Evidence for hyaluronan production in the air pouch model in rats

S. Generini¹, M. Matucci-Cerinic¹, G. Partsch², M. Stancikova³, A. Pignone¹, Y.T. Konttinen⁴, J. Rovensky³, D.J. Baker⁵, H.R. Schumacher Jr.⁵

¹Department of Internal Medicine, Section of Rheumatology, University of Florence, Italy; ²Ludwig Boltzmann Institute of Rheumatology and Balneology, Wien, Oberlaa, Austria; ³Research Institute of Rheumatic Diseases, Piestany, Slovak Republic; ⁴Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; ⁵Rheumatology-Immunology Center, VAMC, Philadelphia, Pennsylvania, USA.

Abstract

Objective
The aim of our work was to investigate the presence of hyaluronan (HA) in the rat air pouch and its behaviour in response to inflammatory stimuli.

Methods
HA levels (by a microplate assay) and the leucocyte count were determined in the fluid obtained from air pouches in which acute or subacute inflammation had been induced by the injection of monosodium urate crystals (MSU) or high density polyethylene (HDPE) debris respectively and in relative controls.

Results
In control pouches of both groups, remarkable levels of HA were found; these levels were higher in the very first hours (2475 and 1850 g/l at 6 hrs) and then gradually decreased. In pouches injected with MSU, HA moderately increased (p < 0.001) after 6 hrs, reached a peak after 12 hrs (p < 0.001) and began to taper at 24 hrs (p < 0.001). The leucocyte count was also increased at 6 hrs (p < 0.001), became higher at 12 hrs (p < 0.001) and tapered at 24 hrs (p < 0.001). In the HDPE pouches, HA levels were significantly reduced with respect to controls after 6 hours (p < 0.001), increasing later (p < 0.001) to reach a peak at 24 hrs (p < 0.001), and returning to the original levels, or even below, in the following 72 hours.

Conclusions
These data confirm that the pouch lining produces fair amounts of HA and provide evidence that, in this system, HA levels seem to be influenced by the degree of inflammation even if with variable behaviour in relation to the different characteristics and phases of phlogosis. The present data suggest that the air pouch is a useful experimental model for studies on HA metabolism in either acute or chronic inflammation.

Key words
Air pouch, hyaluronan, inflammation.

Introduction
The subcutaneous injection of air in the rat back provides a well established experimental system (1) that has been successfully used to test pharmacologic, anti-inflammatory or proinflammatory effects, and carcinogenic and other biological activities of different drugs and agents. In 1981 Edwards et al. (2) showed the rat air pouch to be a structure closely resembling synovium and a suitable site for the study of effects on facsimile synovial tissue under a wide variety of stimuli.

Hyaluronan (HA) is a high molecular weight non-sulfated polymer of the N-acetyl-glucosamine and -D-glucuronic acid disaccharide repeat unit (3) which is synthesised at the level of the cellular plasmatic membrane by a HA-synthetase recently isolated from fibroblasts. HA is present in several biologic fluids and tissues, but is particularly abundant in the synovial fluid, where it is synthesised by B-synoviocytes lining the synovial space (4).

The first aim of this study was to verify how far the air pouch lining actually resembles synovial tissue by evaluating one of its most typical biosynthetic properties: the production of HA. HA production by the air pouch lining was studied both under basal conditions and during acute and subacute inflammation induced by different agents in order to determine whether this experimental animal model may be useful for studies on HA dynamics in response to various stimuli.

Materials and methods
Study design
The study was based on two different models of local inflammation: acute inflammation, induced by the injection of monosodium urate (MSU) crystals, with controls; and sub-acute inflammation, induced by the injection of high density polyethylene (HDPE) debris, with controls. It is well established that the particulate debris of HDPE, a material commonly used for the wear of articular prosthetic surfaces, induces an intense prolonged inflammatory response in vivo in the rat subcutaneous air pouch model (5).

In the acute model of inflammation, 48 Sprague Dawley rats in which a subcutaneous air pouch was formed using the technique of Edwards et al. (2) were divided randomly into 6 groups of 8 rats each. In 3 control groups, 5 ml of normal, non-pyrogenic saline solution was injected into the air pouch and the exudates were withdrawn after 6, 12 and 24 hours. In the other groups, 25 mg of sterile MSU crystals diluted in 5 ml of saline solution was injected into the pouch of each rat, and the exudates were aspirated after 6, 12 and 24 hrs.

In the sub-acute model, 96 Sprague Dawley rats underwent subcutaneous air pouch formation and were randomly divided into 12 groups of 8 rats each. In the first 6 groups (controls), male Sprague Dawley rat albumin/normal saline solution was injected into the pouch. In the rats of the other groups, air pouches were injected with HDPE suspended in rat albumin/saline. Five animals from each group underwent air pouch aspiration at each of six time points (6, 12, 24, 48, 72, and 96 hours).

The aspirated fluids from all the groups for both inflammation models were assayed for volume, leucocyte count and HA concentration.

Preparation of solutions
MSU crystals were prepared according to the method of McCarty and Faires (6), autoclaved for 30 minutes, and resuspended in sterile saline solution just before use. An equal amount of sterile, non-pyrogenic normal saline solution was injected into the pouches of the control group.

Because HDPE particles are extremely hydrophobic, particles were suspended in normal saline containing 0.8 mg/ml rat albumin. Commercially prepared, globulin-free Sprague-Dawley rat albumin was obtained (Sigma Chemical Co., St. Louis, MO). The albumin was mixed in sterile non-pyrogenic normal saline at a concentration of 1.0 mg/ml. The solution was then passed through a 0.2 micron filter to ensure sterility. Prior to injection into control pouches, 4 ml aliquots of control solution were mixed with 1 ml of sterile, non-pyrogenic normal saline to reproduce the
same protein concentration as that of the particle preparations (0.8 mg/ml). Commercially prepared sterile HDPE powder was obtained from Shamrock Technologies (Newark, NJ, USA). The particles had previously undergone commercial SPECTREX analysis by Smith and Nephew Richards Inc. (Memphis, TN, USA) and were found to contain approximately 5 × 10^9 particles per gram. On electron microscopy analysis, the average particle size was found to be 5.67 microns. The particles were sterilized by gamma irradiation. The particles were then weighed out into 1000 mg aliquots which were suspended in 1 ml of sterile non-pyro-genic normal saline. This suspension was subsequently mixed with 4 ml of the control solution to produce a concentration of 1 × 10^8 particles per ml for injection into the pouches. All preparations were tested for the presence of endotoxin by a limulus assay (Sigma Chemical Co., St. Louis, MO, USA).

**Animals**

6 to 8 week old male Sprague Dawley rats weighing 200-250 g were used as experimental animals. The rats were housed 3 to a cage and fed normal rat chow.

**Air pouch formation**

Air pouches were formed as described by Edwards (2): the backs of the rats were shaved, cleansed with alcohol, and 20 ml of air was injected stereilely (through a 0.2 micron filter on a 20 gauge needle) subcutaneously under intraperitoneal anesthesia with 40 mg/ kg of ketamine. Injection of air was repeated as often as required during the first 6 days to keep the cavity inflated. The appropriate particulate or control suspensions were administered into the pouches six days after the first injection of air in 5 ml aliquots per pouch. Each pouch was used for determinations at a single time point.

**Leucocyte count and hyaluronan assay**

White blood cell counts (WBC) were performed in a standard counting chamber. The remaining fluid was centrifuged for 15 min at 600 rpm at 4°C and the supernatant was stored at -70°C for measurements of HA.

Determination of HA was performed with an enzyme linked microplate assay (7). This assay is based on competition between the HA in the sample and the biotinylated HA for binding to HA binding protein which is coated onto microwells (Immuno Plates Maxisorp F96; Nunc, Denmark). Hyaluronan binding protein was prepared from bovine nasal cartilage and purified according to Tengblad (8). Plates were coated with hyaluronan binding protein (0.8 g/well) in a sodium carbonate buffer 0.1 M, pH 9.4 containing 0.2 g/l methiolate for 1 hour at 4°C. The plates were washed 3 times with 0.14 M NaCl containing 0.05% Tween 20 (referred to below as washing solution). Standards and 100 g serum were applied to the wells and incubated for 2 hours at 8°C. The assays were performed in duplicate. Blank wells contained 100 PBS/Tween with 3% albumin. After subsequent washing 100 g biotinylated HA (1 g/ml) in a solution containing 3% albumin was added and incubated for 1 hr at ambient temperature and then washed 3 times with washing solution. A 1:3000 dilution of streptavidin-peroxidase (in washing solution supplemented with 3% albumin) was added to the wells. After an incubation step of 45 minutes at ambient temperature, the wells were washed 3 times again and incubated for 30 minutes with 100 g of substrate solution (0.5 mg/ml o-phenylenediamine containing 0.03% H_2O_2 in 20 mM citric acid plus 50 mM sodium hydrogenphosphate). The reaction was stopped with 100 g of 0.1 M sulfuric acid and absorbance was recorded at 492 nm on a Biomec microplate reader (Beckman, USA).

**Statistical analysis**

Duncan’s multiple range test for analysis of variance was used to evaluate the differences in HA concentration and WBC count at the different times both in controls and in inflamed pouches. The Mann-Whitney non-parametric test was used to compare HA/leucocyte levels between the control and MSU/HDPE groups. Correlations between volumes, leucocyte count and HA levels in all groups (controls, MSU, HDPE) were evaluated using Pearson’s correlation coefficient. All data are expressed as medians and range of values.

**Results**

**HA levels and leucocyte count in the MSU and HDPE groups**

The concentrations of HA and the WBC count detected in the fluids of the acute and subacute models and the statistical significances are shown in Tables I and II.

In the control pouches of both groups, remarkable levels of HA were found; these levels were higher in the very first hours [2475 (1950/3050) g/l and 1850 (1050/2500) g/l at 6 hrs] and then gradually decreased (Fig. 1 and Fig. 2). In pouches injected with MSU, HA was moderately increased [3275 (2350/4600) g/l; p < 0.001] after 6 hrs, reached a peak after 12 hrs [7725 (4900/10500) g/l; p < 0.001] and began to taper at 24 hrs [5825 (4125/7850); p < 0.001], still being significantly higher than controls (Fig. 1). The leucocyte count was also increased at 6 hrs [17425 (13800/24750)/mm^3; p < 0.001], became higher at 12 hrs [42700 (26900/67700)/mm^3; p < 0.001] and tapered at 24 hrs [20750 (10400/33320)/mm^3; p < 0.001] (Fig. 1).

| Table I. The levels of hyaluronan (HA) and the leukocyte count (WBC) in the group of rats treated with MSU (acute model) and the controls (saline solution). |
|-----------------|-----------------|-----------------|
|                  | Control         | MSU             |
| WB C/mm^3        | HA (g/l)        | WB C/mm^3       | HA (g/l) |
| 6 hours          | 450 ± 250       | 250 ± 600       | 18250 ± 6532* | 3280 ± 820* |
| 12 hours         | 1250 ± 400      | 1415 ± 425      | 44262 ± 23438* | 7922 ± 2022* |
| 24 hours         | 650 ± 300       | 870 ± 418       | 20780 ± 12540* | 6230 ± 2040* |

*p < 0.001 (Mann-Whitney).
Table II. The levels of hyaluronan (HA) (g/l) and the leukocyte count (WBC) (/mm\(^3\)) in the group of rats treated with HCDPE (subacute model).

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
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<th>HDPE</th>
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<tr>
<td></td>
<td>WBC</td>
<td>HA</td>
<td>WBC</td>
<td>HA</td>
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<tr>
<td>6 hours</td>
<td>130 ± 70</td>
<td>1719 ± 781</td>
<td>170 ± 130</td>
<td>361 ± 109*</td>
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<tr>
<td>12 hours</td>
<td>1350 ± 950</td>
<td>1620 ± 820</td>
<td>2050 ± 1140*</td>
<td>710 ± 155*</td>
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<tr>
<td>24 hours</td>
<td>1230 ± 1420</td>
<td>1360 ± 980</td>
<td>3740 ± 2760*</td>
<td>2233 ± 167*</td>
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<tr>
<td>48 hours</td>
<td>300 ± 200</td>
<td>420 ± 170</td>
<td>3590 ± 3160*</td>
<td>610 ± 450</td>
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<tr>
<td>72 hours</td>
<td>160 ± 140</td>
<td>318 ± 140</td>
<td>3260 ± 4090*</td>
<td>251 ± 66</td>
</tr>
<tr>
<td>96 hours</td>
<td>140 ± 90</td>
<td>268 ± 320</td>
<td>2300 ± 3050*</td>
<td>221 ± 69</td>
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</table>

*p < 0.001 (Mann-Whitney).

In the HDPE pouches, HA levels were significantly reduced with respect to controls after 6 hours [380 (200/510) g/l; p < 0.001]; they later increased [690 (620/865) g/l at 12 hrs; p < 0.001] reaching a peak at 24 hrs [2250 (1965/2500) g/l; p < 0.001], and returning to the original levels, or even below, in the following hours [470 (280/1060) g/l after 48 hrs, 260 (185/310) g/l after 72 hrs and 215 (170/290) g/l after 96 hrs] (Fig. 2).

The WBC count was low at 6 hours and increased at 12 and 24 hours both in controls and in HDPE; at 48, 72 and 96 hrs the HDPE group pouch aspirates showed significantly higher WBC counts compared with the control group (Fig. 2).

Correlation between HA and WBC in MSU and HDPE groups

In the acute model (MSU group), no statistically significant correlation was detected between HA levels and the WBC count, although the trend of the median values appeared to be similar. In the subacute model, in controls a significant and direct correlation was found at 24 hr (r = 0.36; p < 0.05), 48 hr (r = 0.80; p < 0.001), and 72 hr (r = 0.44; p < 0.05), while an inverse relationship was detected at 96 hrs (r = -0.80; p < 0.001). In the HDPE group, at 6 hr (r = -0.34; p < 0.005), 48 hrs (r = -0.32; p < 0.005) and 72 hrs (r = -0.75; p > 0.001) an inverse relationship was detected while at 12, 24 and 96 hours no significance was found.

No statistical correlation was found between the WBC count, HA and the volume of aspirates.

Discussion

HA has a well established role in the maintenance of the structural and functional characteristics of the extracellular matrix and biologic fluids. Recent studies have suggested a regulatory function on several cellular processes, including motility, adhesion and differentiation, tissue remodelling, the creation of cell-free spaces and tumorigenesis (9-16). The regulatory role of HA in inflammation has received considerable attention as its production seems to be elevated during inflammation,
while other clinical reports claim that HA has anti-inflammatory activity (17). However, several reports showed a decrease of HA concentrations in inflammatory synovial fluids (18). It has also been suggested that high plasmatic levels of HA in rheumatoid arthritis (RA) may be linked to joint inflammation (19).

In 1993 Wilkinson et al. (20) found that, in contrast to synovial tissue, the rat subcutaneous air pouch lining lacks cells showing high activity of uridine diphosphoglucose dehydrogenase, an enzyme involved in HA synthesis. They concluded that the properties of cells on the surface of synovium are not determined simply by tissue cavituation. Francis et al. (21) demonstrated increased production of HA obtained by the injection of polysulphates, in a chronic model of inflammation in the air pouch; HA was considered merely a protective factor and an index to assess the beneficial effects of drugs. As far as we know, in the air pouch model no data on the basal production of HA by normal pouch lining, no correlation with other inflammatory parameters and no outline of the behavioural trend of HA in different kinds, degrees and times of inflammation have been reported in the literature.

Our data indicate that in both the acute and subacute models, HA levels are influenced by the course of inflammation but in different ways. MSU crystals evoked a very potent inflammatory reaction which was evident after 6 hrs, continued to increase up to the 12 hr point, but was decreasing at 24 hrs. Although no further measurements are available, we can presume that the values would have tapered towards normalisation within the following 24-48 hours. This course was clearly shown by the elevation of the WBC count and was paralleled by a significant increase in production of HA in the inflamed pouches.

HDPE particles injection also resulted in an inflammatory response which was not as intense and precocious as that observed with MSU, but was more prolonged and still evident after 96 hrs. Unlike the MSU model, HA levels were significantly reduced with respect to controls after 6 hours, reaching a peak at 24 hrs and, in the following hours, returning to the original low levels or even below (Fig. 2).

The high levels of HA in the very first hours, and probably the raised WBC at 24 hrs in the control groups, were certainly due to the mild inflammatory potential of the different substances used as the control or as the medium of suspension (saline solution and rat albumin/saline solution).

These data suggest that, in the air pouch model, inflammatory stimuli induce the synthesis of HA, even if with differing intensity according to the degree of inflammation. However, it is not clear why in the subacute model inflammation resulted in a reduction in the HA concentration in the early phase of phlogosis, and why HA returned to normal values when active phlogosis was still present (as shown by the elevated WBC count). We may speculate that the first response of the pouch lining to injury is an increased removal of HA from the pouch environment, probably as a result of an increased lymph efflux, followed in time by HA overproduction. In the early phases of inflammation, this overproduction may balance the clearance of HA and maintain normal levels, or, when the inflammation is intense, even lead to an increase in the HA concentration. Later, the production of HA may decline, while the alterations inducing a rapid clearance of HA still persist. In the subacute or chronic phases, the balance would move toward a reduction of HA levels in the pouch fluid.

Analogies with the joint cavity and arthritis may be hypothesized. Activated synovial cells in vitro synthesize greater amounts of HA (22, 23) and HA levels in sera from rheumatoid arthritis patients are notably elevated (24,25), despite analyses of synovial fluids from inflamed joints which have shown that both HA concentration and molecular weights are decreased (18, 26-28). It has been suggested that the increase of HA in serum may be the result of synovitis (19, 29, 30) in association with an abnormal and enhanced HA clearance from the rheumatoid articular cavity. In rheumatoid synovium, structural changes lead to easier passage between the synovial cavity and the interstitial space (31) and to increased lymphatic clearance (32,33).

The different levels of HA obtained by the various types and different phases of phlogosis and still requires elucidation in terms of its pathogenetical meaning.

Healthy synovial fluid contains HA at a level of 2-3 mg/ml, i.e. 10^5 times higher than that found in plasma (4). Although the levels of HA in the air pouch are 400 times lower than in human synovial fluid in basal conditions, therefore resembling the concentrations that one would expect in the ordinary extravascular connective tissue space, they show a trend that may resemble that of the joint space in response to inflammation. This probably reflects the complex dynamics of fluid across the tissue surface and changes in clearance. In conclusion, the air pouch shows some similarities with the joint cavity and may be considered a useful model for the study of inflammatory processes and HA metabolism in acute and chronic inflammation.

References

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