Role of tumour necrosis factor stimulated gene 6 (TSG-6) in the coupling of inter- α -trypsin inhibitor to hyaluronan in human follicular fluid

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Binding of the plasma proteinase inhibitor inter- α -trypsin inhibitor (ITI) to hyaluronan is necessary for normal expansion of the cumulus–oocyte complex. Lack of ITI causes severe infertility. Binding of ITI to hyaluronan depends on calcium ions and coupling activity present in follicular fluid (Ødum *et al.*, 2002). The complexes formed by this process contain ITI heavy chains bound to hyaluronan, and bikunin is detached from ITI during the coupling reaction. In the present study, an electrophoretic technique by which hyaluronan-bound ITI is immobilized was used to demonstrate that tumour necrosis

factor stimulated gene 6 (TSG-6) is necessary for the coupling reaction. Thus, immunoprecipitation of TSG-6 in human follicular fluid eliminates the coupling reaction and re-addition restores the activity. However, it appears that components other than hyaluronan, ITI, calcium ions and TSG-6 are involved in the coupling reaction, as *in vitro* incubation of these components does not generate stable complexes between ITI heavy chains and hyaluronan unless some follicular fluid is added. In conclusion, TSG-6 is necessary for the coupling of ITI to hyaluronan, but at least one additional component in follicular fluid is essential.

Introduction

The cumulus–oocyte complex (COC) undergoes a profound expansion before ovulation in response to the mid-cycle surge of gonadotrophins. This expansion 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). The ovulatory surge induces synthesis of hyaluronan by the cumulus cells (Chen *et al.*, 1993) and an increased 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). Furthermore, LH appears to induce formation of a coupling activity that supports the binding of ITI to hyaluronan (Ødum *et al.*, 2002). The formed complexes stabilize the extracellular matrix (Chen *et al.*, 1992, 1994). Studies have shown that ITI is essential for normal reproduction. Thus, female mice lacking ITI are infertile, but infertility can be reversed by injection of ITI (Sato *et al.*, 2001; Zhuo *et al.*, 2001).

Human ITI consists of three different polypeptide chains named 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).

ITI is synthesized in the liver (Mizushima *et al.*, 1998) and transported via the blood to the follicles. Upon LH stimulation, the blood–follicle barrier opens to allow influx of ITI into the follicle (Hess *et al.*, 1998). Inside the follicle, ITI forms complexes with hyaluronan in a coupling reaction in which bikunin is detached from ITI. Thus, complexes in follicular fluid formed *in vivo* contain only ITI heavy chains linked to hyaluronan (Jessen *et al.*, 1994; Eriksen *et al.*, 1999). When the coupling reaction is performed *in vitro*, ITI, hyaluronan, calcium ions and at least one unidentified protein produced locally in the follicle are required for successful coupling to take place (Ødum *et al.*, 2002).

Tumour necrosis factor-stimulated gene 6 (TSG-6) is a 35 kDa glycoprotein. TSG-6 gene expression has been shown to be upregulated in cumulus cells during expansion of the mouse COC *in vivo* (Fülöp *et al.*, 1997) and in the ovaries 4–8 h after stimulation with hCG (Yoshioka *et al.*, 2000). Immunohistochemical studies have revealed that TSG-6 and ITI co-localize in the hyaluronan-rich matrix around cumulus cells of the expanded mouse COC (Carrette *et al.*, 2001). A 125 kDa firm complex between TSG-6 and either of the heavy chains of ITI has been extracted from mouse COC by hyaluronidase digestion (Mukhopadhyay *et al.*, 2001). This result strongly indicates that a complex between TSG-6 and the heavy chains of ITI is bound to hyaluronan as a structural part of the COC. However, it is not known whether the binding between ITI and hyaluronan

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is dependent on an interaction with TSG-6. These observations made it relevant to investigate whether TSG-6 is involved in the coupling of ITI to hyaluronan. The purpose of this study was therefore to investigate whether TSG-6 participates in the coupling reaction. The effect of anti-TSG-6 antibodies on the coupling reaction was determined using immunoelectrophoresis (Ødum *et al.*, 2002). Subsequently, TSG-6 protein was added to investigate whether activity could be restored.

Materials and Methods

Reagents and chemicals

Ovine testicular hyaluronidase (EC 3.2.1.35, specific activity 49130 national formulary (NF) units (as defined in United States Pharmacopeia) per mg protein) and human umbilical cord hyaluronic acid 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) (Jessen *et al.*, 1994). Agarose LSL 4000 was obtained from FMC BioProducts (Rockland, ME). ITI was purified from serum using the method described by Jochum and Bittner (1983) and appeared on SDS-PAGE as a single band with a molecular mass of 240 kDa. Recombinant TSG-6 and rabbit antibodies directed against TSG-6 were kindly provided by H-G. Wisniewski (DNYU School of Medicine, New York). Pooled preovulatory human follicular fluids were obtained as described by Andersen *et al.* (1999). The use of human follicular fluid for research purposes was approved by the Ethical Committee for the counties of Copenhagen and Frederiksberg, and patients gave consent after having received information orally and in writing. Immunoprecipitated human follicular fluids were obtained by addition of anti-TSG-6 antibodies to a pool of human follicular fluid followed by incubation for 18 h at 4°C, and centrifugation (14 000 *g* for 15 min). The amount of anti-TSG-6 antibody added was adjusted to give a supernatant devoid of TSG-6. All other reagents and chemicals were of analytical grade.

Demonstration of coupling activity by immunoelectrophoresis

Experiments were performed by mixing the following components: (i) ITI source: one volume of normal human serum (1 U ITI ml⁻¹) or purified ITI (1.5 U ml⁻¹), (ii) buffer: one volume of 45 mmol Tris-HCl l⁻¹, pH 7.4; 4.5 mmol CaCl₂ l⁻¹; 150 mmol NaCl l⁻¹; 4.5 mg hyaluronan ml⁻¹, with or without 18 µg or 36 µg TSG-6 ml⁻¹ and (iii) follicular fluid: one volume of human follicular fluid or immunoprecipitated human follicular fluid. In some experiments follicular fluid was replaced with saline. The mixtures were incubated at 37°C.

Aliquots were withdrawn (at 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. The visualization of hyaluronan-bound (immobilized) heavy chains was performed by a two-step electrophoresis (Jessen *et al.*, 1994; Ødum *et al.*, 2001). Heavy chains bound to hyaluronan were retained in the application well during removal of unbound proteins by electrophoresis. The retained hyaluronan was then degraded and the released hyaluronan-bound proteins detected by electrophoresis in an antibody-containing gel. In step 1, separation electrophoresis of 10 µl 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, 1 h, 37°C) 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 (1.1 µl 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). All experiments were performed at least twice.

The coupling activity was expressed as the concentration of immobilized ITI heavy chains formed during incubation for 1 h (mU ml⁻¹ h⁻¹), where 1 U corresponds to the total amount of ITI heavy chains in 1 ml normal human serum. Coupling activity was measured during the first 3 h of the incubation period, when the substrates ITI and hyaluronan were in excess.

Results

Involvement of TSG-6 in the coupling reaction

Coupling activity in follicular fluid (Fig. 1a, lanes 1–3) was expressed as the increase in heavy chain–hyaluronan complexes formed during 24 h after mixing of human follicular fluid, human serum and hyaluronan. The ITI immunoprecipitates seen at 0 h were due to ITI–hyaluronan complexes preformed *in vivo* in human follicular fluid. The coupling activity was 9.6 mU ml⁻¹ h⁻¹ at the start of the incubation period (Fig. 1a, lanes 1,2). After immunoprecipitation of TSG-6 in follicular fluid, no coupling activity was detected (Fig. 1a, lanes 4–6). The immunoprecipitates did not increase in size, demonstrating that no ITI heavy chains were coupled to hyaluronan during the incubation period. However, if TSG-6 was subsequently added to the immunoprecipitated follicular fluid (6 µg ml⁻¹ or 12 µg ml⁻¹), the coupling activity was restored in a dose-response manner (Fig. 1a, lanes 7–12) to 14.3 mU ml⁻¹ h⁻¹ (lanes 7,8) and 24.3 mU ml⁻¹ h⁻¹ (lanes 10,11), respectively. Consequently, TSG-6 must be essential for the coupling of ITI to hyaluronan.

The coupling reaction was attempted with purified components to examine whether TSG-6 is identical

Fig. 1. Involvement of tumour necrosis factor-stimulated gene 6 (TSG-6) in the coupling reaction of inter- α -trypsin inhibitor (ITI) to hyaluronan in human follicular fluid. Complexes between ITI and hyaluronan were demonstrated by a two-step immunoelectrophoresis in which hyaluronan and hyaluronan-bound proteins were retained in electrophoretic wells. After removal of unbound proteins, hyaluronan-bound proteins were released by enzymatic degradation of hyaluronan. (a) Coupling activity in the presence of follicular fluid. Lanes 1–3: control experiment with mixture of human follicular fluid, hyaluronan and serum incubated for 0, 3 and 24 h, respectively. Lanes 4–6: mixture of TSG-6-immunoprecipitated human follicular fluid, hyaluronan and serum incubated for 0, 3 and 24 h, respectively. Lanes 7–9: mixture of TSG-6-immunoprecipitated human follicular fluid, hyaluronan and serum, with addition of $6 \mu\text{g TSG-6 ml}^{-1}$, incubated for 0, 3 and 24 h, respectively. Lanes 10–12: mixture of TSG-6-immunoprecipitated human follicular fluid, hyaluronan and serum, with addition of $12 \mu\text{g TSG-6 ml}^{-1}$, incubated for 0, 3 and 24 h, respectively. (b) Coupling activity with purified components without follicular fluid. Lanes 1–3: mixture of hyaluronan and ITI incubated for 0, 3 and 24 h, respectively. Lanes 4–6: mixture of hyaluronan and ITI, with addition of $12 \mu\text{g TSG-6 ml}^{-1}$, incubated for 0, 3 and 24 h, respectively.

to the recently proposed coupling factor in follicular fluid (Ødum *et al.*, 2002). Hyaluronan was incubated with purified human ITI under physiological conditions with and without TSG-6. Only minute amounts of ITI bound to hyaluronan under these conditions (Fig. 1b). Thus, even though TSG-6 is necessary for the coupling of ITI to hyaluronan, it can account for only a part of the coupling activity of follicular fluid.

Discussion

Binding of ITI to hyaluronan is of great importance for normal expansion of the cumulus–oocyte complex. However, the processes leading to this binding are mainly unknown. Complexes between ITI and hyaluronan generated *in vivo* contain only the heavy chains of ITI (Jessen *et al.*, 1994), so a detachment of bikunin seems to take place in connection with the binding of ITI to hyaluronan. Experiments *in vitro* have shown that complexes between ITI and hyaluronan contain bikunin shortly after their formation, but that bikunin is absent in the complexes after 24 h (Ødum *et al.*, 2002). Thus, the

initial binding of ITI to hyaluronan seems to be possible when bikunin is still part of the ITI molecule.

Human and bovine follicular fluids contain unidentified protein factors that are necessary for the binding of ITI heavy chains to hyaluronan *in vitro* (Ødum *et al.*, 2002). A factor secreted by granulosa cells possesses the same properties (Chen *et al.*, 1996). Thus, a mixture of purified ITI and hyaluronan alone does not generate stable complexes between ITI heavy chains and hyaluronan. Follicular fluid containing the unidentified coupling activity has to be added (Ødum *et al.*, 2002).

TSG-6, which can bind both ITI and hyaluronan (Wisniewski *et al.*, 1994), is synthesized in the follicle by the cumulus cells, and its gene expression is upregulated during expansion of mouse COC *in vivo* (Fülöp *et al.*, 1997). Complexes between TSG-6, ITI heavy chains and hyaluronan have been detected in the hyaluronan-rich matrix around the cumulus cells of the expanded mouse COC (Carrette *et al.*, 2001). Therefore, TSG-6 was considered to be a possible coupling factor involved in the physiological binding of ITI to hyaluronan.

The present study has shown that the coupling activity of human follicular fluid disappears when endogenous TSG-6 is immunoprecipitated. Furthermore, coupling activity can be restored in a dose-response manner by addition of exogenous TSG-6 to the immunoprecipitated follicular fluid. Consequently, TSG-6 must be essential for the coupling of ITI to hyaluronan. This result is in accordance with the observation that the coupling activity in follicular fluid increases after the mid-cycle surge of gonadotrophins (Ødum *et al.*, 2002). This occurs simultaneously with the upregulation of TSG-6 gene expression (Fülöp *et al.*, 1997, Yoshioka *et al.*, 2000). The concentration of TSG-6 in follicular fluid has, to the authors' knowledge, not been described, but concentrations of up to $20 \mu\text{g ml}^{-1}$ have been detected in synovial fluid from patients with arthritis (Wisniewski *et al.*, 1993). In the present study, similar concentrations were effective *in vitro*, $12 \mu\text{g ml}^{-1}$ giving a higher response than $6 \mu\text{g ml}^{-1}$.

Although TSG-6 is necessary for the normal coupling of ITI to hyaluronan, it is not sufficient. Thus, a mixture of purified components (including calcium ions) does not generate ITI-hyaluronan complexes.

In conclusion, TSG-6 is necessary for the coupling of ITI to hyaluronan, but at least one additional component in follicular fluid essential for the coupling reaction remains to be identified.

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