Concentration and Degradation of Hyaluronic Acid in Knee Synovial Fluid from Carrageenin-Induced Rabbit Arthritis

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The concentration and degradation of hyaluronic acid in the synovial fluid of carrageenin-induced arthritic joints of rabbits was studied. A 0.5-ml volume of 1% λ-carrageenin was intra-articularly injected three times into a right knee joint, and saline into a left. After 5 d from the last injection, inflammatory changes were observed in the synovial membrane and synovial fluid, but not in the articular cartilage. In the inflammatory synovial fluid, lipid peroxide content, phosphatase activity and cell counts were significantly increased, but the copper concentration was not changed. Concentration of polymeric hyaluronic acid and total hyaluronic acid were determined by high-performance liquid chromatography using gel-permeation columns. Total hyaluronic acid was appreciably decreased in the inflammatory fluid. The polymeric hyaluronic acid determined was 38% of the total hyaluronic acid in the inflammatory fluid and 74% in the control fluid. This suggests that in the inflammatory fluid, molecular weights of hyaluronic acid are distributed in the broader range. The concentration of chondroitin sulphates was similar in both the inflammatory fluid and the control fluid, but the content ratio of chondroitin sulphates to hyaluronic acid was higher in the inflammatory fluid. In the inflamed synovial membrane, synthesis of hyaluronic acid as measured by incorporation of [14C]glucosamine into glycoconjugates was increased by about twice that in the control membrane.

Keywords hyaluronic acid; synovial fluid; carrageenin; arthritus

Carrageenin is known to induce acute and chronic inflammatory responses. The intra-articular injection produced an experimental condition resembling the early stages of rheumatoid arthritis. In the synovial membrane and fluid, the increases of lysosomal enzymes, lipid peroxides and uronic acid as cartilage breakdown products appeared as inflammatory responses. Superoxide anion has been suggested as a mediator of the inflammatory process.

Hyaluronic acid is a principal component of glycosaminoglycan, synthesized in synovial membrane and released into synovial fluid. In rheumatoid arthritic synovial fluid, the concentration, intrinsic viscosity, molecular weight and size have been significantly smaller than those obtained from normal fluid. Moreover, it is known that hyaluronic acid is readily depolymerized by superoxide anion and other oxygen-derived free radicals. Small amounts of chondroitin sulphate have also been detected in normal and rheumatoid synovial fluid.

In carrageenin-induced arthritic synovial fluid, the concentration and molecular weight of hyaluronic acid and the chondroitin sulphate content have not yet been elucidated. In this study, we have attempted to analyze the synovial fluid, synovial membrane and articular cartilage from a carrageenin-injected joint, compared to those from a normal joint.

Experimental

Materials λ-Carrageenin and the acid phosphatase assay kit (Acid-Phospha K-Test) were purchased from Wako Junyaku. Streptomyces hyaluronicidus was obtained from Amano Seiyaku, and chondroitinase ABC of Proteus vulgaris from Seikagaku Kogyo. d-L[1-14C]Glucosamine (specific activity 259 GBq/mol) was obtained from New England Nuclear. Eagle's minimum essential medium was from Grand Island Biological Company. Penicillin and streptomycin were from Meiji Seika. A dye reagent for protein assay was from Bio-Rad. All other chemicals were of the highest grade available.

Injection of Carrageenin into the Rabbit Knee Joints Male Nihon white rabbits weighing between 2.2 and 2.5 kg were used for intra-articular injection of carrageenin. When the carrageenin was simultaneously injected into the left and right hind knee joints of the same rabbit, there were no significant differences in the inflammatory response in both joints. The individual rabbits, however, differed significantly in the inflammatory response as previously described. Accordingly, one hind knee joint was used for carrageenin treatment and the other joint for saline treatment. The right joint was intra-articularly injected three times with 0.5 ml of 1% (w/v) λ-carrageenin suspension in saline at 5-d intervals. The control left joint was treated with 0.5 ml saline in a similar manner. The rabbit was killed 5 d later and each knee joint was intra-articularly injected with 1.0 ml saline and washed. The synovial fluid was then drawn into a 1-ml syringe and immediately expelled into a polystyrene tube in ice water. The assay of synovial fluid was performed within the same day except for the determination of uronic acid. The synovial membrane was excised from the joint, backed by a Millipore membrane filter as previously described, and placed in a culture tube. The articular cartilage was then removed from the knee joint and stored frozen until immediately before use.

High Performance Liquid Chromatography (HPLC) of Synovial Fluid

For the direct separation and quantification of hyaluronic acid polymer in synovial fluid, a HPLC analysis was carried out using Shodex OHpak B-806 and B-805 columns (50 cm x 8 mm i.d.) and a precision differential refractometer. A 100-μl volume of synovial fluid was diluted to 300 μl with saline, filtered, and then chromatographed. HPLC assays for the total concentration of hyaluronic acid and trace concentration of chondroitin sulphates in synovial fluid were performed using two aliquots of the fluid individually digested with chondroitinase ABC and Streptomyces hyaluronicidus as previously described. The fluid containing unsaturated disaccharides produced by chondroitinase ABC digestion was analyzed at 232 nm using Shodex Ionpak KS-802 and KS-801 columns (30 cm x 8 mm i.d.) at 80°C. The system was run at a flow rate of 0.7 ml/min with 0.2 M NaCl. The other fluid containing unsaturated oligosaccharides produced by Streptomyces hyaluronicidus digestion was chromatographed at a flow rate of 1.0 ml/min using only a KS-802 column. Peak heights, peak areas, and retention times were measured by a Shimadzu chromatograph integrator.

Synovial Fluid Assays

Acid phosphatase activity was measured by following the release of phenol from phenylphosphate using a Wako acid phosphatase assay kit. A 100-μl volume of the synovial fluid was used. Protein contents were determined at 595 nm using 10 μl of synovial fluid and 5.0 ml of 5-fold diluted Bio-Rad dye reagent solution. Cells were counted using a hemocytometer. Lipid peroxides were colorimetrically measured by a modification of the Yagi method. To 60 μl of synovial fluid, 0.6 ml of 0.05 M HCl and 0.2 ml of 0.67% thiobarbituric acid aqueous solution–acetic acid (1:1) were added and mixed. The reaction mixture was heated for 60 min in boiling water. After cooling, 0.8 ml of ethanol-n-butanol (3:17) was added and the mixture was shaken. After
centrifugation at 1500 × g for 15 min, the n-hexanisol layer was measured at 533 nm using a micro black cell. Tetraethoxypropane was used as the standard. Uronic acid was determined by the carbazole method of Bitter and Muir.23) Copper analysis was carried out by flameless atomic absorption spectrometry.

In Vitro Incubation of Synovial Membrane with [14C]Glucosamine The synovial membrane was placed into a culture tube containing 1 ml Eagle’s minimum essential medium, supplemented with 3.7 MBq of [14C]glucosamine, 100 units of penicillin and 200 μg of streptomycin. The membrane stretched with the filter was incubated at 37°C for 6 h under a 5% CO2 atmosphere. After incubation, the membrane was removed from the culture medium and washed twice with 1 ml saline. The medium and washings were combined and dialyzed against saline. The synovial membrane was detached from the filter, defatted with washing with 5 ml each of cold acetone and ether, and dried in vacuo. The extraction of [14C]glycoconjugates from the defatted, dried membranes was performed using 4 M guanidinium chloride in 0.1 M Tris-HCl buffer, pH 7.5, as previously described.23) Radioactivity was determined in a liquid scintillation spectrophotometer, Aloka LSC-700.

Preparation of Articular Cartilage Extracts An average of 100 mg of articular cartilage was removed from each joint, defatted by washing with cold acetone and ether, and dried in vacuo. The glycoconjugates of the cartilage were extracted in a similar manner as described above. The uronic acid contents of the extracts were determined by the carbazole method.23)

Statistical Analysis Differences between saline- and carrageen-in-treated joints were assessed by the Student’s paired t-test.

Results Response of Carrageenin-Injected Rabbit Knee Joints After carrageenin treatment, the joint tissue exhibited hypertrophy in the synovial membrane, yellow coloration in the synovial fluid, and little histologic changes in the cartilage. The synovial fluids from carrageenin-injected joints and saline-injected joints were analyzed for cell counts, acid phosphatase activity, and contents of protein, lipid peroxide, and copper. Uronic acid was determined in both the synovial fluid and cartilage. As shown in Table I, in the synovial fluid from carrageenin-injected right joints, cells, phosphatase activity and lipid peroxide were significantly increased (p < 0.01 for the first and second and p < 0.05 for the last). In contrast, uronic acid was decreased (p < 0.01). The copper content, however, showed no significant changes between the right synovial fluid and the control left fluid. The uronic acid contents of articular cartilage were also the same in the right and in the control left.

Assay of Synovial Fluid by HPLC Polymeric hyaluronic acid in synovial fluid was directly determined using a combination of two gel-permeation columns, B-806 and B-805.19a) The representative chromatograms of the fluids from carrageen-injected joints and saline-injected joints are illustrated in Fig. 1. The first peaks were hyaluronic acid. The other components in synovial fluid were eluted near the total volume (40 ml). The inflammatory fluid from the carrageen-injected joint showed a broader molecular weight distribution of hyaluronic acid. The molecular weight

![Fig. 1. Representative Chromatograms of Rabbit Synovial Fluids from a Carrageenin-Injected Right Joint and from a Saline-Injected Left Joint](image)

Each synovial fluid was diluted three times and chromatographed. Column, Shodex OHPack B-806 + B-805; eluent, 0.02 M NaCl; flow rate, 0.5 ml/min; detection, refractive index detector, 1 x 10-4 refractive index units full-scale.

<p>| Table I. Effect of Intra-Articular Injection of Carrageenin on Synovial Fluid and Articular Cartilage in Rabbit Knee Joints |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>Cell (count/ml × 10^-4)</th>
<th>Acid phosphatase (phenol, μg/ml)</th>
<th>Lipid peroxide (nmol/ml)</th>
<th>Copper (μg/ml)</th>
<th>Uronic acid (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.89 ± 1.33</td>
<td>0.47 ± 0.25</td>
<td>0.50 ± 0.49</td>
<td>0.53 ± 0.42</td>
<td>0.06 ± 0.02</td>
<td>144.8 ± 40.2</td>
</tr>
<tr>
<td>Carrageenin</td>
<td>4.52 ± 3.68</td>
<td>7.30 ± 4.78</td>
<td>4.66 ± 3.95</td>
<td>1.81 ± 1.50</td>
<td>0.07 ± 0.04</td>
<td>86.9 ± 24.0</td>
</tr>
<tr>
<td>NS</td>
<td>p &lt; 0.01⁴</td>
<td>p &lt; 0.01⁴</td>
<td>p &lt; 0.01⁴</td>
<td>p &lt; 0.05⁹</td>
<td>NS</td>
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</tr>
</tbody>
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Table II. The Concentration of Hyaluronic Acid and Chondroitin Sulphates in Synovial Fluids from Saline- and Carrageenin-Injected Rabbit Knee Joints

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<tr>
<th>Treatment</th>
<th>Carbazole reaction HA (μg/ml)</th>
<th>B-806 + B-805 HA polymer</th>
<th>HPLC (μg/ml)</th>
<th>KS-802 Oligosaccharide</th>
<th>KS-802 + KS-801 ΔD-HA⁴</th>
<th>KS-802 + KS-801 ΔD-4S⁴</th>
<th>Ratio ΔD-4S/ΔD-HA</th>
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<tr>
<td>Saline</td>
<td>291.6 ± 80.2</td>
<td>129.9 ± 21.8</td>
<td>175.6 ± 17.8</td>
<td>174.7 ± 21.5</td>
<td>6.4 ± 1.3</td>
<td>8.9 ± 2.9</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>Carrageenin</td>
<td>156.4 ± 48.1</td>
<td>34.6 ± 11.6</td>
<td>90.2 ± 34.7</td>
<td>86.5 ± 34.6</td>
<td>8.9 ± 2.9</td>
<td>NS</td>
<td>p &lt; 0.01⁴</td>
</tr>
</tbody>
</table>

Table III. The Concentration of Hyaluronic Acid and Chondroitin Sulphates in Synovial Fluids from Saline- and Carrageenin-Injected Rabbit Knee Joints

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<td>NS</td>
<td>p &lt; 0.01⁴</td>
</tr>
</tbody>
</table>

a) The content of hyaluronic acid (HA) was based on a 40% uronic acid (carbazole) content. b) 2-Acetamido-2-deoxy-3-O-(β-D-gluco-6-epenepyranoxylocuronic acid)-o-glucose. c) 2-Acetamido-2-deoxy-3-O-(β-D-gluco-6-epenepyranoxylocuronic acid)-4-O-sulpho-o-galactose. d) Significant differences in saline vs. carrageenin (paired t-test). NS: not significant (paired t-test). After saline treatment of a left joint and carrageenin of a right, each joint cavity was washed with 1 ml saline and the synovial fluid was then taken out. The concentration of hyaluronic acid polymer was determined using B-806 and B-805 columns. The fluid digested with Streptomyces hyaluronidase was assayed for oligosaccharides from HA. The fluid digested with chondroitinase ABC was used for the determination of unsaturated disaccharide from HA and unsaturated sulphated disaccharides from chondroitin sulphate. The contents of HA and oligosaccharide, and chondroitin sulphate are given in ΔD-HA and ΔD-4S units, respectively. Each value represents mean ± S.D. (n = 9).
can be determined from the relationship between the elution volume and the logarithm of the molecular weight. For all the synovial fluids from carrageenin-injected joints and saline-injected joints measured, the elution volumes of the hyaluronic acid were a little less than that of a hyaluronic acid standard having a molecular weight of 10$^6$. Accordingly, the molecular weights of the hyaluronic acid were estimated to be a few million. Table II shows the determined concentrations of hyaluronic acid polymer.

The total concentration of hyaluronic acid in the synovial fluid was assayed for unsaturated hexasaccharide and unsaturated tetrasaccharide from hyaluronic acid specifically digested with Streptomyces hyaluronidase. The synovial fluid digested with chondroitinase ABC was used for the determination of trace concentrations of chondroitin sulphates. Figure 2 shows the representative chromatograms. The content of unsaturated sulphated disaccharides from chondroitin sulphates was calculated from the first peak areas, and the content of unsaturated non-sulphated disaccharide from hyaluronic acid from the second peak areas. Table II shows the data obtained by the HPLC analyses, together with the results of the carbazole reaction method. In the inflammatory fluids from carrageenin-injected right joints, the concentrations of polymeric hyaluronic acid and total hyaluronic acid measured as unsaturated oligosaccharides were significantly lower ($p<0.01$) than those in the control fluid; while the contents of chondroitin sulphates showed little difference. After carrageenin treatment, the ratio of unsaturated sulphated disaccharide to unsaturated non-sulphated disaccharide, however, increased ($p<0.01$) as a result of the decrease of unsaturated non-sulphated disaccharide, i.e., hyaluronic acid.

**In Vitro Incorporation of $[^14C]$Glycosamine into Glycoconjugates by Synovial Membrane**

The radioactivity of the synovial membranes isolated from carrageenin- and saline-injected joints and their mediums was measured. The results are shown in Table III. The radioactivity of synovial membrane extracts from carrageenin-injected joints was increased by about twice ($p<0.05$) that of the control extracts, and the radioactivity of its medium slightly increased ($p<0.05$). The major part of $[^14C]$Glycosamine-labeled glycoconjugates extracted from synovial membranes and secreted into culture medium was hyaluronic acid as previously described. Accordingly, the elevated radioactivity in the inflamed synovial membrane indicates an increased synthesis of hyaluronic acid.

**Discussions**

Carrageenin is rapidly taken up by migrating macrophages and other monocytes and generally induces the rupture of lysosomes and the subsequent release of hydrolytic enzymes. After intra-articular injection of carrageenin, the predominant type of cells which appeared in the inflammatory synovial fluid were polymorphonuclear leukocytes. Macrophages and polymorphonuclear leukocytes release superoxide anion and hydrogen peroxide when undergoing phagocytosis. Therefore, in the synovial fluids from carrageenin-injected joints, the elevated cell counts suggest the increase of leukocyte infiltration and the enhanced production of superoxide anion and hydrogen peroxide by the leukocytes. The inflammation of the synovial membrane is initiated with an infiltration of leukocyte and other cellular material containing large numbers of lysosomes. Accordingly, the elevated concentration of lipid peroxide indicates that cell membrane lipids underwent more oxidative damage. Moreover, the increased activity of acid phosphatase demonstrates the rupture of lysosomes from the inflammatory cells. Therefore, the results of this experiment suggest that the injection of carrageenin into a joint induced inflammation in the synovial fluid.

During the carrageenin-induced arthritis, the hyaluronic acid of synovial fluid decreased in concentration and became broader in molecular weight distribution. The polymeric hyaluronic acid consisted of 38% of the total concentration of hyaluronic acid in the inflammatory fluid and 74% in the control fluid (Table II). The results suggest that in the inflammatory synovial fluids, degradation of the parent hyaluronic acid and production of the smaller fragments occurred. In *in vivo* leukocyte-dependent inflammatory reaction, activated leukocytes are known to release superoxide anion and hydroxyl radical, and their products, hydrogen peroxide and $\text{OCl}^-$ Moreover, it has been suggested that in the synovial fluid from rheumatoid arthritis, hyaluronic acid is degraded by a hydroxyl radical.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n$</th>
<th>[${}^14\text{C}$]Glycosamine incorporated (dpm/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>9</td>
<td>243 ± 44</td>
</tr>
<tr>
<td>Carrageenin</td>
<td>9</td>
<td>413 ± 127</td>
</tr>
</tbody>
</table>

(3)$p<0.05\quad(4)$

$[^14\text{C}]$Glycosamine was used in the range of 30–50 mg from knee joints and cultured at 37°C for 6 hr in 1 ml of Eagle’s minimal essential medium containing 3.7 MBq of $[^14\text{C}]$Glycosamine under a 5% CO$_2$ atmosphere. The radioactivity was measured as described in the experimental. Each value represents mean ± S.D.
generated during inflammation. 28–30 Recently, two groups have suggested that hyaluronic acid is degraded by OCl− produced through the reaction of hydrogen peroxide and myeloperoxidase from activated neutrophils. 31,32 In the present arthritis, active oxygen species derived from stimulated leukocyte probably also degraded hyaluronic acid of the synovial fluid. On the other hand, the degradation of hyaluronic acid seems to play an active role in protecting articular tissues from oxidative damage. 33 The degradation of hyaluronic acid increases the lower molecular weights. The decreased concentration of hyaluronic acid in the arthritic fluid might be a consequence of the enhanced outflow rate from the synovial cavity dependent on the low molecular weight. Moreover, in the inflamed synovial membrane, the enhanced synthesis seems to be induced by the decreased concentration. There has indeed been an increased production of hyaluronic acid in rheumatoid tissue lining the synovial cavity as compared to normal synovial tissue. 9

The concentrations of chondroitin sulphates determined in both the inflammatory fluids and control fluids showed similar value (Table II). It has been suggested that chondroitin sulphates in the synovial fluid mainly originate from the synovial membrane, but in rheumatoid joints with osteoarthritic changes, they are also probably released by a breakdown of the cartilage matrix. 17 Moreover, it is known that the content ratio of chondroitin sulphate to hyaluronic acid is higher in rheumatoid fluid than in normal fluid. 11,17 In the carrageenin-induced arthritic fluid, the ratio was similarly higher, but in the cartilage, the uronic acid content was scarcely changed. Therefore, the origin of chondroitin sulphate is probably from the synovial membrane alone.

In rheumatoid arthritic synovial fluid, the total copper concentration significantly increases. 34 Ceruloplasmin (0.34% Cu) accounts for more than 90% of the total copper and is found to increase with an increasing duration of arthritis. The rise of ceruloplasmin is suggested to be a protective acute response to inflammation because of its antioxidative activity. In this experiment, the copper concentration was scarcely changed. This result suggests that ceruloplasmin was still not increased.

The concentration of hyaluronic acid based on uronic acid content measured by the carboxazole reaction was much higher than the total concentration of hyaluronic acid determined by the HPLC method (Table II). To investigate the difference, the synovial fluid was eluted through gel-permeation columns (Fig. 1) and fractionated at 1 ml each. When all the fractions were tested for the carboxazole reaction, the first peak and near total volume were positive. Their positive fractions were further investigated for the presence of hyaluronic acid. After treatment with pronase, deproteinization and dialysis, the concentrated fractions were electrophoresed. The fractions of the first peak contained hyaluronic acid, but the fractions of the near total volume contained no glycosaminoglycans. Accordingly, the concentration of hyaluronic acid obtained from the carboxazole reaction is not accurate.

In the present carrageenin-induced arthritis, hyaluronic acid of the synovial fluid indicated similar changes, that is, decreased concentration and broader molecular weight distribution, to that in rheumatoid arthritis.

References