosa cells together with at least four membrane-associated HSPG core proteins, the membrane-spanning syndecan-1, -2, and -4 and the GPI-anchored HSPG glypican-1. Early studies by Yanagishita and Hascall described the synthesis by rat granulosa cells of two HSPG membrane-bound species, one membrane-spanning and one GPI-anchored HSPG core proteins (Yanagishita and Hascall, 1992). Based on the apparent M_r reported for these HSPGs, we propose that they can be accounted for by syndecan-1 and glypican-1. Recently, Ishiguro *et al.* (1999) reported the synthesis of the four members of the syndecan family in mouse postovulatory follicles. They report levels of syndecan-3 expression lower than that of syndecan-4 in follicles before ovulation and suggest that syndecan-3 might be involved in angiogenesis in corpora lutea. By analogy, we assume that low levels of syndecan-3 could also be synthesized by rat granulosa cells. In addition, Veugelers *et al.* (1999) recently reported that glypican-6 is strongly expressed in the human ovary; however, *in situ* hybridization on mouse ovary showed mainly glypican-6 expression on mesenchymal cells. Granulosa cells are epithelial cells (Rodgers *et al.*, 1999), a cell lineage that often contains glypican-1 expression, unlike glypican-6, which is mainly expressed in mesenchymal cells. Moreover, Eriksen *et al.* (1999) purified HSPGs in human follicular fluid that are immunologically related but not identical to perlecan, suggesting that additional HPSG might be also present. Hence, rat ovarian granulosa cells express multiple HSPG core proteins. The core proteins target HS chains to their strategic positions on the cell surface or in the extracellular matrix, and they determine their accumulation and turnover at these sites. It remains to be determined if aHS chains are attached to all core proteins synthesized by granulosa cells. Whether cells can specifically direct the attachment of HS chains with particular biological activities on given HSPG core proteins is not firmly established, but mounting evidence suggests some degree of specificity in HS matching to core proteins. aHSPGs have been found on all HSPG core proteins synthesized by endothelial cells, with some preference for glypican (Mertens *et al.*, 1992). Moreover, it has been recently shown that cells can direct the attachment of HS chains with antiproliferative and adhesion properties to syndecan-1 and glypican-1, respectively (Liu *et al.*, 1998).

In view of the important variation observed in aHSPG production during the ovarian cycle, we analyzed the expression of perlecan; syndecan-1, -2, and -4; and glypican-1 in preovulatory and postovulatory stages. In total ovary extracts from naturally cycling adult rats, we did not see any striking differences in the signal obtained for HSPG core proteins in proestrus, estrus, or metestrus stages, nor did we detect consistent differences in the expression of core proteins mRNAs in granulosa cells isolated from gonadotropin-stimulated ovaries before or after ovulation. Similar observations were reported for syndecan-4 expression in mouse granulosa cells, except that syndecan-4 was up-regulated during apoptosis of atretic follicles (Ishiguro *et al.*, 1999). Granulosa cells from gonadotropin-stimulated ovaries contain little apoptotic cells until ovulation, due to the rescue action of FSH (Chun *et al.*, 1996). We observed low syndecan-4 mRNA expression before ovulation, close to our limit of detection, consistent with the low expression reported in healthy mice follicles. After ovulation, the syndecan-4 signal was also slightly higher in granulosa cells, probably due to the presence of small atretic follicles that failed to develop fully. This small increase is explained by the minor contribution of apoptotic granulosa cells in the cell population, which is dominated by healthy luteinizing granulosa cells from large postovulatory follicles (data not shown).

Thus, the remarkably stable pattern of mRNA expression of HSPG core proteins in granulosa cells before and after ovulation does not reflect the extensive variations in aHSPGs observed on granulosa cells both *in vitro* and *in vivo*. These data indicate that the expression of aHSPGs on rat granulosa cells is regulated at levels distinct from the transcription of HSPG core proteins. Additional modulation could be exerted at the level of the core proteins translation or by alterations of their catabolic pathways (Yanagishita, 1992), including the rapid removal of HSPGs from the cell surface by shedding, a regulatory mechanism that releases intact extracellular domains of the syndecan family members (Subramanian *et al.*, 1997). Such regulation could explain the reported increase in total HSPG output by stimulated granulosa cells (Salustri *et al.*, 1989; Yanagishita *et al.*, 1981), while the mRNA levels of core proteins remain stable. Furthermore, aHSPGs can also be modulated by post-translational modifications, in agreement with observations by Shworak *et al.* (1994) that syndecan-4 core protein was not the limiting factor in aHS chains production. Moreover, the expression of D-glucosaminyl-3-O-sulfotransferase-1 and the assembly of adequate aHS chain precursors have been shown to be limiting factors in the biosynthesis of aHSPGs (Shworak *et al.*, 1996; Zhang *et al.*, 1999), and these factors are likely to be involved in the hormonal modulation of aHSPG expression in the ovary. Our observation that D-glucosaminyl-3-O-sulfotransferase-1 is expressed in granulosa cells supports this view.

The ovarian follicle constitutes the only known example of aHSPG expression outside of the vascular bed, and the complex biosynthetic pathway necessary to generate AT-binding sequences suggests that aHSPGs play a key role in the regulation of proteolytic activities in this extravascular compartment. The present demonstration of the highly regulated expression of aHSPGs according to the follicular cycle strengthens the notion that aHSPG function is not restricted to their antithrombotic properties inside the vascular bed. Further studies are needed to identify aHSPG functional partners and to reveal their physiological functions in the reproductive tract.

Materials and methods

Materials

hCG was purchased from Serono. DES was obtained from Asta Medica (Switzerland). Purified ovine FSH and LH were a generous gift from Michel Aubert (Geneva University Medical School). Bovine vitronectin and tissue culture reagents were purchased from Gibco-BRL (Life Technologies, Inc.). Purified

human AT was obtained from Cutter Biological. ^{125}I-AT was prepared as previously published with a specific activity of 5×10^4 d.p.m./ng (de Agostini *et al.*, 1994). Flavobacterium heparitinase (EC 4.2.2.8.) was purchased from Seikagaku (Tokyo). Chondroitinase ABC and PMSG were from Sigma. Porcine mucosal heparin was from Diosynth Inc. (Chicago, IL) (Atha *et al.*, 1987). Pure heparin AT-binding pentasaccharide was kindly supplied by Sanofi Recherche (France). Autoradiographic emulsion type NTB2 and Dektol developer were from Kodak. Terminal deoxynucleotididyl transferase kit was from Boehringer-Mannheim, and rat endothelial cell specific monoclonal antibody RECA-1 (Duijvestijn *et al.*, 1992) was purchased from Immuno Quality Products (Groningen, Netherlands). All other chemicals used were of the highest grade available.

Heparan sulfate core protein probes

The plasmid pBR322-BPG5 containing rat perlecan cDNA was generously provided by John Hassell (Shriners Hospital, Tampa, FL) (Noonan *et al.*, 1988). We subcloned the probe prPer01, a 583-bp *Eco*RI–*Sac*II fragment of BPG5 rat perlecan cDNA, into pBluescript-KS. Probes for syndecan-1 (pNWS127) and syndecan-4 (pNWS126) were gifts from Robert D. Rosenberg (MIT, Cambridge, MA). Probe pNWS127 is a 585-bp blunt-*Bsa*HI fragment of rat syndecan cDNA inserted into pBluescript-KS. Probe pNWS126 is a 228-bp *Acc*I–*Eco*RV fragment of rat syndecan-4 cDNA inserted into pBluescript-KS (Kojima *et al.*, 1992). The probe for syndecan-2 (C17) was kindly provided by John T. Gallagher (University of Manchester, GB) (Pierce *et al.*, 1992). Probe C17 is a 2153-bp full-length cDNA of rat syndecan-2 digested by *Eco*R1 and inserted into pBluescript-SK. Probes for glypican-1 (4X1 and 4P2) were kindly provided by Arthur Lander (University of Irvine, CA) (Litwack *et al.*, 1994). Probe 4X1 is a 162-bp *Eco*RI–*Xho*I fragment of rat glypican (coding region) cDNA inserted into pBluescript-SK (Stratagene). Probe 4P2 is a 332-bp *Pst*I fragment of rat glypican (3′ untranslated region) cDNA inserted into pBluescript-SK. The plasmid pmGAPDH.FL, containing the complete cDNA of mouse GAPDH amplified by PCR and inserted into pBluescript-KS, was a gift from Pierre-Alain Menoud (Institute of Histology, Fribourg, Switzerland).

RNA extraction and Northern analyses

Total RNA was isolated from homogenized rat tissues and primary granulosa cells by the guanidium isothiocyanate method as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The extract was buffered by 0.1 volume sodium acetate 2 M and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). RNA were precipitated with an equal volume of isopropanol for 1 h at –20°C and recovered by centrifugation. Pellets were resuspended in sodium isothiocyanate 4 M, sodium citrate 25 mM, sodium-sarcosyl 0.5%, and β-mercaptoethanol and admixed with 1 volume of isopropanol. After centrifugation, pellets were washed with 75% ethanol, resuspended in RNase-free water, and their concentration determined by optical density. RNA samples were denatured in 10 mM sodium phosphate buffer, pH 6.8, containing 1 M glyoxal and 50% dimethyl sulfoxide and resolved on 1.2% agarose gels. RNA samples were transferred to Hybond-N membrane (Amersham) by capillary blotting. The membranes were vacuum backed for 2 h at 80°C. RNA size markers (Promega) were used as size and transfer efficiency markers. For quantitative comparisons of mRNA levels, hybridization signals were compared between the tested mRNA and the housekeeping gene GAPDH mRNA (Belin, 1997).

Northern blot analysis

Perlecan. Prehybridization, hybridization, and washes were carried out according to the method described by Nikkari *et al.* (1994). cDNA probe prPer01 was labeled with ^{32}P-dCTP by using the Prime-a-Gene labeling system (Promega) and hybridized at 2×10^6 c.p.m./ml.

Syndecan-1. Prehybridization and hybridization were carried out in 0.25 M sodium phosphate buffer, pH 7.2, containing 7% SDS, 1 mM EDTA, and 1% BSA. pNWS127 cDNA probe was labeled with ^{32}P-dCTP with the Prime-a-Gene labeling system and hybridized at 1×10^6 c.p.m./ml. Hybridization was carried out at 48°C for 15–20 h. The blots were then washed twice at 48°C in 2% SDS, 0.1 M sodium phosphate buffer, 1 mM EDTA for 15 min.

Syndecan-2. Prehybridization, hybridization, and washes were carried out according to the method described by Pierce *et al.* (1992). The C17 probe was labeled with ^{32}P-dCTP using the Prime-a-Gene labeling system and hybridized at 2×10^6 c.p.m./ml.

Syndecan-4. Prehybridization, hybridization, and washes were carried out according to published procedures (Belin, 1997). pNWS126 plasmid was linearized by XbaI, and pNWS126 cRNA probe was labeled with ^{32}P-UTP using T3 polymerase. The probe was hybridized at 2×10^6 c.p.m./ml.

Glypican-1. Prehybridization, hybridization and washes were carried out according to the method described by Litwack *et al.* (1994). 4X1 and 4P2 cDNA probes were labeled with ^{32}P-dCTP and ^{32}P-dATP (Hartmann, Switzerland) using the Prime-a-Gene labeling system and hybridized at 2×10^6 c.p.m./ml. The two probes were hybridized simultaneously.

All radiolabeled probes were separated from unincorporated nucleotide using G-50 Sephadex home-made 1-ml columns. All filters were exposed to Kodak XAR-5 film with two intensifying screens at –80°C for 24–48 h. Quantification of hybridization signal intensity was performed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RT-PCR detection of D-glucosaminyl-3-O-sulfotransferase-1

First-strand cDNA was generated in a 20 µl volume from 1 µg total RNA of different organs primed with gene specific primer (3A rat primer : 5′-TTGGGATCTACTTGAGGGTGCGC-3′, 1 pM) using a reverse transcriptase (RT, Life technologies) according to manufacturer's protocol. Touchdown PCR reaction conditions (10 µl) were carried out according to the method described by Shworak *et al.* (1997), using 1S rat primer: 5′-CCTGGCCCGGGACTCAAACAGCAGGG-3′ and 2A rat primer: 5′-TCCCAGTCAAAGAAATGGACCTCGTTTTC-3′ (rat cDNA sequence accession number AF177430), giving a 219-bp band resolved on 2% agarose gel electrophoresis with SYBR green staining (Molecular Probes). Rat

microvascular endothelial cells (RFP), used as positive control, were described previously (de Agostini *et al.*, 1994).

Animals

Immature 23-day-old female Sprague-Dawley rats, purchased from Iffa-Credo (L'Arbresle, France) were maintained on a 12 h day/night cycle and treated with gonadotropins to induce ovulation by sequential injection of PMSG (20 IU) and hCG (10 IU) 48 h later according to published procedures (Peng *et al.*, 1993). The animals were sacrificed by decapitation, and the ovaries were recovered before treatment (immature control), 48 h after PMSG injection, and 6 h, 12 h, 24 h, or 72 h after hCG injection. Alternatively, gonadotropin-independent granulosa cell proliferation was induced by treating 21-day-old female rats by daily subcutaneous injections of 1 mg DES in sesame oil for 4 days; the animals were sacrificed at day 25, and ovaries were used to isolate granulosa cells for culture or were frozen for microscopic analysis (Hosseini *et al.*, 1996). Regular cycles in adult female rats were documented by daily vaginal cytology for 10 days. Animals were sacrificed, and ovaries recovered at proestrus (preovulatory phase), estrus (ovulation), and metestrus (postovulatry phase).

Granulosa cell culture

Ovaries from DES-treated animals were dissected, granulosa cells isolated as described (Hosseini *et al.*, 1996), seeded in parallel in 96-well plates and on microscope slides precoated with 1 mg/ml bovine vitronectin at 0.5×10^6 cells/cm^2 and cultured for 48 h in McCoy's medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.03 mM isobutylmethylxanthine, 2.6 mM 19-hydroxyandrostendione, and 50 ng/ml ovine FSH in humidified 5% CO_2 /95% air at 37°C. For LH stimulation, the FSH-primed cells were subsequently incubated for an additional 48 h with fresh medium containing 0.5 µg/ml ovine LH. The response of cultured granulosa cells to FSH and LH was verified by quantifying estradiol and progesterone in spent medium by RIA, respectively (data not shown). Cell-bound and secreted aHSPGs were measured at the cell surface and in the medium by ^{125}I-AT cell- and ligand-binding assays as described (de Agostini *et al.*, 1994). The protein content of control wells was measured using the BCA protein assay (Pierce), and results were normalized as c.p.m./mg protein. For RNA extraction, granulosa cells were obtained as outlined above, recovered by centrifugation, and pellets were immediately extracted as described.

Tissues

Ovaries for microscopic localization of aHSPGs were rapidly excised from sacrificed animals, dissected free of ovarian fat pad, and embedded in Tissue Tek OCT compound (Miles Inc., IN, USA), frozen in precooled 2-methyl-butane and stored at –80°C. Five-micrometer cryosections were cut from frozen ovaries using a Microm cryostat, mounted on poly(L-lysine)-coated slides, and stored at –20°C until used. Duplicate serial sections were used for incubations with ^{125}I-AT, histologic staining with hematoxylin/eosin, and immunohistochemistry. For RNA extraction, dissected ovaries were snap-frozen in liquid nitrogen and kept at –80°C until extraction.

aHSPG localization

aHSPGs were localized by ^{125}I-AT-binding on ovary cryosections followed by microscopic autoradiography. Cryosections were air-dried and then preincubated in phosphate-buffered saline (PBS) (NaCl 0.15 M, sodium phosphate buffer 10 mM, pH 7.4) containing 50 µg/ml BSA (Sigma) for 15 min. The sections were incubated with 35 µl of ^{125}I-AT diluted in the same buffer (15,000 c.p.m./µl) for 1 h at 4°C in humidified chambers. Excess unbound ^{125}I-AT was removed by five washes at 4°C, with three changes of PBS containing 100 µg/ml BSA and two changes of PBS. After fixation for 10 min in ethanol at –20°C, the section were rehydrated in 70% ethanol and distilled water. After drying, the slides were immersed in NTB-2 photographic emulsion and developed after 3–5 or 6–10 days' exposure for dark field and light field illustrations, respectively (Sappino *et al.*, 1989). aHSPG localization on cultured granulosa cells was done using the same technique on live cells. The cells were fixed in ethanol at –20°C after the washes and counterstained in methylene blue for 40 s. The specificity of ^{125}I-AT binding to aHSPGs was shown by competition with soluble sulfated polysaccharides and by preincubation with glycosidases. Control slides were incubated with ^{125}I-AT in the presence of 10 or 100 µg/ml heparin or 100 µg/ml dextran sulfate. Alternatively, control cryosections were preincubated for 1 h at 37°C in PBS containing heparitinase (20 mUI/ml) or chondroitinase ABC (0.1 U/ml) prior to the incubation with ^{125}I-AT.

Endothelial cell staining

Endothelial cells were stained by indirect immunohistochemistry using the specific rat endothelial cell-specific monoclonal antibody (RECA-1) and a peroxidase-conjugated sheep anti-mouse second antibody (Amersham) and revealed with diaminobenzidine as described (Yanagishita *et al.*, 1989). The sections were counterstained with hematoxilin. When the first antibody was replaced by a control non-immune antibody, no staining was observed (data not shown).

Observation and photography

Stained preparations were examined using a Nikon Optiphot-2 microscope equipped with Nikon E-plan 10/0.25 Ph1DL and E-plan 40/0.65 Ph3DL objectives (Nikon, Japan) for ovary sections and cultured cells, respectively. Micrographs shown in dark field exposure of autoradiogram of ^{125}I-AT-labeled ovary sections were taken with a photo camera system HFX-DX attachment using Kodak Ektachrome film. Micrographs shown in bright field exposure of stained ovary sections were taken using a Zeiss Axiophot photomicroscope (Carl Zeiss) equipped with Plan Neofluar 10/0.30 and Ph2 40/0.75 objectives and with a high-sensitivity Coolview color digital camera (Photonic Science, London, UK). All pictures were compiled by using PhotoShop version 5.0 (Adobe System, Mountain View, CA) and printed with a digital Fujifilm Pictography 4000 printer (Fujifilm, Tokyo, Japan) (Christen *et al.*, 1999).

Acknowledgments

We are grateful to Nicholas Shworak for his help in Northern blot analysis of D-glucosaminyl-3-O-sulfotransferase-1. We thank Prof. Aldo Campana for his support of this work. We acknowledge the contributions of Daniela Birsan and Cristina

Cagide in the localization experiments and of M. Julien Barroche for subcloning the perlecan probe. We thank Marie-Andrée Ramus and Isabelle Dentand-Quadri for outstanding technical assistance. This work was supported by Grants 32-39587.93 and 32-49646.96 from the Swiss National Fund for Scientific Research. Marc Princivalle was supported in part by fellowships from the Sir Jules Thorn Trust and from the Fondation pour Recherches Médicales, Geneva. Shereen Hasan was the recipient of a Swiss Federal Fellowship and Ghamartaj Hosseini was recipient of a fellowship from the Carlos and Elsie de Reuter Foundation.

Abbreviations

AT, antithrombin III; ^{125}I-AT, ^{125}I-labeled AT ; DES, diethylstilbestrol; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; HS, heparan sulfate glycosaminoglycan chain, HSPG, heparan sulfate proteoglycan; aHSPG, anticoagulant HSPG; LH, luteinizing hormone; PBS, phosphate-buffered saline; PMSG, pregnant mare serum gonadotropin.

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