Characterization of the coupling activity for the binding of inter- α -trypsin inhibitor to hyaluronan in human and bovine follicular fluid

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The plasma proteinase inter- α -trypsin inhibitor is necessary for normal expansion of the cumulus–oocyte complex (COC) and lack of inter- α -trypsin inhibitor results in severe infertility. After diffusion from the circulation into the follicles, inter- α -trypsin inhibitor is incorporated into the extracellular hyaluronan network of the expanding COC. However, mixing isolated inter- α -trypsin inhibitor with hyaluronan *in vitro* does not result in coupling to hyaluronan. Other components must be present. A recently developed electrophoretic technique by which hyaluronan-bound inter- α -trypsin inhibitor is immobilized was used to demonstrate coupling activity in human and bovine follicular fluid that is necessary for the formation of a firm binding between inter- α -trypsin inhibitor heavy chains and hyaluronan, as observed *in vivo*. No coupling activity could be detected in human serum. Coupling occurred only in the presence of follicular fluid. The coupling activity of

follicular fluid was irreversibly destroyed by heat treatment, lowering of pH or tryptic digestion, indicating that the coupling activity is associated with a protein. Calcium ions are essential for the coupling reaction. The binding reaction *in vitro* using intact inter- α -trypsin inhibitor is slow and occurs over 24 h. The early-formed complexes between inter- α -trypsin inhibitor and hyaluronan contain small amounts of bikunin, whereas the end product contains heavy chains and essentially no bikunin. The heavy chains released from inter- α -trypsin inhibitor by NaOH treatment bound immediately to hyaluronan, indicating that the dissociation of heavy chains from inter- α -trypsin inhibitor is the rate-limiting step. In conclusion, at least four components are essential for the covalent binding of heavy chains to hyaluronan: inter- α -trypsin inhibitor and calcium from plasma, hyaluronan and one or more proteins found in follicular fluid.

Introduction

Normal development of the cumulus–oocyte complex (COC), which undergoes a profound expansion before ovulation in response to the mid-cycle surge of gonadotrophins, is important for normal ovulation and collection of the COC by the oviduct (Salustri *et al.*, 1989; Hess *et al.*, 1999; Lam *et al.*, 2000). FSH stimulates hyaluronan production in follicular cells (Eppig, 1979) and the ovulatory surge induces an increase in the permeability of the blood–follicle barrier leading to an influx of plasma-derived proteins including inter- α -trypsin inhibitor (ITI) (Powers *et al.*, 1995; Hess *et al.*, 1998). ITI is connected to hyaluronan (Chen *et al.*, 1992, 1994; Jessen *et al.*, 1994) and the complex formed stabilizes the extracellular matrix (Chen *et al.*, 1992, 1994). Studies in mice have shown that ITI is essential for normal reproduction, as female mice lacking ITI suffer from severe infertility; however, infertility can be reversed by injection of ITI (Sato *et al.*, 2001; Zhuo *et al.*, 2001).

Human ITI is synthesized by the liver and consists of three different polypeptide chains: bikunin, heavy chain 1 and heavy chain 2 (Ødum, 1990; Salier *et al.*, 1996; Fries and Blom, 2000). Bikunin, a Kunitz type proteinase inhibitor, is covalently linked to both of the heavy chains via a chondroitin sulphate bridge (Jessen *et al.*, 1988; Enghild *et al.*, 1989).

A complex between hyaluronan and the two heavy chains is generated (Huang *et al.*, 1993) similar to the complex detected *in vivo* in follicular fluid and synovial fluid (Jessen *et al.*, 1994). Consequently, bikunin is detached just before, during or after the coupling of ITI to hyaluronan.

Oocytes in mice lacking bikunin–ITI complexes sometimes lack zona pellucida (Sato *et al.*, 2001). As the zona pellucida contains bikunin (Ødum and Nielsen, 1997), bikunin might be important for normal zona pellucida development. Bikunin is a proteinase inhibitor of plasmin among others, but several functions have been ascribed to bikunin as reviewed by Fries and Blom (2000).

Tumour necrosis factor-stimulated gene 6 (TSG-6) can form a stable complex with ITI consisting of TSG-6, heavy

chain 2 (HC2) and bikunin (Wisniewski *et al.*, 1994). TSG-6, ITI and hyaluronan co-localize in mouse cumulus extracellular matrix (Carrette *et al.*, 2001). A 120 kDa complex between TSG-6 and apparently heavy chain 1 or heavy chain 2 has been extracted from mouse COCs. Furthermore, the complex seems to bind hyaluronan as it is released from COCs only by hyaluronidase treatment (Mukhopadhyay *et al.*, 2001). The binding of TSG-6 to ITI appears to increase the plasmin inhibitory activity of ITI (Wisniewski *et al.*, 1996).

The heavy chain-hyaluronan complexes found *in vivo* are connected covalently by an ester bond (Zhao *et al.*, 1995). In the present study, mixing of serum including ITI and hyaluronan caused no stable coupling of ITI to hyaluronan. As cultured granulosa cells produce a factor mediating the coupling (Chen *et al.*, 1996), it was assumed that the stabilization of the extracellular matrix induced by ITI depends on more locally produced compounds in the follicle than on hyaluronan.

Development of the separation electrophoresis (Jessen *et al.*, 1994) made it possible to test for 'coupling activity' in unpurified material. This method was used in the present study to determine whether a protein in human and bovine follicular fluid was necessary for the firm coupling of ITI to hyaluronan. Furthermore, investigation of the reaction conditions demonstrated that calcium ions are essential for the coupling reaction and that free heavy chains participate in the coupling reaction at a faster rate than intact ITI. Finally, the coupling activity has been quantified in human follicular fluid collected before or after the normal LH surge or hCG administration.

Materials and Methods

Reagents and chemicals

Ovine testicular hyaluronidase (EC 3.2.1.35, spec. activity: 49130 national forumulatory (NF) units (as defined in United States Pharmacopeia) per mg protein (art. no. 38594) and human umbilical cord hyaluronic acid (art. no. 385902) were obtained from Calbiochem (Novabiochem Corporation, Darmstadt). Rabbit anti-human ITI immunoglobulin directed against all peptides of ITI (that is, bikunin, heavy chain 1 and heavy chain 2) was obtained from Dako A/S (Copenhagen; code A 301) (Jessen *et al.*, 1994). Trypsin from bovine pancreas (activity: 12700 benzoyl-L-arginine ethyl ester (BAEE) units per mg, T-8253) (one BAEE unit = A_{253} of 0.001 pr. min with BAEE as substrate at pH 7.6 at 25°C), and proteinase inhibitors: phenylmethylsulphonyl fluoride (PMSF), *p*-chloromercuribenzoic acid (PCMB), 1,10-phenanthroline monohydrate, 6-amino-hexanoic acid (EACA), benzamidide hydrochloride and soybean trypsin inhibitor (activity: 1.6 mg will inhibit 1.6 mg trypsin, T-9003) were obtained from Sigma (Aldrich Chemie GmbH, Steinheim). Agarose LSL 4000 was obtained from FMC BioProducts (Rockland, ME). Hyaluronidase-digested bikunin was prepared by incubating partly purified bikunin (200 mg l⁻¹) (Ødum and Nielsen, 1997) with ovine

testicular hyaluronidase (2000 U ml⁻¹). ITI was purified from serum using the method described by Jochum and Bittner (1983) and appeared on SDS-PAGE as a single band of 240 kDa (not shown). The metal ion chelators EDTA and EGTA as well as all other reagents and chemicals were of analytical grade.

Sample collection

Human follicular fluid that represented four different types of preovulatory follicular fluid was obtained as described by Andersen *et al.* (1999). In brief, in group 1, follicles were aspirated from women ($n = 3$) just before the induction of ovulation by the administration of hCG (that is, 36 h before expected ovulation). When the largest follicle was ≥ 17 mm in diameter, all follicles ≥ 10 mm in diameter were aspirated on the following day, except for the two or three largest follicles. After sampling, the couple were advised to have intercourse 24–48 h after the injection of hCG. In group 2, women ($n = 9$) received ovarian stimulation according to the flare-up protocol, including GnRH agonist administration in combination with exogenous human menopausal gonadotrophin (Andersen, 1993). Ovulation was induced with hCG (10 000 iu) when at least two follicles measured ≥ 17 mm in diameter and oocyte retrieval was performed 36 h later. Group 3 consisted of women who had normal menstrual cycles with a normal endogenous mid-cycle of gonadotrophins inducing ovulation. Each woman ($n = 11$) developed one follicle and ovulation was predicted by measurement of LH in urine. Group 4 consisted of women ($n = 9$) with natural menstrual cycles in whom ovulation was induced with hCG (10 000 iu) when the preovulatory follicle measured ≥ 18 mm in diameter. Aspiration of the oocyte was performed 36 h after injection of hCG. In all cases, follicular fluid was collected into a syringe by manual suction ensuring no contamination of flushing fluid. The use of human follicular fluid for research purposes was approved by the Ethical Committee for counties of Copenhagen and Frederiksberg, and the women gave their informed consent after having received information orally and in writing.

Pooled bovine follicular fluids were produced from ovaries as described by Avery *et al.* (1998).

Testing for coupling activity

One volume of bovine follicular fluid was mixed with one volume of buffer (10 mmol phosphate buffer l⁻¹, pH 7.4, with or without 4 mg ml⁻¹ hyaluronan) and with two volumes of normal human serum (Fig. 1). The solutions were then incubated at 37°C. Aliquots were withdrawn (0, 1, 3 and 24 h) and stored at -20°C until analysis.

Experiments were performed by mixing the following components: (i) follicular fluid: one volume (human or bovine) to be tested for coupling activity; (ii) buffer: one volume of 45.0 mmol Tris-HCl l⁻¹, pH 7.4, 4.5 mmol CaCl₂ l⁻¹, 150.0 mmol NaCl l⁻¹, $\pm$ 4.5 mg hyaluronan ml⁻¹, with or without inhibitors; (iii) ITI source: one volume of

Table 1. Components required for formation of heavy chain–hyaluronan complexes

Experiment	Component	Heavy chains (mU ml ⁻¹) bound to hyaluronan ^a after:			
		0 h	3 h	24 h	24 h – 0 h ^b
1	Hyaluronan + serum	0	0	0	0
2	Hyaluronan + ITI	0	0	0	0
3	Follicular fluid (human) + ITI	24	19	17	0
4	Follicular fluid (human) + hyaluronan	53	79	57	0
5	Follicular fluid (human) + ITI + hyaluronan	59	79	160	101
6	Follicular fluid (human) + serum + hyaluronan	63	92	140	77
7	Follicular fluid (bovine) + serum + hyaluronan	11	83	98	87
8	Follicular fluid (bovine) + serum ^c + hyaluronan	84	84	87	3
9	Follicular fluid (human) + heavy chain ^d + hyaluronan	145	152	148	0
10	Follicular fluid (trypsin ^e) + serum + hyaluronan	0	0	21	21

^aCoupling activity is detected by an increasing concentration of heavy chain complexes after 3 and 24 h.

^bThe increase in hyaluronan-bound heavy chains during the first 24 h is shown, that is, the value after 24 h minus the value at 0 h (mU ml⁻¹ day⁻¹).

^cNormal human serum treated with 75 mmol NaOH l⁻¹ to release the heavy chains of inter-alpha-trypsin inhibitor (ITI).

^dPurified ITI treated with 25 mmol NaOH l⁻¹ to release the heavy chains of ITI.

^eBovine follicular fluid pretreated with trypsin.

Fig. 1. Occurrence of coupling activity in bovine follicular fluid. Heavy chain–hyaluronan complexes were demonstrated by the two-step electrophoresis in which hyaluronan and hyaluronan-bound proteins are retained in the electrophoretic wells. After removal of unbound proteins, hyaluronan-bound proteins were released by enzymatic degradation of hyaluronan. Lanes 1–4: mixture of bovine follicular fluid, hyaluronan and serum incubated for 0, 1, 3 and 24 h, respectively; lanes 5–8: mixture of bovine follicular fluid and serum without hyaluronan incubated for 0, 1, 3 and 24 h, respectively.

normal human serum (ITI concentration = 1 U ml⁻¹) or purified ITI (1.5 U ml⁻¹) (Table 1, Figs 2 and 3). The mixtures were incubated at 37°C. Aliquots were withdrawn (0, 3 and 24 h) and 1.5 mol NaCl l⁻¹ (one-third of the withdrawn volume) was added before storing at –20°C. In some experiments, one (or two) component was replaced with saline as shown (Table 1). The amount of heavy chains bound to hyaluronan was determined as described below.

One volume of bovine follicular fluid was mixed with one volume of 500 mmol phosphate buffer l⁻¹, pH 7.4 (with or without 5 mmol Ca–EGTA l⁻¹ or Mg–EGTA in the buffer), 2.5 volumes of normal human serum and one volume of hyaluronan (5 mg ml⁻¹ in physiological saline) (Fig. 4). The solutions were incubated at 37°C. Aliquots were withdrawn at 0, 2 and 4 h, and stored at –20°C until analysis. Samples were centrifuged at 10 000 *g* for 10 min at 4°C just before electrophoresis.

Qualitative experiments (Figs 1–4) were performed several times. The quantitative results (Table 1) represent one experiment, but the overall conclusions have been confirmed by similar experiments using alternative incubation periods. The quantitations of coupling activity in follicular fluid from women with different hormone status were all determined in duplicate and the mean value was used (Table 2).

Quantitation of hyaluronan-bound ITI polypeptides

The quantitation of hyaluronan-bound (immobilized) heavy chains was performed by two-step electrophoresis (Jessen *et al.*, 1994; Ødum *et al.*, 2001). In principle, heavy chains bound to hyaluronan are retained in the application

Fig. 2. Bikunin content of hyaluronan inter-alpha-trypsin inhibitor (ITI) complexes. A combination of bovine follicular fluid, serum and hyaluronan was incubated for 0 (lane 1), 3 (lane 2) and 24 (lane 3) h and tested electrophoretically with 2.5 μ l purified bikunin (200 mg l^{-1}) included in a 3 mm narrow intermediate gel giving a line that crosses the rockets. Upward deflections are due to bikunin released after digestion of hyaluronan in the sample wells.

well during removal of unbound proteins by electrophoresis. The retained hyaluronan is then degraded and the released hyaluronan-bound proteins are detected by electrophoresis into an antibody containing gel. In step 1, separation electrophoresis of 10 μ l samples was carried out at 10 V cm^{-1} for 60 min. After electrophoresis, sample wells were treated with testis hyaluronidase (50 U per well) for detection of hyaluronan-bound protein. In step 2, after digestion of hyaluronan in sample wells, the gel at the anodic side of the wells containing the anodic migrating proteins of the first run was replaced by anti-ITI gel (1.2 μ l cm^{-2}). Electroimmunoassay of the ITI polypeptides released by hyaluronidase treatment was run overnight at 2.5 V cm^{-1} into the anti-ITI gel (Axelsen and Bock, 1983).

For the digestion of hyaluronan, the use of hyaluronidase from ovine testes was preferred, because of its ability to degrade both hyaluronan and the chondroitin sulphate bridge of ITI. One arbitrary unit of heavy chain was defined as the total amount of heavy chain in 1 ml normal human serum.

It is important to change the electrophoresis buffer before each run to avoid buffer-ion exhaustion and thereby to

improve precision. The intra-assay coefficient of variation was 16% for the determination of hyaluronan-bound ITI.

Coupling activity

The coupling activity was expressed as the concentration of immobilized heavy chains formed during incubation for 1 h ($mU\ ml^{-1}\ h^{-1}$) and was determined as the mean increase in rocket height during the first 3 h of the incubation period, in which the substrates (ITI and hyaluronan) were in excess.

NaOH degradation of ITI

Purified ITI in saline was added to a final concentration of 25 mmol $NaOH\ l^{-1}$, incubated for 30 min at 25°C and finally neutralized by acetic acid as described by Zhao *et al.* (1995) to release the two heavy chains of ITI from the chondroitin sulphate bridge. The concentration of $NaOH$ was increased to 75 mmol l^{-1} to detach heavy chains from ITI in serum.

Tryptic digestion of coupling factor

Bovine follicular fluid (1000 μ l) was mixed with 100 μ l phosphate buffer (50 mmol l^{-1} , pH 8.0) containing 10 mg trypsin ml^{-1} . After incubation at 37°C for 6 h, 100 μ l soybean trypsin inhibitor (10 mg ml^{-1} in phosphate buffer) was added. Finally, coupling activity was tested as described above.

Inhibitors

Coupling assays were performed as described above: (i) 100 μ l bovine follicular fluid; (ii) 100 μ l buffer with hyaluronan and inhibitors; and (iii) 100 μ l human serum were incubated at 37°C, and aliquots were withdrawn after 0, 2 and 4 h. Concentrations of inhibitors during the coupling reaction were 1.5 mmol PMSF l^{-1} , 1.0 mmol PCMB l^{-1} , 1.0 mmol 1,10-phenanthroline l^{-1} , 10.0 mmol benzamidine l^{-1} , 25.0 mmol EACA l^{-1} and 10.0 mmol EDTA l^{-1} , 5.0 mmol Ca-EGTA l^{-1} and Mg-EGTA (PMSF was added directly into the mixture dissolved in isopropanol).

Measurement of steroids

Progesterone and oestradiol were monitored using a commercially available direct radioimmunoassay kit (Orion, Turku). The interassay coefficient of variation was 6–8% and results were considered valid only when two internal standards provided by the manufacturer were in the given range. Samples were diluted 1:500 or 1:1000 in steroid-free serum so that values were within the range of the standard curve.

Statistical analysis

As the material consisted of four groups of women who received different treatments and from whom one to four follicular fluid samples were tested, a random effect-multi-level model was used to compare the effects of ovarian

Fig. 3. Coupling activity is inhibited by EDTA. Combinations of bovine follicular fluid, serum and hyaluronan were incubated for 0 (lanes 1, 4, 7 and 10), 3 (lanes 2, 5, 8 and 11) and 24 h (lanes 3, 6, 9 and 12). Lanes 1–3: control; lanes 4–6: addition of proteinase inhibitors (phenylmethylsulphonyl fluoride (PMSF), benzamidine, 1,10-phenanthroline, p-chloromercuribenzoate and 6-amino-hexanoic acid (EACA) without EDTA; lanes 7–9: addition of proteinase inhibitors plus EDTA; and lanes 10–12: EDTA was added after incubation (without inhibitors) and formation of complexes.

Fig. 4. Coupling activity depends on calcium ions. Lanes 1–3: controls containing a combination of bovine follicular fluid, human

stimulation on follicular concentrations of coupling activity and of preformed ITI-hyaluronan complexes. This model accounts for the inter-individual variation between women.

Results

Occurrence of coupling factor

Coupling activity was found in follicular fluid (Fig. 1). After mixing of bovine follicular fluid, human serum and hyaluronan, increasing amounts of heavy chain-hyaluronan complexes were formed during 24 h (Fig. 1; lanes 1–4). In the absence of hyaluronan, no complexes were formed, indicating that hyaluronan was necessary for complex formation (Fig. 1, lanes 5–8). As the antibody used did not precipitate bovine ITI, no genuine hyaluronan-bound ITI was observed (Fig. 1, lane 1). The ITI components released after hyaluronidase treatment showed antigenic non-identity with bikunin after 24 h of incubation (Fig. 2, lane

serum and hyaluronan incubated for 0, 2 and 4 h; lanes 4–6: as lanes 1–3 with the addition of Mg-EGTA; lanes 7–9: as lanes 1–3 with addition of Ca-EGTA.

Table 2. Coupling activity and preformed heavy chain–hyaluronan complexes in follicular fluids obtained from women with different hormone status

Hormone status	Number of women	Number of follicles	Coupling activity ^{ab} (mU l ⁻¹ h ⁻¹)	Oestradiol μmol l ⁻¹	Progesterone μmol l ⁻¹	Preformed heavy chain–hyaluronan complexes ^b (mU l ⁻¹)
Group 1 Women stimulated with exogenous FSH before the LH surge	3	12	0.2 (0.4)	16.6 (2.4)	12.1 (2.4)	34 (5.4) ^c
Group 2 Women stimulated with exogenous FSH and hCG	9	18	4.3 (0.7)	2.1 (0.2)	31.0 (2.6)	87 (7.6)
Group 3 Normal women after LH surge	11	11	3.0 (0.7)	5.6 (0.9)	39.8 (3.7)	84 (9.4)
Group 4 Normal FSH plus hCG	9	9	6.3 (1.0)	6.2 (0.6)	43.4 (2.6)	91 (5.6)

^aTested by a random effect–multi level model; concentrations in group 1 were significantly lower than in groups 2–4 ($P = 0.0001$), and concentrations in group 4 were significantly higher than in groups 1–3 ($P = 0.0025$).

^bMean follicular values. The values in parentheses are the SEM.

^cConcentrations in group 1 were significantly lower than in groups 2–4 ($P < 0.00005$).

3), whereas the released ITI components that had only been incubated for a short period or for 3 h gave a small deviation of the bikunin line, indicating that bikunin is present in the early formed complexes (Fig. 2, lanes 1 and 2). As the stable end product contains essentially no bikunin it is referred to as heavy chains.

Various mixtures of follicular fluid, hyaluronan and purified ITI or serum were tested to examine further the components necessary for complex formation. No coupling activity was detected by single incubation of each of hyaluronan, serum, ITI or follicular fluid (human as well as bovine). Furthermore, no formation of heavy chain–hyaluronan complexes took place after mixing serum (including ITI) and hyaluronan alone (Table 1, Expt 1). This finding was verified with normal sera from three males and three women obtained at the time of ovulation. Formation of significant amounts of new heavy chain–hyaluronan complexes occurred only when ITI, hyaluronan and components of follicular fluid were present together (Table 1, Expts 5–7). The formation was slow and took place within 24 h. Extension of the incubation period to 48 h resulted in almost no further increase in heavy chain–hyaluronan complex formation. It was seen that human follicular fluid contained *in vivo* formed hyaluronan-bound ITI (Table 1, Expts 3–6, 0 h). As the antibodies used did not precipitate bovine ITI, the small rocket was assumed to be due to

complexes formed within minutes before and after freezing of the assay mixture (Table 1, Expt 7, 0 h).

Integrity of ITI

Purified ITI pretreated with 25 mmol NaOH l⁻¹ bound immediately to hyaluronan in the presence of bovine follicular fluid (Table 1, Expt 9 compared with Expt 5). Similar results were obtained using NaOH-treated serum (Expt 8 compared with Expt 7). The NaOH treatment is known to detach the heavy chain from the chondroitin sulphate bridge of ITI. When NaOH-treated ITI was incubated with hyaluronan alone, no coupling took place.

Evidence for a protein component

After tryptic digestion of bovine follicular fluid, the coupling activity almost disappeared (Table 1, Expt 10), that is, no increase in rocket height was observed. This effect was blocked by addition of a trypsin inhibitor (SBTI). Heating for 15 min at 60°C reduced the coupling activity of bovine follicular fluid to about 20% and heating for 15 min at 75°C reduced coupling activity to 11%.

The pH stability of the coupling activity was tested after reducing the pH for 1 h in bovine follicular fluid. The coupling activity was not affected at pH 4.0, but at pH 3.0 the activity was reduced to 50%, and at pH 2.0 to 17.0%.

Inhibitors

The effect of various proteinase inhibitors was tested on the ITI coupling reaction.

The two serine proteinase inhibitors PMSF (1 mmol l⁻¹) and benzamidine (25 mmol l⁻¹), the plasmin inhibitor EACA (10 mmol l⁻¹), the cysteine proteinase inhibitor *p*-chloromercuribenzoate (1 mmol l⁻¹) and the metalloproteinase inhibitor 1,10-phenanthroline (1 mmol l⁻¹) were all unable to inhibit the coupling reaction when tested individually (not shown) or together (Fig. 3, lanes 4–6).

Only EDTA inhibited the coupling reaction, indicating that a di- or tri-valent metal ion was essential (Fig. 3, lanes 7–9). If EDTA was added after the coupling reaction, it had no influence on the preformed ITI–hyaluronan complexes (Fig. 3, lanes 10–12).

The influence of equimolar amounts of Mg²⁺ and EGTA (Mg–EGTA) or Ca²⁺ and EGTA (Ca–EGTA) was tested on the coupling reaction to characterize further the necessary ions for coupling activity. Mg–EGTA chelates all di- and tri-valent metal ions, but allows free Mg²⁺ (Holloway and Reilley, 1960). No coupling was observed under these conditions (Fig. 4, lanes 4–6). Calcium saturated with EGTA allows both free Mg²⁺ and Ca²⁺, and this was sufficient for coupling to take place (Fig. 4, lanes 7–9). Therefore, Ca²⁺ must be essential for the coupling reaction, whereas other metal ions such as Fe³⁺, Cu²⁺, Zn²⁺, Fe²⁺ and Mg²⁺ should not influence the coupling reaction at physiological concentrations.

Coupling activity and preformed heavy chain–hyaluronan in follicular fluid samples

The coupling activity and the preformed heavy chain–hyaluronan complexes were examined in follicular fluid samples obtained from four groups of women that received different types of ovarian stimulation (Table 2). The group of women (group 1) in which the follicular fluid samples were obtained before initiation of final follicular maturation by either an exogenous bolus of hCG or by an endogenous mid-cycle surge of gonadotrophins showed significantly lower coupling activity and fewer preformed heavy chain–hyaluronan complexes than any of the three other groups. The three groups (2–4) with follicular fluids obtained just before ovulation all showed similar amounts of preformed heavy chain–hyaluronan complexes and were about three times higher than the amounts seen in fluids before the induction of ovulation. The increase in coupling activity was even more pronounced and more than ten times higher after the induction of ovulation (group 1 compared with groups 2–4). The natural menstrual cycle that had an endogenous mid-cycle surge of gonadotrophins (group 3) produced lower amounts of coupling activity than the hCG-stimulated groups, and women with natural menstrual cycles in whom ovulation was induced by a large bolus of hCG (group 4) expressed significantly higher concentrations than the other groups. Concentrations of oestradiol and progesterone confirm that follicular fluid

samples were obtained before ovulation was induced (group 1) or close to ovulation (groups 2–4).

Discussion

The coupling of ITI to hyaluronan is important for normal oocyte development and ovulation (Hess *et al.*, 1999), and is necessary for normal fertility (Sato *et al.*, 2001; Zhuo *et al.*, 2001). However, the mechanisms involved in coupling are mainly unknown. Bikunin is not a part of the ITI–hyaluronan complex (Jessen *et al.*, 1994; Zhuo *et al.*, 2001), but whether the release of bikunin occurs before, during or after the coupling process of heavy chains to hyaluronan is unclear. Furthermore, the nature of the connection has not been fully elucidated (Chen *et al.*, 1996). The covalent binding between heavy chains and hyaluronan in synovial fluid has been described as an ester bond (Zhao *et al.*, 1995), although other types of binding may participate. Possibly, an ion interaction is involved in the primary association between ITI and hyaluronan (Chen *et al.*, 1994). However, in the present study no coupling was observed between ITI and hyaluronan in the presence of physiological salt concentrations, indicating that a possible charge-based interaction is weak. In contrast, stable complexes were formed only in the presence of ITI, hyaluronan and calcium ions in combination with an unknown component of follicular fluid. Zhuo *et al.* (2001) proposed that an enzymatic factor in mouse serum was necessary for the coupling process. However, in the present study, no activity was found in human serum (male as well as female), indicating that the coupling activity is produced locally by the follicle.

The coupling activity was unaffected by proteinase inhibitors, indicating that a proteolytic cleavage is not involved in the coupling reaction. The coupling activity was found to be sensitive to tryptic digestion, heating and low pH, indicating that the activity may be attached to a protein structure.

The coupling reaction proceeds slowly for many hours. The results from the present study demonstrate that bikunin is present in the early formed complexes between ITI and hyaluronan, indicating that there is a two-step reaction mechanism initiated by the binding of intact ITI followed by a dissociation of bikunin. In agreement with this mechanism, in the present study it was found that heavy chains released from the chondroitin sulphate bridge by treatment of ITI with 25 mmol NaOH l⁻¹ bound quickly to hyaluronan, but only in the presence of the coupling activity of follicular fluid. Consequently, the chondroitin sulphate might be a rate-limiting factor in the coupling of heavy chains to hyaluronan and possibly needs to be displaced in agreement with the lack of bikunin in stable complexes. A possible mechanism is a displacement of the chondroitin sulphate bridge by hyaluronan in a trans-ester reaction, in which one or two heavy chains shift from the chondroitin sulphate bridge in ITI to hyaluronan as suggested by Chen *et al.* (1996) and Zhuo *et al.* (2001). A

coupling factor could in this case mediate the reaction by bringing the two reactants (ITI and hyaluronan) in close contact with one another or act as an esterase. As chelation of Ca^{2+} by EDTA was shown in the present study to have no influence on existing ITI–hyaluronan complexes, Ca^{2+} might be essential for establishing the contact between the reactants. In support of this contention, a gradual reduction was observed in the formation of heavy chain–hyaluronan complexes when the ionic strength of the reaction media was increased to 0.6 mol NaCl l^{-1} or higher (data not shown).

The coupling could possibly depend on more proteins working in concert. Some proteins like TSG-6 (Wisniewski *et al.*, 1994) and hyaluronic acid binding protein (Hirashima *et al.*, 1997) have been shown to bind ITI and hyaluronan, and thus may be possible coupling factor candidates. The most likely is TSG-6 as a 120 kDa complex extracted from mouse COCs consisted of TSG-6 and either of the heavy chains bound to hyaluronan (Mukhopadhyay *et al.*, 2001). Furthermore, TSG-6 is detected in the extracellular matrix co-localized with ITI and hyaluronan (Carette *et al.*, 2001). The TSG-6–hyaluronan complex contains no bikunin and is resistant to treatment with chondroitinase and hyaluronidase (Mukhopadhyaya *et al.*, 2001). This finding is in contrast to complexes formed *in vitro* between ITI and TSG-6 that appear to contain the chondroitin sulphate and bikunin plus heavy chain 2 (HC2) (Wisniewski *et al.*, 1994). These findings indicate that a complex between ITI and TSG-6 is modified further in follicular fluid, perhaps through the action of coupling activity.

TSG-6 gene expression is upregulated in cumulus cells during expansion of mouse COCs *in vivo* (Fülöp *et al.*, 1997) and in the ovaries 4–8 h after stimulation with hCG (Yoshioka *et al.*, 2000). The results from human follicular fluid samples in the present study are similar to the TSG-6 expression results, as the mid-cycle surge of gonadotrophins or administration of a large bolus of hCG for ovulation induction also caused accumulation of coupling activity. Compared with before ovulation induction, the coupling activity is more than ten times higher and may therefore reflect an increased expression of TSG-6. It is interesting to note that although deposition of hyaluronan in the extracellular matrix seems to be governed mainly by FSH, coupling activity seems to be induced mainly by LH–hCG. The highest concentration of coupling activity was found in the hCG-stimulated group with normal FSH production. This probably reflects a higher ovulatory stimulus by hCG as compared with the mid-cycle surge of gonadotrophins and demonstrates that LH–hCG is involved in stimulation of synthesis of coupling activity.

As coupling of ITI to hyaluronan is essential for normal fertility in mice (Sato *et al.*, 2001; Zhuo *et al.*, 2001), defects in ITI may play a role in infertility. However, defects in the coupling activity are likely to have similar severe implications for fertility, and isolation and further characterization of the coupling activity is therefore of interest.

In vitro maturation of COCs is becoming increasingly popular to avoid gonadotrophin treatment of women as in conventional IVF treatment. Sufficient amounts of serum and calcium ions need to be present during *in vitro* conditions to obtain COC expansion of the *in vivo* unstimulated oocytes. As with calcium, the concentration of ITI in follicular fluid from follicles obtained after stimulation with hCG close to ovulation is almost similar to the concentration in plasma (Ødum *et al.*, 2001). Therefore, it could be of interest to investigate whether addition of follicular fluid from mature follicles obtained close to ovulation would improve COC expansion and subsequent fertilization, and embryo development in connection with *in vitro* maturation of oocytes.

In conclusion, binding of ITI heavy chains to hyaluronan requires the presence of calcium ions and proteins in the follicular fluid. These appear to be produced locally and stimulated by LH–hCG.

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