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TSG-6 and calcium ions are essential for the coupling of inter-alpha-trypsin inhibitor to hyaluronan in human synovial fluid

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Summary

Objective: To investigate the role of tumor necrosis factor stimulated gene 6 (TSG-6) and metal ions in the coupling of inter-α-trypsin inhibitor (ITI) to hyaluronan in human synovial fluid.

Design: The concentration of ITI heavy chains bound to hyaluronan was determined by a two-step electrophoretic technique. Synovial fluid, TSG-6 depleted synovial fluid and metal chelated synovial fluid were tested for their ability to support the coupling of ITI heavy chains to hyaluronan.

Results: When synovial fluid was mixed with an ITI-source (serum or purified ITI), coupling of ITI heavy chains to hyaluronan took place. TSG-6 immunodepleated synovial fluid lost the coupling activity, but addition of recombinant TSG-6 restored the activity. EDTA inhibited the coupling activity, but combinations of the metal-ion chelators Mg-EGTA and Ca-EGTA demonstrated, that Ca^{++} is essential for the coupling of ITI heavy chains to hyaluronan.

Conclusions: Tumor necrosis factor stimulated gene 6 (TSG-6) and calcium ions are both essential for the coupling of inter-α-trypsin inhibitor to hyaluronan in human synovial fluid.

Key words: Coupling-factor, ITI, Hyaluronan, Inter-alpha-trypsin inhibitor, TSG-6, Hyaluronan network, Synovial fluid, ITI coupling, Coupling activity.

Introduction

Hyaluronan is of great significance for the hydrodynamic and viscoelastic properties of synovial fluid and is therefore considered to be responsible for the lubricating and shock-absorbing properties of synovial fluid[1]. Hyaluronan is a high molecular weight linear polysaccharide, and in synovial fluid it forms an entangled network throughout the solution. Small molecules like water and inorganic ions can diffuse unhindered through the network, while large molecules and cells are excluded or restricted in their free migration by the hyaluronan network[2]. Hyaluronan is synthesized by hyaluronan synthase isoenzymes, HAS1, HAS2 and HAS3[3] located in fibroblastic synovial lining cells, from where it is excreted into the synovial cavity.

Proteins in synovial fluid are mainly derived from the blood plasma. The mean concentration of plasma proteins in normal human synovial fluid is 13 g/l[4]. The plasma proteins enter the joint space by infiltration from the blood-stream across the synovial barrier and they return to the circulation through lymphatic vessels[5]. One plasma protein, inter-α-trypsin inhibitor (ITI), is known to form firm complexes with hyaluronan[6,7]. ITI being synthesized in the liver[8], is a protein with a unique structure consisting of polypeptide chains covalently crosslinked by carbohydrate[9]. Two heavy chains H1 (Mr 65 kDa) and H2 (Mr 70 kDa) and one light chain named bikunin (Mr 30 kDa) are linked together by chondroitinsulfate[10]. In synovial fluid inter-α-trypsin inhibitor exists both free in the solution and bound to the hyaluronan network[6]. The ITI fraction bound to hyaluronan consists only of ITI heavy chains coupled by covalent bindings to hyaluronan[7]. The unbound ITI fraction include bikunin and other ITI metabolites[6]. Therefore, one or more metabolic processes in the synovial fluid must transform the infiltrated plasma ITI into ITI heavy chains coupled to hyaluronan, and to unbound bikunin. The heavy chains is supposed to be transferred to hyaluronan by a transester reaction[7],but the molecular mechanism leading to the biological coupling of ITI heavy chains to hyaluronan are only partly resolved.

In-vitro incubation of ITI with hyaluronan does not lead to formation of firm complexes between ITI heavy chains and hyaluronan[11]. Recent investigations of an analogous binding reaction between ITI and hyaluronan in human follicular fluid have shown that calcium ions and tumor necrosis factor stimulated gene 6 protein (TSG-6) are essential components for the coupling of ITI heavy chains to hyaluronan[12,13].

Human tumor necrosis factor stimulated gene 6 (TSG-6) originally cloned from human fibroblasts, encodes a 277 amino acid prepropeptide that is secreted as a glycoprotein with a Mr of 35 000. TSG-6 is expressed in many cell types including synovial cells, chondrocytes, mononuclear cells and fibroblasts, and high levels of TSG-6 protein are found in synovial fluids of patients with rheumatoid arthritis (RA),

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osteoarthritis, Sjögren's syndrome, polyarthritic gout and osteomyelitis[14–16]. The expression of TSG-6 is induced by cytokines such as tumor necrosis factor-α and interleukin-1[17]. TSG-6 has an anti-inflammatory activity and up-regulation of the gene as well as therapeutic injections of recombinant TSG-6 reduces symptoms of inflammation and joint destruction in mouse models[17–19].

These observations made it relevant to investigate if TSG-6 and calcium ions in synovial fluid are involved in the coupling of ITI to hyaluronan, like in follicular fluid[12]. As demonstrated in the present work this was in fact the case: TSG-6 antibodies and calcium chelating agents blocked the coupling activity of synovial fluid, and subsequent addition of recombinant TSG-6 protein and free calcium ions, respectively, to depleted synovial fluid restored the activity, showing that both TSG-6 and calcium ions are necessary for the coupling of ITI to hyaluronan.

Materials and methods

REAGENTS AND CHEMICALS

Ovine testicular hyaluronidase (EC 3.2.1.35, specific activity 49 130 national formulary (N.F.) units (as defined in United States Pharmacopeia) per mg protein, (art. no. 38 594) and human umbilical cord hyaluronic acid (art. no. 385 902) 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)[6]. 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 substrate at pH 7.6 at 25°C), 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). ITI was purified from serum using the method described by Jochum and Bittner[20] and appeared on SDS-PAGE as a single band with Mr 240 kDa (not shown). Recombinant TSG-6 protein[21] and rabbit antibodies directed against TSG-6 were kindly provided by Hans-Georg Wisniewski (DNYU School of Medicine; Department of Microbiology; 550 First Avenue; New York, NY 10016). Immunoprecipitated human synovial fluid were obtained by addition of TSG-6 antibodies to a pool of human synovial fluid followed by incubation 18 h at 4°C, and centrifugation (14 000 g, 15 min). The amount of anti-TSG-6 added was adjusted to give a supernatant devoid of TSG-6. Trypsin digestion was prepared by mixing synovial fluid (1000 µl) with 100 µl phosphate buffer (50 mmol l^{-1}, pH 8.0) containing 10 mg trypsin ml^{-1}. After incubation at 37°C for 6 h, 100 µl soybean trypsin inhibitor (10 mg ml^{-1} in phosphate buffer) were added. Hyaluronidase-digested bikunin was prepared by incubating partly purified bikunin (200 mg l^{-1})[22] with ovine testicular hyaluronidase (2000 U ml^{-1}). The metal ion chelators ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) as well as all other reagents and chemicals were of analytical grade.

SAMPLES

Synovial fluids were kindly donated by Dr Michael Sonne, Department of Rheumatology, Roskilde University Hospital, DK-4000 Roskilde. Synovial fluids were from patients with RA treated for effusions of the knee-joint. Synovial fluids were collected in tubes without anticoagulants and centrifuged at 3000 g for 10 min before use. Pools of synovial fluids were made by mixing equal amounts of synovial fluids from 5 to 10 patients.

DEMONSTRATION OF COUPLING ACTIVITY BY IMMUNOELECTROPHORESIS

Experiments were performed by mixing the following components: (i) ITI source: one volume of normal human serum (ITI concentration=1 U ml^{-1}) or purified ITI (0.8 U ml^{-1} or 1.5 U ml^{-1}), (ii) buffer: one volume of 45 mmol Tris-HCl l^{-1}, pH 7.4, 4.5 mmol $CaCl_2$ l^{-1}, 150 mmol NaCl l^{-1}, 4.5 mg hyaluronan ml^{-1}, with or without 9 µg or 36 µg TSG-6 ml^{-1}, (iii) synovial fluid: one volume of human or immunoprecipitated human synovial fluid. In some experiments synovial fluid or the ITI-source was replaced with saline. The mixtures were incubated at 37°C. Aliquots were withdrawn (0, 3, 24 and 42 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[6,23]. 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-volt cm^{-1} for 60 min in a 1.5 mm thick horizontal 1% agarose gel. After electrophoresis, sample wells were treated with testis hyaluronidase (50 U per well, 1 h, 37°C) to release hyaluronan-bound proteins. 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}). Electro-immunoassay of the ITI polypeptides released by hyaluronidase treatment was run overnight at 2.5 V cm^{-1} into the anti-ITI gel[24]. The concentration of ITI heavy chains bound to hyaluronan is expressed in mU ml^{-1}, where 1 U correspond to the total amount of ITI heavy chains in 1 ml normal human serum[23].

All experiments have been performed at least twice.

Results

OCCURRENCE OF COUPLING ACTIVITY

Coupling activity was present in synovial fluid (Fig. 1, lane 1–4) expressed as increasing amounts of HC-hyaluronan complexes formed during 42 h after mixing of human synovial fluid, human serum and hyaluronan. The ITI immunoprecipitates seen at 0 h (Fig. 1 lane 1, 5, 9) is due to *in vivo* preformed ITI-hyaluronan complexes in human synovial fluid plus the ITI bound to hyaluronan in the few minutes from mixing of the reactants to the aliquots were frozen. When serum was replaced by purified ITI, similar results were seen (Fig. 1 lane 5–8), but coupling of ITI to hyaluronan reached a maximum after 3 h of incubation, probably due to exhaustion of the ITI-source. No coupling took place if the ITI-source was omitted, i.e. mixing only synovial fluid and hyaluronan (Fig. 1 lane 9–12). The ITI released from the electrophoresis wells after hyaluronidase treatment showed antigenic non-identity with bikunin and are therefore referred to as heavy chains (HC)

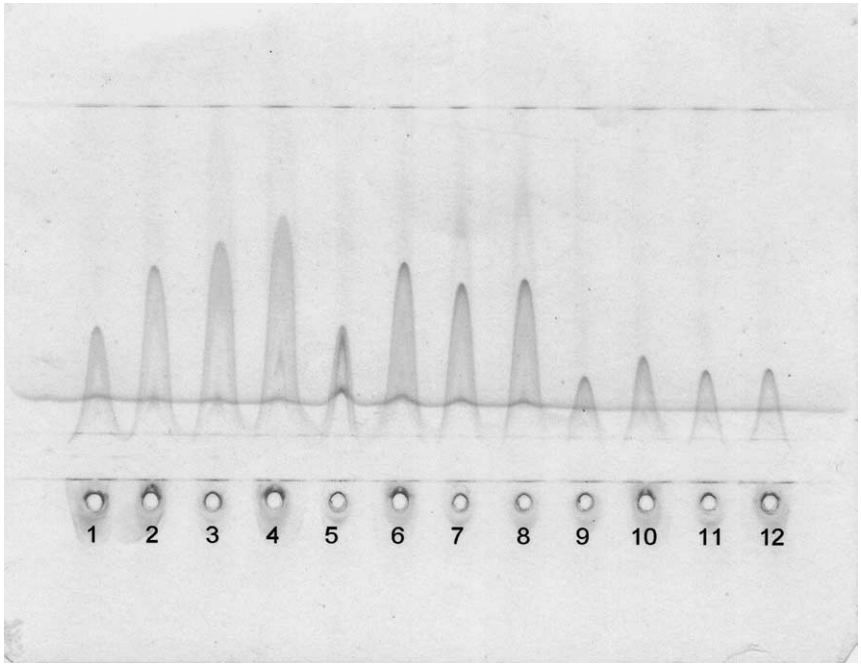

Fig. 1. Demonstration of coupling activity in synovial fluid. Complexes between ITI and hyaluronan were demonstrated by the 2 step-immuno-electrophoresis by which hyaluronan and hyaluronan bound proteins are retained in the electrophoretic wells. Following removal of unbound proteins, hyaluronan bound proteins were released by enzymatic degradation of hyaluronan, and ITI was visualized as 'rocket' immunoprecipitates by electrophoresis into an anti-ITI containing gel. Before the final 'rocket' immunoelectrophoresis, a narrow (5 mm) intermediate gel containing 8 µl purified bikunin (200 mg l^{-1}) was placed between the anti ITI gel and the sample wells, to give a horizontal precipitation line. Lane 1–4: Coupling activity in the presence of serum and synovial fluid**:** control experiment with mixture of human synovial fluid, hyaluronan and human serum incubated for 0, 3, 24 and 42 h. Lane 5–8: Coupling activity in the presence of purified ITI and synovial fluid: as in lane 1–4, but with human serum substituted by purified ITI (0.8 U ml^{-1}). Lane 9–12: No coupling activity in synovial fluid without exogenous ITI: as in lane 1–4, but with human serum substituted by saline.

(Fig. 1 lane 1–4), i.e. the weak horizontal precipitation line passes the rockets without interaction. Only when using purified ITI (Fig. 1 lane 5–8) a weak deflection of the precipitation line is seen (mostly in lane 5 & 6) suggesting that the ITI hyaluronan complexes shortly after their formation contain some bikunin.

Mixing solely serum or ITI with hyaluronan did not result in formation of HC hyaluronan complexes (Table I, experiment 1 & 2). Only when synovial fluid was mixed with an ITI-source (serum or purified ITI), coupling took place (Table I, experiment 4–7). The fact that addition of purified ITI to synovial fluid leads to formation of ITI hyaluronan complexes (Table I, experiment 4) demonstrates that the pool of synovial fluid from RA patients contains all the substances needed for coupling, except ITI. Synovial fluid must therefore contain hyaluronan in excess, accessible to take part in further coupling of ITI. However, synovial fluid must also contain unidentified factors involved in the coupling reaction. A pre-treatment of synovial fluid with trypsin destroyed the coupling activity (Table I experiment 8), suggesting that a protein factor in synovial fluid is involved in the coupling.

Table I
Components required for formation of heavy chain-hyaluronan complexes

Experiment	Component	Heavy chains (mU ml^{-1}) bound to hyaluronan after 0 h	3 h	24 h
1	Hyaluronan+serum	0	0	0
2	Hyaluronan+ITI*	0	0	0
3	Synovial fluid+hyaluronan	90	98	80
4	Synovial fluid+ITI*	116	135	275
5	Synovial fluid+serum	108	145	183
6	Synovial fluid+ITI*+hyaluronan	118	129	237
7	Synovial fluid+serum+hyaluronan	98	129	188
8	Synovial fluid†+serum+hyaluronan	89	79	71

*ITI concentration=1.5 U ml^{-1}.
†Human synovial fluid pre-treated with trypsin.

INVOLVEMENT OF TSG-6 IN THE COUPLING REACTION

Synovial fluid was incubated with polyclonal rabbit antibodies directed against human TSG-6 protein in order to immunoprecipitate TSG-6.

After immunoprecipitation, coupling activity decreased in a dose dependent manner (Fig. 2) and addition of anti TSG-6 antibody diluted 1:2 completely inhibited the coupling activity, i.e. the immunoprecipitates did not increase in size showing that no ITI-heavy chain were coupled to hyaluronan during the incubation period.

However, if recombinant TSG-6 was subsequently added to the immunopreciptated synovial fluid (Fig. 3), the coupling activity was restored in a dose-response manner (Fig. 3 lane 7–12) using 3 µg/ml or 6 µg/ml TSG-6. Consequently, TSG-6 must be essential for the coupling of ITI to hyaluronan in synovial fluid.

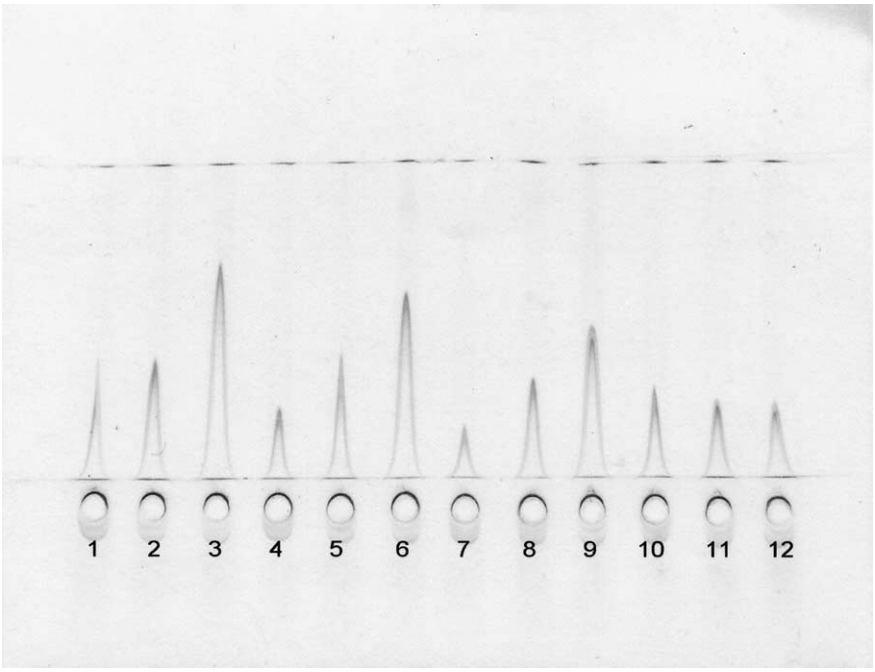

Fig. 2. Coupling activity in synovial fluid is removed by anti-TSG 6. Lane 1–3: Coupling activity in the presence of serum and synovial fluid**:** control experiment with mixture of human synovial fluid, hyaluronan and human serum incubated for 0, 3 and 24 h. Lane 4–6: Immunoprecipitation with anti TSG 6 (diluted 1:8)**:** as in lane 1–3, but after pre-treatment of synovial fluid with anti-TSG 6. Lane 7–9: Immunoprecipitation with anti TSG 6 (diluted 1:4)**:** as in lane 1–3, but after pre-treatment of synovial fluid with anti-TSG 6. Lane 10–12: Immunoprecipitation with anti TSG 6 (diluted 1:2): as in lane 1–3, but after pre-treatment of synovial fluid with anti-TSG 6.

To test the influence of Calcium ions known to be essential in follicular fluid, 10 mM EDTA was added to remove free Ca^{++}. As seen in Fig. 4 (lane 4–6) this blocked the coupling activity. To further characterize the necessary ion Mg-EGTA (lane 7–9) was added. Mg-EGTA will chelate Ca^{++}, Fe^{++}, Fe^{+++}, Zn^{++} and Cu^{++}, but allow free Mg^{++}. No coupling was seen in this case. Addition of Ca-EGTA (lane 10–12) chelates Fe^{++}, Fe^{+++},Zn^{++} and Cu^{++}, but allow free Ca^{++} and Mg^{++}. In this case the coupling activity of synovial fluid was maintained, showing that Ca^{++} is essential for the coupling reaction in synovial fluid.

Discussion

The focus of ITI binding to hyaluronan has in the last few years been on normal oocyte development and ovulation[25] as ITI is necessary for normal fertility[26]. The binding of ITI to hyaluronan was first discovered in pathological synovial fluid[27], but it is not until the last decade that attention has been drawn to the similarities between the coupling of ITI to hyaluronan in synovial fluid and follicular fluid[6].

One important question is: does ITI simply bind to hyaluronan if it gets access to body-compartments with hyaluronan or is other components necessary? When our binding assay was performed at physiological salt concentration, we observed no binding by mixing only ITI and hyaluronan. Addition of synovial fluid to the reaction mixture generated complexes between ITI and hyaluronan progressing over a period of hours, as demonstrated in Fig. 1. As we found no coupling of ITI to hyaluronan if synovial fluid was substituted by normal human serum, we concluded that synovial fluid must contain at least one component distinct from normal plasma constituents, but essential for the coupling reaction.

For the binding to take place in follicular fluid, ITI, and Ca^{++} plus hyaluronan and TSG-6 supplied by follicular fluid must be present together to form stable complexes[12]. TSG-6 is an anti-inflammatory protein[28] detected both in synovial fluid of arthritic patients[14] and in ovarian follicles that have been induced to ovulate[29].

In the present study we have demonstrated that TSG-6 and Ca^{++} are necessary for the coupling of ITI to hyaluronan in synovial fluid. This is in accordance with the recently discovered role of TSG-6 in follicular fluid as mentioned above[12]. The molecular mechanism behind the coupling of ITI to hyaluronan and the role of TSG-6 and Ca^{++} in this biochemical reaction is not fully understood. TSG-6 is, however, known both as a hyaluronan binding and an ITI binding protein[21], and one possible function of TSG-6 in the coupling reaction might therefore be to establish contact between ITI and hyaluronan as a first step in the coupling reaction. A cross-linking of hyaluronan could be established by a possible secondary displacement of bikunin and the chondroitin sulphate chain in a trans-ester reaction leading to the formation of an ester bond between the C-terminal aspartate of HC and hyaluronan[7].

There are many similarities between the coupling of ITI to hyaluronan in synovial fluid and in follicular fluid. Both reactions take place in the extra-cellular space of the body compartment in question with an internal synthesis of hyaluronan and TSG-6, and an external supply of ITI from the blood circulation. In both cases the reaction is dependent on calcium ions, and the final coupling product consists of ITI heavy chains without bikunin. Recently reported experiments[12] showed that incubation of Hyaluronan, TSG-6, and Ca^{++} with purified ITI did not lead to coupling of ITI to hyaluronan. Synovial fluid and follicular fluid may therefore contain unidentified components essential for the

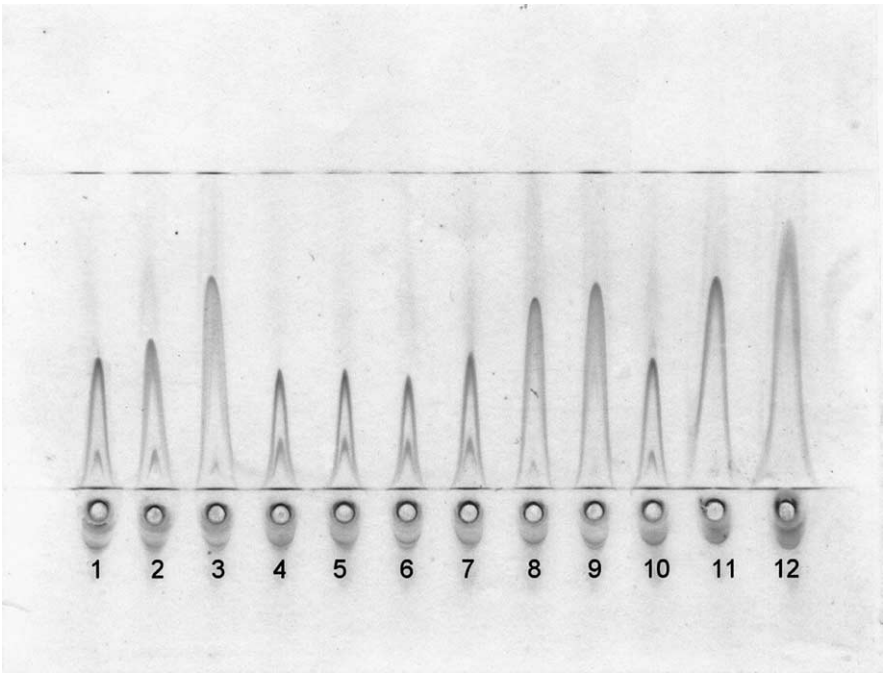

Fig. 3. Restoration of coupling activity in immunoprecipitated synovial fluid by addition of recombinant TSG 6. Lane 1–3: Coupling activity in the presence of serum and synovial fluid: control experiment with mixture of human synovial fluid, hyaluronan and human serum incubated for 0, 3 and 24 h. Lane 4–6: Immunoprecipitation of synovial fluid with anti TSG 6 (diluted 1:2): as in lane 1–3, but after pre-treatment of synovial fluid with anti-TSG 6. Lane 7–9: Addition of TSG 6 to immunoprecipitated synovial fluid: as in lane 4–6, but with addition of TSG 6 (3 μg/ml). Lane 10–12: Addition of TSG 6 to immunoprecipitated synovial fluid: as in lane 4–6, but with addition of TSG-6 (12 μg/ml).

coupling reaction, or the lack of coupling might be due to non-optimal reaction conditions.

We find it most likely to be the same reaction mechanism behind the coupling of ITI to hyaluronan in synovial fluid and follicular fluid. There are, however, differences between the coupling potential of synovial fluid and follicular fluid. In synovial fluid from RA patients, ITI seems to be the limiting component in the coupling reaction, as purified ITI added to synovial fluid causes further coupling of ITI heavy chains to hyaluronan. In follicular fluid no further coupling is generated when ITI is added, but addition of hyaluronan to follicular fluid causes further coupling[11]. Therefore, the influx of ITI to the synovial cavity seems to be the limiting factor for the coupling of ITI to hyaluronan in synovial fluid from RA patients, while intrafollicular synthesis of hyaluronan seems to be the limiting factor in preovulatory follicles.

The physiological function of the coupling of ITI heavy chains to hyaluronan is much better understood in the ovaries than in joints. A profound expansion of the cumulus cell-oocyte complex (COC) in the follicle takes place just before ovulation. The COC is a mucilaginous network of hyaluronan, hyaluronan producing cumulus cells, ITI heavy chains and TSG-6[30]. *In-vitro* experiments have shown that when ITI is not present at the time of COC expansion, the hyaluronan network surrounding the ovum cannot maintain its integrity, and no stable COC matrix is obtained[31]. In agreement with this, *in-vivo* experiments have shown that female mice lacking ITI are infertile, but infertility can be reversed by injection of ITI[26,32]. Deficiency of TSG-6 appears to have the same consequences. Female mice lacking TSG-6 are sterile, and fail to produce expanded COC, but fertility is regained by injection of recombinant TSG-6[13]. Thus both ITI and TSG-6 are indispensable for female fertility, and the important role of the two proteins in the ovulation process seems to be a stabilization of the COC and an incorporation of newly synthesized hyaluronan molecules into the expanding hyaluronan network of the COC.

The physiological function of the hyaluronan network in synovial fluid is considered to be of great importance for its high viscosity and for its lubricating and shock-absorbing functions[2]. TSG-6 is an acute phase protein[15] and one might speculate if the formation and maintenance of the ITI-hyaluronan network are of especially importance in inflammatory joint disorders with influx of plasma and joint swelling. In various models of experimentally induced arthritis TSG-6 is reported to exhibit a strong anti inflammatory and chondroprotective effect preventing cartilage proteoglycan from metalloproteinase-induced degradation[18,19,33]. Thus, in inflamed joints TSG-6 could be important both due to its anti-inflammatory and chondroprotective activity, but also in collaboration with ITI through stabilization and rebuilding of the hyaluronan network in order to maintain the rheological properties of the synovial fluid.

Intra-articular injections of native hyaluronan or a cross-linked hyaluronan derivative have been used in the treatment of patients with osteoarthritis to relieve pain and improve the function of the knee[34]. Inflammatory symptoms are reduced, and the rheological properties of synovial fluid are to some degree restored by this treatment[35]. Since the concentration of TSG-6 is raised in inflamed joints, a combination of hyaluronan and ITI might be a far more effective therapeutic agent compared to pure hyaluronan, because the coupling of ITI heavy chains to hyaluronan in inflamed joints seems according to this study mainly to be limited by the influx of plasma ITI into the synovial cavity.

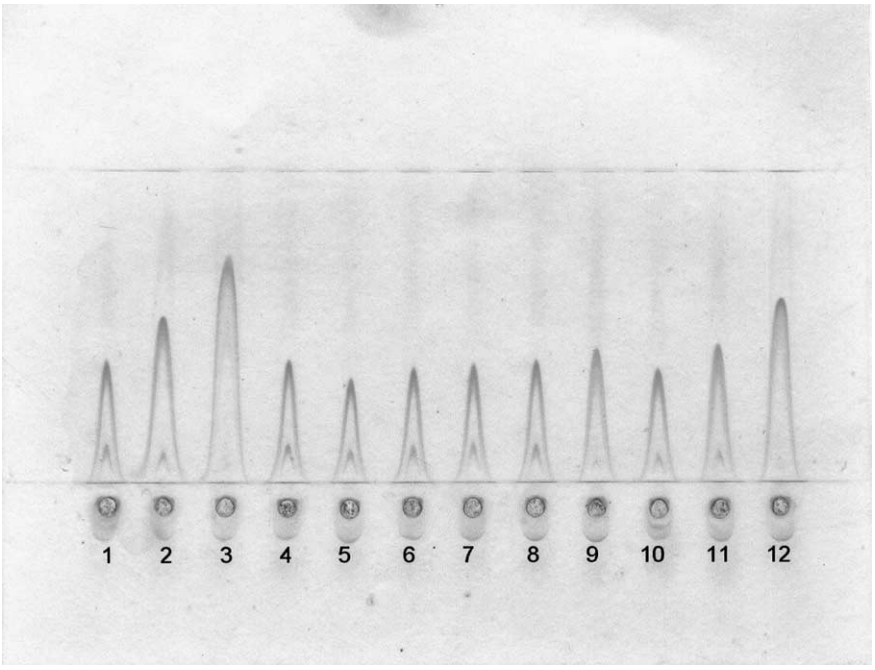

Fig. 4. Coupling activity in synovial fluid depends on calcium ions. Lane 1–3: Coupling activity in the presence of serum and synovial fluid**:** control experiment with mixture of human synovial fluid, hyaluronan and human serum incubated for 0, 3 and 24 h. Lane 4–6: Coupling activity is blocked by EDTA: as in lane 1–3, but with addition of EDTA (10 mmol/l). Lane 7–9: Coupling activity is blocked by Mg-EGTA**:** as in lane 1–3, but with addition of Mg-EGTA (10 mmol/l). Lane 10–12: Coupling activity is not blocked by Ca-EGTA**:** as in lane 1–3, but with addition of Ca-EGTA (10 mmol/l).

In conclusion, the present study shows that TSG-6 and calcium ions are both necessary for the coupling of ITI to hyaluronan.

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