SPANDIDOS PUBLICATIONS

Pro- and anti-inflammatory cytokines increase hyaluronan production by rat synovial membrane *in vitro*

ANNA HYC, ANNA OSIECKA-IWAN, JUSTYNA NIDERLA-BIELINSKA, EWA JANKOWSKA-STEIFER and STANISLAW MOSKALEWSKI

Department of Histology and Embryology, Medical University of Warsaw, Chalubinskiego 5 St, PL02-004 Warsaw, Poland

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Abstract. Synovial membrane consists of fibroblasts and macrophages forming the synovial lining supported by vascularized subsynovium. Each of these components may specifically react to a particular stimulus. Thus, reactions of isolated synovial cells may not correspond to that of intact tissue. We characterized the production of hyaluronan (HA) by rat synovial membrane exposed *in vitro* to pro- and anti-inflammatory cytokines and compared it with previous results obtained with isolated fibroblasts. Synovial membrane dissected from one knee joint served as a control to that from the opposite knee exposed to IL-1ß, TGF-ß1, TNF-α, IFN-γ or IL-4 for 24 h. The HA content was determined by ELISA, and hyaluronan synthase (HAS) mRNA by real-time PCR. The size distribution of the HA chain was evaluated by agarose gel electrophoresis. The HA content in the freshly dissected synovial membrane was ~1 μg and decreased to 0.1 μg after incubation, while in the medium it increased from 0 to 3 to 5 μg. All cytokines stimulated production of HA. The strongest effect was observed in the case of TNF-α. The level of HAS1 and HAS2 mRNA increased 2-fold during a 12-h incubation while that of HAS3 decreased. The distribution of the HA chain length did not differ in the medium from the control and stimulated membranes. Transfer of the synovial membrane from the HA-rich synovial fluid into the medium stimulated release of HA from the membrane and increased HAS expression and HA production. Thus, the synovial membrane acts as a sensor reacting to changes in HA concentration in its environment. Pro-inflammatory cytokines stimulate production of HA in intact synovial membranes similarly as in cultures of rheumatoid fibroblasts. In contrast, our results suggest that the response to anti-inflammatory cytokines (TGF-ß1 and IL-4) of the whole synovial membrane differs from that of isolated fibroblasts.

Correspondence to: Dr Justyna Niderla-Bielinska, Department of Histology and Embryology, Medical University of Warsaw, Chalubinskiego 5 St, PL02-004 Warsaw, Poland
E-mail: justynan@ib.amwaw.edu.pl

Abbreviations: HA, hyaluronic acid; HAS, hyaluronic acid synthase

Key words: anti-inflammatory cytokines, hyaluronan, synovial membrane

Introduction

Hyaluronan (HA) is a high-molecular-mass polysaccharide present predominantly in the extracellular matrix (1). In extracts obtained from tissues, it is polydispersed with regard to molecular mass with an average M_r of several million (2). HA, bound to cell receptors, is also the major component of pericellular matrices exhibited by various cell types and is susceptible to the action of hyaluronidase (3-5). HA has also been found to occur intracellularly, particularly in cells subjected to endoplasmic reticulum stress, forming a ring around the nucleus and passing into the extracellular space in the form of cable-like extensions (6-8).

HA present in synovial fluid is synthesized by synovial membrane (9) defined in anatomy textbooks as vascular connective tissue which produces synovial fluid (10). Synovial membrane is composed of synovial lining and subsynovium (11).

Synovial lining (synovium) consists of macrophage-like type A cells and fibroblast-like B cells (12,13). Synovial fibroblasts show a subintimal and intimal phenotype, depending on their position in the synovium (14). The cells displaying the latter phenotype are surrounded by HA (15) and express high activity of uridine diphosphoglucose dehydrogenase (UDPGD) which indicates that they are responsible for HA synthesis (16). Furthermore, type-B synoviocytes, without distinct subintimal and intimal forms, were reported to express hyaluronan synthases (HASs) (17,18). HA is the major macromolecular component of the synovial fluid and has an important role in joint lubrication (1,19,20). It protects joint tissues inhibiting the stimulatory effect of pro-inflammatory cytokines on the production of matrix metalloproteinases (21,22). HA in the synovial fluid may be degraded within the joint cavity since synoviocytes produce hyaluronidase active at a neutral pH (23).

Synovial fibroblasts, either obtained from inflammatory synovium (24) or aspirated from joints of patients with rheumatoid arthritis (RA) or osteoarthritis (OA) (25) produce a panel of cytokines similar to that secreted by macrophages (26,27). Thus, both populations of synovial cells may affect

each other, and their combined response could differ from that produced by a particular class of synoviocytes. Moreover, the synovial lining (synovium) is supported by a highly vascularized subsynovium, consisting of loose alveolar tissue, dense fibrous tissue and fat pad (11). Hormones or cytokines produced by adipocytes (28-31) and cytokines produced by endothelial cells (32) present in subsynovium may also contribute to the total response of the synovial lining to particular stimuli.

*In vitro s*tudies of the reaction of the synovial membrane to different agents have been limited to the pathological material either from patients with RA (33,34) or from the hind feet joints of rats with adjuvant arthritis (35,36). We recently described a culture of rat synovial membrane dissected from knee joints and demonstrated that synovial membrane stimulated *in vitro* by lipopolysaccharides (LPS) for 24 h had a significantly stronger cytokine response than the synovial membrane from the opposite (control) knee (37). This *in vitro* system seems to be particularly suitable for analysis of the joint response of synovial membrane tissue components. There are several reports indicating that pro-inflammatory cytokines stimulate production of hyaluronan by fibroblasts (38-42). As far as we can establish, however, no reports on the *in vitro* stimulation of HA production by normal synovial membrane have been published. In view of the importance of HA in joint physiology, in this study we investigated the production of HA by synovial membrane, either non-stimulated or stimulated by pro- (IL-1ß, TNF-α, IFN-γ) or anti-inflammatory (IL-4, TGF-ß1) cytokines. The study also aimed to detect hyaluronan synthase (HAS) expression in synovial membranes, to examine neutral hyaluronidase activity in the culture medium and to determine whether HA undergoes degradation in cultures exposed to cytokines.

Materials and methods

Animals. Inbred, 3-month-old Lewis male rats served as donors of synovial membranes. Animals, kept in pathogen-free conditions, were obtained from the Animal Unit of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw. The study and the methods were approved by the Animal Ethics Committee of the Medical University of Warsaw.

Preparation of rat synovial membranes. After the opening of the knee joint cavity, the synovial membrane was excised together with the patella, patellar ligament and joint capsule. Next, the synovial membrane with infrapatellar fat pad was separated from the other tissues according to a previously described method (37).

Incubation of synovial membranes. Synovial membranes were put into flat-bottomed 24-well plates (Corning, NY USA) in 1 ml of RPMI medium with 1% antibiotic and antimycotic Solution (Sigma-Aldrich Chemie, Diesenhofen, Germany) for 24 h of incubation on a slow-motion shaker at 37˚C in 5% CO_2 in air. The synovial membrane from one knee joint served as a control to the synovial membrane from the opposite knee. Experimental synovial membranes were exposed to 10 ng/ml of IL-1ß, 10 ng/ml TGF-ß1, 20 ng/ml TNF-α (Sigma), 100 ng/ml IFN-γ or 10 ng/ml IL-4 (PromoKine, PromoCell GmbH, Heidelberg, Germany). After incubation, the media were collected and stored at -20˚C. Synovial membrane lysates were prepared on ice with lysis buffer [CHAPS 1% w/v, NaCl 150 mM, Tris 50 mM, EDTA 5 mM, PMSF 1 mM (pH 7.8); 30-min incubation) and stored at -20˚C (43).

Total RNA isolation. Synovial membrane RNA for HAS expression determination was isolated immediately after dissection or after a 12-h incubation in cytokine-free medium. Two synovial membranes (from two rats) were pooled. RNA was isolated with the NucleoSpin®RNA II Kit (Macherey-Nagel, Duren, Germany) according to he manufacturer's protocol. The quality and quantity of isolated total RNA were measured with PicoDrop (Picodrop Ltd., Walden, UK). Quality of isolated RNA was also checked by electrophoresis in 1% agarose denaturing gel containing 6% formaldehyde and buffered with MOPS (Sigma). After electrophoresis, the gel was scanned with Digital Imaging System GDS9000 using GRAB-IT.2.0 software (UVP, Cambridge, UK).

Reverse transcription. RT was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cheshire, UK) according to the manufacturer's protocol in Eppendorf Master Cycler Gradient. cDNA samples were stored at -20˚C.

Real-time PCR. Real-time PCR was performed in ABI PRISM 7500 (Applied Biosystems) in 96-well optical plates. Each sample was tripled and supplied with endogenous control (Rat GAPDH Endogenous Control VIC®/MGB Probe). For gene expression, TaqMan Expression Assays were used: for HAS1, Rn00597231_m1; for HAS2, Rn00565774_ml; and for HAS3, Rn01643950_m1. All probes were stained with FAM (Applied Biosystems). Reactions were run in a 25-μl volume with TaqMan Universal Master Mix, appropriate primer set, MGB probe and 50 ng of cDNA template. Universal thermal conditions: 10 min at 95˚C and 40 cycles of 15 sec at 95˚C and 1 min at 60˚C were used. Data analysis was conducted with sequence detection software version 1.2 (Applied Biosystems).

ELISA assay for HA determination. The HA level in supernatants and lysates was evaluated with Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (Echelon Biosciences Inc., Salt Lake City, UT, USA). HA ELISA is a competitive assay. Concentration of HA in the sample was determined using a standard curve of a known amount of HA.

Agarose gel electrophoresis of HA. The length of the HA chains in the control and cytokine-stimulated synovial membrane cultures was compared by agarose gel electrophoresis. Supernatants from particular experimental groups were pooled, desalted using PD-10 columns (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) and lyophilized. Each sample contained 5 μg of HA. Then the samples were dissolved in 30 μl 0.15 M NaCl and digested with pronase E to avoid protein contamination. Each sample

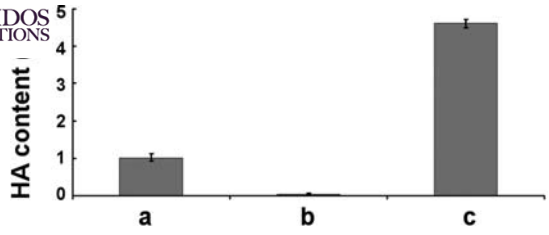

Figure 1. HA content in the synovial membrane lysates and in culture medium. Mean value ± SE of HA content in the lysates of freshly isolated synovial membranes (a), in lysates (b) and medium (c) after 24 h of incubation. Each group, n=6. Differences between groups were significant according to the Wilcoxon matched pairs test at $p<0.05$.

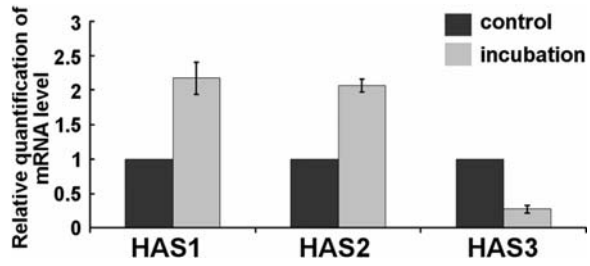

Figure 2. HAS1, HAS2 and HAS3 mRNA levels in the synovial membrane. Mean value ± SE of HAS1, HAS2 and HAS3 mRNA levels in the synovial membrane immediately after isolation and after 12 h of incubation, measured with real-time PCR. Each group, n=3. Relative expression was related to the reference gene, GAPDH. Analysis was conducted as a relative quantification study using the freshly isolated synovial membrane gene expression as a calibrator (value = 1).

was mixed with 5 μl of pronase solution (Sigma) at a concentration of 76 mg/ml of 0.15 M NaCl and incubated with gentle swirling at 37˚C for 5 h.

Electophoresis of 35-μl samples mixed with 5 μl of 0.02% bromophenol blue solution and 2 M sucrose in TAE buffer was performed in 0.5% HGT agarose gel (SeaKem, Lonza Rockland, ME, USA) in TAE buffer at room temperature for 4 h with a constant voltage of 50 V. After electrophoresis, gels were placed in a solution containing 0.005% Stains-All, 10% formamide, 25% isopropanol, 15 mM Tris-HCl (all from Sigma) and 65% H_2O (pH 8.8) for 24 h. The gels were destained in distilled water and stored in the dark for 48 h (44). Then the gels were exposed to ambient room light for 30 min and scanned with the Digital Imaging System GDS9000 using GRAB-IT 2.0 software.

Detection of hyaluronidase activity. Hyaluronidase activity was detected by hyaluronic acid zymography according to Miura *et al* (45) with some modifications. Supernatants were concentrated 10-fold and mixed with an equivalent volume of Laemmli's sample buffer (46) containing 4% SDS and no reducing agent. Bovine testis hyaluronidase was used as a standard at a concentration of 125 U/sample/line (Sigma). Samples, without heating, were applied to 8% SDS-polyacrylamide gel containing 0.17 mg/ml HA from rooster's comb (Sigma). Stacking gel did not contain HA. After electrophoretic run, the gel was rinsed with 2.5% Triton X-100 (Sigma) at room temperature for 1 h in order to remove SDS and incubated in 50 mM citrate-phosphate buffer (Sigma) (pH 7.0) at 37˚C overnight. To visualize digestion of the HA, the gels were stained with 0.5% Alcian blue (Sigma) in 20% ethanol-10% acetic acid for 1 h and destained with 20% ethanol-10% acetic acid.

Statistical analysis. Differences between the control and experimental groups were evaluated by the Wilcoxon matched-pairs test.

Results

To assess the quality of the dissection and the preparation of synovial membranes, the difference in HA content between the synovium from the left and right knee joint of the same rat was evaluated. There was no significant difference in HA content both in the synovium lysates and the incubation media (data not shown).

The mean HA content in the lysates of the synovial membrane immediately after dissection was 1.06 (SE ±0.09) μg per one synovium. After 24 h of incubation, the HA content in the lysates of the synovial membranes decreased to <0.1 μg. HA accumulated, however, in the incubation medium, exceeding its initial value in the explant 2- to - 5-fold after 24 h of incubation (Fig. 1).

The increase in HA content in the medium during synovial membrane incubation was in accord with the results of our experiment concerning hyaluronan synthases. The level of mRNA for HAS1 and 2 increased 2-fold in the synovial membranes maintained in the culture medium for 12 h in comparison with freshly dissected samples. At the same time, the level of HAS3 mRNA decreased after 12 h of incubation (Fig. 2). Expression of mRNA for HAS3 synthase, was, however, very low initially. In the case of HAS1 and HAS2 mRNA, the curve started to increase between 20 and 30 real-time PCR cycles. In the case of HAS3, the curve increased only slightly in 35 PCR cycles. To prove that the latter result was not an artifact we repeated the experiment using a double amount of RNA. The HAS3 mRNA curve started to increase in 29 PCR cycles whereas the final result was the same, a decrease in the HAS3 mRNA level.

Exposure of the synovial membranes to TNF-α, IL-1ß, IFN-γ, TGF-ß1 or IL-4 significantly increased the HA content in the medium above that of the control. The highest increase, exceeding 50%, occurred after TNF-α stimulation and the lowest, barely reaching 10%, occurred in the synovial membranes exposed to IFN-γ (Fig. 3). The HA content in the synovial membrane lysates, independently of cytokine stimulation, remained at the control level and did not exceed 2.5% of that in the medium (data not shown).

Subsequently, we compared the size distribution of the HA chains in the control and cytokine-stimulated synovial membrane cultures by agarose gel electrophoresis followed by Stains-All staining. HA in the gel formed broad bands with a predominance of material in the high molecular weight range (Fig. 4). No difference in the HA chain size distribution between the control and cytokine-treated samples was noticed.

Activity of hyaluronidase was studied by HA zymography at pH 6.0. Hyaluronidase from bovine testis, used as a standard, exhibited activity against HA and was detected as a 65-kDa white band against a blue background. Similar

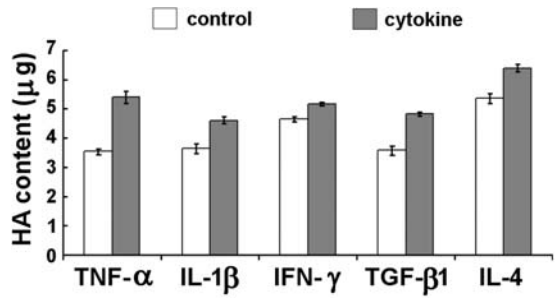

Figure 3. HA content in the media of cytokine-stimulated synovial membrane cultures. Mean value ± SE of HA content in the medium after 24 h of synovial membrane incubation without (control) or with cytokine stimulation. Values for synovial membrane lysates are omitted since their HA content did not exceed 2.5% of that in the medium. Each group, n=6. All differences between the control and experimental values were significant according to the Wilcoxon matched pairs test; $p<0.05$.

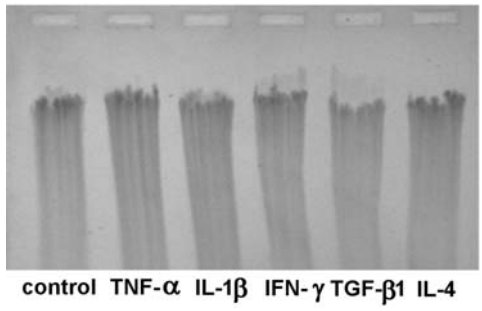

Figure 4. Size distribution of HA chains in the media from the synovial membrane cultures. Size distribution of HA chains in the media from synovial membrane cultures without (control) or with cytokine stimulation. Agarose gel, Stains-All staining.

activity was undetectable in the media in which synovial membranes, either control or cytokine-treated, were incubated for 24 h, although samples of media were concentrated 10-fold before electrophoresis (data not shown).

Discussion

The content of HA in tissues is controlled in mammals by two classes of enzymes: HA synthases (HAS1, HAS2 and HAS3) (47) and hyaluronidases (48,49). Particular isoforms of HAS probably synthesize HA of different sizes (50). Hyaluronan synthesis takes place at the inner surface of the plasma membrane, and nascent hyaluronan is extruded onto its outer surface while it is still attached to the synthase that produced it (47,50,51). Thus, hyaluronan remains in close contact with the cell surface and can participate in the formation of the pericellular hydrated zone (3).

Studies on the enzymes responsible for HA synthesis in human synovial fibroblasts suggest that HAS2 and HAS3 are constitutively activated, while HAS1 is the only gene responding to stimulation by pro-inflammatory cytokines (17,18). In the synovium obtained from OA patients, expression of HAS3 mRNA was significantly greater than that in the control synovium while expression of HAS1 and HAS2 mRNA decreased (52). Our results suggest that the expression of HASs in synovial lining cells may differ between species, since in rat synovial membrane, expression of HAS3 mRNA was much lower than that of HAS1 and HAS2 and further declined during *in vitro* incubation.

In the synovial membrane, HA is produced by fibroblast-like synoviocytes and must be secreted continuously to maintain the effective concentration needed for lubrication of articular surfaces (53). The rate of secretion is significantly higher in joints expanded by injection of saline which suggests existence of a compensatory mechanism which aids to combat a fall in HA concentration (54,55). This mechanism may involve static stretch since HA secretion is mechanosensitive (56). This property of synoviocytes is probably involved in the rise in circulating HA levels, occurring after resuming physical activity after a period of rest (57). Furthermore, an adequate concentration of HA may also be important for maintenance of the synovial membrane itself, particularly in pathological states, since HA has been shown to exert a protective influence on fibroblast-like synovial cells exposed to LPS (58).

Results obtained in this study are in accord with the above-mentioned observations and suggest that an equilibrium between concentrations of HA in the synovial membrane and in the synovial fluid exists in the intact joint. Retention of HA in synovial membrane is caused by its binding to collagen type VI (59) present in the subsynovium (60), other interstitial hyaladherins (4) or cell surface HA receptors (61,62). The content of HA per freshly dissected rat synovial membrane was ~1 μg and decreased to <0.1 μg after 24 h of incubation. The content of HA in the medium increased in various experiments from a zero value at the beginning of the experiment to 3-5 μg after 24 h of incubation. Since the drop in the HA content in the incubated synovial membranes was accompanied by its marked increase in the medium, we suggest that HA was detached from hyaladherins present in the synovial membrane and was released into the medium as a reaction to the disturbed equilibrium between the synovial membrane and its environment and not as a result of the metabolic impairment of the tissue. This conclusion is supported by the increased expression of HAS1 and HAS2 in the synovial membranes maintained in incubation medium. Taking into account the large volume of incubation medium (1 ml) in comparison to the volume of synovial fluid in the joint cavity, the equilibrium between the synovial membrane and its surroundings could not be restored to the *in vivo* level, and the HA content in the synovial membrane could not return to its initial value even when HASs were strongly activated. In general, our results agree with theories (54,55) that synovial lining cells contain a sensor which detects changes in HA concentration in synovial fluid and activates HASs when it drops below a physiological level.

The size distribution of HA chains produced by synovial membranes did not change upon treatment with cytokines which is in good agreement with the lack of detectable neutral hyaluronidase activity in the medium after 24 h of incubation. Previous results (63) showed that hyaluronidase was also not detected in synovial (equine) fluid which contained only hyaluronidase ultrafiltrated from blood and active at pH 4.0.

Our observations that pro-inflammatory cytokines, TNF-α, IL-1ß and IFN-γ, stimulate synthesis of HA in rat synovial

e incubated *in vitro* are in agreement with previous of such stimulation in monolayer cultures of fibroblasts from lung (39,64,65), human skin (40,42) and rheumatoid human synovium (41,66). This suggests that these effects, at least in a general tendency, do not depend on the source of fibroblasts, conditions of culture and presence of other cells in synovial membrane.

In our experiments TGF-ß1 and IL-4, generally recognized as anti-inflammatory cytokines (67), significantly stimulated production of HA. Response to TGF-ß1 and IL-4 reported in previous studies varied and depended on cell type and culture conditions. Thus, TGF-ß1 enhanced production of HA in lung but not in skin fibroblasts (68). In another study TGF-ß1 was found to inhibit HA synthesis in subconfluent cultures of adult dermal fibroblasts maintained in plastic dishes but stimulated at high cell density. Production of HA by subconfluent cultures of these fibroblasts on a collagen substratum was also inhibited by TGF-ß1, but remained unaffected in confluent cultures (38). TGF-ß1 in one report inhibited HA production in human rheumatoid fibroblastic synovial lining cells (41), but in another report it increased expression of HAS1 and production of hyaluronan but suppressed HAS3 activity (17). In fibroblasts derived from rabbit synovial membrane, TGF-ß1 increased production of HA and expression of HAS2 mRNA (56,69).

IL-4 exerted an inhibitory effect on HA production in lung (39) and in retroorbital fibroblasts (70). IL-4 up-regulated HA synthesis and expression of HAS2 in retroorbital fibroblasts stimulated by IL-1ß (71) but inhibited HA production by synovial cells from RA patients exposed to the same cytokine (66).

RA synovial fibroblasts in many respects differ from normal fibroblasts (72). Thus, it is difficult to say whether inhibition of HA production by TGF-ß1 in such cells (41) and its stimulation in the whole synovial membrane observed in this study, are dependent on the difference between rheumatoid and normal synoviocytes, species difference, or the type of culture. More interesting is the comparison with synoviocytes obtained from normal rabbit synovium cultured in monolayer. These synoviocytes responding to TGF-ß1 treatment increased the HA content both in cell layer and in the medium, particularly at a dose >30 ng/ml. However, at the dose of 10 ng/ml, used in our study, the response was insignificant (69). When species specificity is ignored, it appears that synoviocytes in the synovial membrane are more sensitive to TGF-ß1 than in a monolayer culture. Furthermore, the loss of HA from synovial membrane in an organ culture and its accumulation in monolayer cultures suggest that the reaction to the decline in HA content requires preservation of tissue integrity and disappears when synoviocytes are separated from their natural habitat.

Acknowledgements

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