Molecular Weight-Dependent Effects of Hyaluronate on the Arthritic Synovium

Akira ASARI¹, Satoshi MIYAUCHI¹, Satoshi MATSUZAKA¹, Tomomi ITO¹, Eiki KOMINAMI² and Yasuo UCHIYAMA³

Tokyo Research Institute¹, Seikagaku Corporation, Tokyo; Department of Biochemistry², Juntendo University School of Medicine, Tokyo; and Department of Cell Biology and Anatomy³, Osaka University Medical School, Suita, Osaka, Japan.

Received February 27, 1998

Summary. Intra-articular injection of hyaluronate (HA) is widely used in the treatment of arthropathies. However, the mechanism of the effects of HA preparations on the arthritic synovium and the relationship between their effects and molecular weights (MW) remains unknown. The objectives of this study were to compare the effects of two hyaluronate preparations, HA84 (MW: 84x10⁴) and HA230 (MW: 230x10⁴), on the synovium of an arthritis model and to examine the mechanism of the effects of HA. The HA preparations were intra-articularly injected in a model of canine arthritis induced by anterior cruciate ligament transection for a total trial of 5 weeks. To define the accessibility of HA preparations to the synovial lining layers, fluorescein-labeled HA84 or HA230 was injected at the last administration. Pathological changes analyzed included increases in volumes and prostaglandin E₂ concentrations in synovial fluids, thickening of the synovial lining layers, vacuolar alterations in the lining cells, and stainability of HA in the synovium. Expression levels of Heat shock protein 72 (Hsp72) were immunohistochemically detected in the tissues to investigate the ability of the cells to survive the degeneration. The pathological changes described above were more significantly suppressed in the HA84-treated than in the HA230-treated groups. In most cases of the HA84-treated group (five cases out of six), fluorescein particles were intensely distributed in the synovial lining layers, but only two cases in the HA230-treated group showed a weak distribution of fluorescein particles in the layers, indicating a certain difference in the accessibility of HA preparations to the lining cells between the two HA molecules. Moreover, the immunoreactivity for Hsp72 in the lining cells as more intense in the HA84-treated than in the HA230-treated groups. The difference in the accessibility of HA molecules corresponded well with that in the inducibility of Hsp72 in the lining cells. These results suggest that the up-regulation of Hsp72 may offer a new concept concerning mechanism of the effects of HA preparations on the arthritic synovium.

Intra-articular injection of hyaluronan (HA) is now widely used in the treatment of human arthropathies (NAMIKI et al., 1982; BALAZS and DELINGER, 1985; DIXON et al., 1988). The molecular weight (MW) of the preparation used for intra-articular therapy is known to vary among manufactures; however, the mechanism of the effect of HA molecules on arthritis remains controversial. Particularly, little is known about how the effect of HA molecules on the arthritic synovium differs depending on molecular size.

To examine the mechanism of the effect of HA molecules on arthropathies, we made a slowly progressive model of early osteoarthritis (OA) or traumatic arthritis (TA) induced in canine knee joints by anterior cruciate ligament transection (ACLT) according to the method of KENNETH et al. (1991). The ACLT model is well known to mimic the early stage of human osteoarthritis (KENNEH et al., 1991). We have shown that cartilage changes indicated by the increased concentration of chondroitin-6-sulfate in synovial fluids, the proliferation of synovial cells, and hydrarthrosis occur in the joints of traumatic arthritis patients (ASARI et al., 1994). In the course of the study using the ACLT model, we noticed that the canine ACLT model shows cartilage changes similar to those seen in traumatic arthritis patients, suggesting that the ACLT model is useful as an early stage model of OA or TA.

Using this arthritis model, we have previously shown that HA84 up-regulates heat shock protein 72 (Hsp72) to suppress vacuolar degeneration in synovial cells (ASARI et al., 1996). It has been suggested that cells usually react by increasing the expression
of Hsps when they experience adverse changes in their environment (ASHBURNER and BONNER, 1979; LINDQUIST, 1986). In cells experiencing metabolic stress, newly synthesized proteins unable to fold properly become stably bound to Hsp72 (BECKMAN et al., 1992). Hsps are well known to be essential to the ability of cells to survive environmental insult (BAIER et al., 1992).

One of pathological signs in the canine arthritis model is known to be hydrarthrosis. It has been shown that prostaglandin E_2 (PGE_2) enhances vascular permeability, which is associated with hydrops (IKEDA et al., 1975). It is therefore important to determine changes in PGE_2 concentrations in synovial fluids to understand the degree of arthritis.

In the present study, we examined whether the effects of HA preparations on the arthritic synovium of canine knee joints induced by the ACLT operation differ depending on the molecular size of HA preparations. Since an HA preparation with MW of 230 \times 10^4 (HA230) is close to MW of endogenous HA in human normal and TA joints (ASARI et al., 1997), HA230 was used as a larger molecular sized HA preparation. As a lower molecular sized HA preparation, we used an HA preparation with 84 \times 10^4 (HA84) which functions as a drug rather than as the complement to synovial fluids. After treatment with these two HA preparations, pathological alterations in the arthritic synovium were analyzed using morphological, immunohistochemical, and histochemical techniques. Volumes and PGE_2 concentrations in synovial fluids were also biochemically determined. The present results clearly demonstrate that therapeutic effects of HA preparations on synovial cell alterations, such as proliferration and vacuolization, and hydrarthrosis differ depending on their molecular size, and that the smaller sized molecule, HA84, is more effective than the larger sized molecule, HA230.

**MATERIALS AND METHODS**

**HA84 and HA230**

HA84 and HA230 (10 mg/ml), prepared from rooster combs, were obtained from the Seikagaku Corporation (Tokyo, Japan). HA84 is marketed as a drug for OA, Arzt® in Japan (Seikagaku Corporation). HA230 is marketed as an ophthalmologid drug (Seikagaku Corporation), but not as a drug for OA in Japan. HA preparation, whose mean molecular weight is about 200 \times 10^4, is marketed as Healonid® world-wide for veterinary medicine (Kabi Pharmacia, Sweden). Both HA84 and HA230 are highly pure, sterile, and pyrogenfree. The HA-salt was dissolved in phosphate-buffered physiologic saline (PBS) to obtain a 1% solution. The protein content is less than 0.1%. Electrophoresis detected no glycosaminoglycans other than HA.

**Experimental animals**

Male beagle dogs aged between 5 and 8 months, weighing approximately 7.0–9.0 kg, were used in the present study. Three dogs were used as non-operated normal controls (non-operated group).

Arthritis was induced in both the right and left sterile joints by an ACLT in 12 dogs. The whole procedure was carried out under general anesthesia (25 mg/kg body weight pentobarbitone).

Six operated dogs received a single intra-articular injection (0.5 mg/50 μl/kg) of HA84 into the left-knee joints (HA84-treated group) once a week for a total trial of 5 weeks, 4 weeks after the ACLT. The other 6 operated dogs received an injection of HA230 in the same manner as in HA84 described above (HA230-treated group). Fluorescein-labeled HA84 or HA230 was injected at the last administration to define the localization of these molecules in the tissues. Sterile phosphate buffered saline (PBS) was intra-articularly injected (50 l/kg) into the right knee joints of the dogs and HA84 or HA230 into the left knee joints. The PBS-treated group consists of 12 joints.

<table>
<thead>
<tr>
<th>Table 1. Organization of experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operation</td>
</tr>
<tr>
<td>No. of dogs</td>
</tr>
<tr>
<td>No. of joints</td>
</tr>
<tr>
<td>Injected materials</td>
</tr>
</tbody>
</table>

Sterile PBS was intra-articularly injected (50 l/kg) into the right knee joints of the dogs and HA84 or HA230 into the left knee joints. The PBS-treated group consists of 12 joints.
injected (50 µl/kg) into the right knee joints (PBS-treated group) of the animals receiving HA84 or HA230 according to the same schedule as described above. All dogs were sacrificed 1 week after the last intra-articular injection, and then synovial fluids, cartilage and synovial tissues were collected from their joints.

The organization of the experimental groups and schedule of the experiments are summarized in Table 1 and Figure 1, respectively.

**Synovial fluid**

**Volume**

The volume of synovial fluids obtained from the knee joints of the animals was determined. The synovial fluids were collected in plastic tubes after 3 intra-articular injections of 2 ml physiological saline and then centrifuged at 10,000 g for 15 min to remove cells and tissue debris. The supernatants were stored in aliquots at −20°C until used. Synovial fluid volume was calculated using the formula of the Donnan equilibrium (GIANYI et al., 1988): synovial fluid volume = total calcium amount in the lavage of the joint cavity (g/joint)/calcium concentration in the plasma (g/l). The calcium concentrations in the plasma and synovial fluids were colorimetrically determined by the method by GITELMAN (1967), using the O-cresolphthalein complexion method (Calcium C-test Wako Kit, Wako Pure Chemical Industrial, Ltd., Osaka, Japan).

**Prostaglandin E2 (PGE2) concentration**

The PGE2 content in the synovial fluids was measured using a PGE2 (125I) RIA kit (Dupont-New England Nuclear, Boston, MA, USA).

**Histopathology in synovial tissues**

All synovia, which are up to 30 mm in length, were removed from certain portions of the medial parapatella synovium in the knee joints one week after the last administration. The synovial tissues were fixed with 5% cetylpylidinium chloride-10% acetic acid for 24 h. According to routine procedures, these samples were embedded in an O. C. T. compound (Miles Inc., Elkhart, USA.) at -80°C or in paraffin. Cryosections or paraffin sections of the samples were then cut at 5 µm by a cryostat or microtome.

**Morphometry in hematoxylin-eosin staining**

After deparaffinization, the sections were stained with hematoxylin and eosin. Degeneration and inflammation were confined to the transition area between dense and loose connective tissues. The incidence (%) of vacuolar synovial cells in 200 synovial lining cells in each animal was counted in the confined area. The thickness of synovial lining layers was measured as an indication of the proliferation of synovial lining cells. The thickness indicates the distance from the synovial surface to the bottom of the synovial lining cells at the deepest layer.

**Electron microscopy**

To determine the cell type of the vacuolar degenerative synovial cells, the synovial tissue in the PBS group was observed by ordinary electron microscopy. Synovial cells were divided into three types: macrophage-like type A and fibroblast-like types B and S, according to the criteria as previously reported (GRAABÆK, 1982; ASARI et al., 1994). Type S cells were large in size and variable in shape, being characterized by abundant profiles of rough endoplasmic reticulum, a well-developed Golgi apparatus, and small dense granules (GRAABÆK, 1982; ASARI et al., 1994), but not by large vacuoles or slender cytoplasmic processes. Based on their morphology, type S cells are considered to be an activated form of type B cells (ASARI et al., 1994).
Fig. 2. Synovial tissues stained with hematoxylin-eosin in the non-operated (N; a, b), PBS- (c, d), HA84- (e, f) and HA230-treated groups (g, h). A mitotic cell (arrowhead) is observed in the PBS-treated groups (d inset). Arrows: synovial cells with vacuolar degeneration. a, c, e, g: ×115; b, d, f, h: ×580; d inset. ×1,500
HA staining

HA was detected in sections as previously described (ASARI et al., 1994, 1995). Briefly, biotinylated HA binding protein (HABP) used in HA staining was prepared from bovine nasal cartilage proteoglycans using affinity chromatography on immobilized HA by the procedure by TENGBLAD (1979). Cryosections were incubated with biotinylated HABP (2 μg/ml) at room temperature for 1 h, and then treated with 3,3-diaminobenzidine (DAB) solution followed by a streptavidin-peroxidase solution, or with a streptavidin-peroxidase solution (Southern Biotechnology Associates, Inc., AL, USA). Control sections were pre-treated with hyaluronidase (HAase) derived from Streptomyces hyalurolyticus (Amano Pharmaceutical, Nagoya, Japan) prior to HA staining. No HA staining was observed in the control. The intensity of the HA staining in 3 areas of synovial lining layers (20×50 μm² in each) from each experimental animal was evaluated with an image analyzer (Pias, Osaka, Japan).

Immunostaining of Hsp72

For immunostaining of Hsp72, cryosections were incubated with anti-Hsp72 monoclonal antibody (Amersham, 1: 500) at 4°C overnight. The sections were then treated with peroxidase-labeled anti-mouse IgG (Jackson Lab., PA, USA) followed by a DAB solution. The incidence (%) of Hsp72 positive cells was counted in 1000 synovial lining cells from each experimental animal under a light microscope. The intensity of the immunostainability of Hsp72 in 3 areas of synovial lining layers (20×50 μm² in each) from each experimental animal was measured using an image analyzer.

Double immunostaining of Hsp72 and cathepsin B

Double immunostaining of Hsp72 and cathepsin B was performed to determine the cell types of Hsp72-immunopositive cells in the synovium. Since macrophage-like type A cells possess numerous lysosomes in the cytoplasm, cathepsin B, a representative lysosomal cysteine proteinase, was used to define the cells (ASARI et al., 1995). Sections stained for Hsp72 followed by Cy5-labeled-anti-mouse IgG were incubated with anti-rat cathepsin B at room temperature overnight, and then further incubated with Texas red-conjugated anti-rabbit IgG (Jackson Laboratory), as previously described (ASARI et al., 1995).

The intensity of DAB staining for HA and Hsp72 in the cryosections was expressed by the glow mode.

Table 2. Volume and prostaglandin E₂ concentration in synovial fluids

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonoperated</th>
<th>PBS</th>
<th>HA84</th>
<th>HA230</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of joints</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>0.93±0.03</td>
<td>1.50±0.06*#</td>
<td>1.21±0.12</td>
<td>1.57±0.08*#</td>
</tr>
<tr>
<td>PGE₂ (pg/ml)</td>
<td>39.8±4.97</td>
<td>89.8±19.5†</td>
<td>75.0±18.7</td>
<td>95.2±10.6*</td>
</tr>
</tbody>
</table>

Mean±standard error; Nonoperated: Non-operated non-injected group; *: P<0.01 versus non-operated group; †: P<0.05 versus non-operated group; #: P<0.05 versus HA84 group.

Table 3. Histopathological examinations in synovium

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonoperated</th>
<th>PBS</th>
<th>HA84</th>
<th>HA230</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of joints</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Va</td>
<td>N. D</td>
<td>18.4±3.31</td>
<td>4.17±2.93*</td>
<td>7.67±3.71</td>
</tr>
<tr>
<td>Thickness</td>
<td>13.2±0.84</td>
<td>31.0±1.22†</td>
<td>24.3±1.57 †</td>
<td>26.4±1.54 † *</td>
</tr>
<tr>
<td>HA</td>
<td>29.8±2.87</td>
<td>18.8±1.24†</td>
<td>27.8±2.56*</td>
<td>20.5±2.20</td>
</tr>
</tbody>
</table>

Mean±standard error; Nonoperated: Non-operated noninjected group; Va: incidence of vacular synovial cells in synovial lining cells (%); Thickness: thickness of synovial lining layers (μm); HA: relative optical intensity of hyaluronic staining in synovial lining layers; †: P<0.05 versus Non-operated group, †: P<0.01 versus Non-operated group, *: P<0.05 versus PBS group, †: P<0.01 versus PBS group.
130 A. ASARI et al.

Distribution of injected HA preparations

Fluorescein-labeled HA preparations were cryosectioned and observed, using the confocal laser scan microscope (Leica).

Since HABP dose not react with oligosaccharides (HASCALL and HEINAGARD, 1974), localization of the fluorescein-labeled HA84 was examined in the cryosections which were further stained with biotinylated HABP, followed by streptaridin-conjugated Texas red, and viewed with the confocal laser scan microscope in order to detect whether fluorescein-labeled HA84 is degraded into oligosaccharide forms.

Statistical analysis

The data were blindly obtained to insure the accuracy and reproducibility of the image analytical and morphometrical methods. The data were statistically analyzed by the TUKEY multiple-comparison test.

RESULTS

Synovial fluids

Changes in volumes and PGE2 concentrations of synovial fluids obtained from the ACLT-operated joints of the HA84-, HA230- and PBS-treated groups and the non-operated joint were examined and compared among the groups. Both volumes and PGE2 concentrations were elevated in the operated groups, compared with the non-treated group. In the operated groups, they were clearly suppressed in the HA84-treated group, but not in the HA230-treated group (Table 2).

Synovial tissues

Since volumes and PGE2 in synovial fluids were largely altered in the operated groups, we further examined how synovial tissues were damaged by the operation and protected from damage by the treatment with HA preparations.

Proliferation of synovial cells

By the ACLT operation, synovial lining cell layers became thickened and mitotic cells often appeared in the layers (Fig. 2). After treatment with HA preparations, however, the synovial lining cell layers clearly appeared thinner than after treatment with PBS (Fig. 2; Table 3). The results suggest that the proliferation of synovial lining cells was suppressed in the HA84 and HA230 groups.

Vacuolar degeneration

In addition to the proliferative change in synovial lining cells, many cells possessing large and small vacuoles in the cytoplasm appeared in the synovial lining layers after the ACLT operation (vacuolar degeneration) (Figs. 2, 3). By electron microscopy, large and small vacuolar structures were often seen in the cytoplasm of synovial lining cells which had abundant profiles of the rough endoplasmic reticulum (fibroblast-like type B cells), but those structures were not detected in those possessing numerous dense bodies and cytoplasmic processes (macrophage-like type A cells) (Fig. 3). The lining cells with vacuoles had a smooth cell surface, so that they were fibroblast-like type S cells. Since the membrane of vacuolar structures in the cells was attached by ribosomes, vacuoles were rough endoplasmic reticula.
with dilated cisternae. When the number of vacuolated lining cells were counted, the incidence of the cells was significantly lower in the HA84-treated than in the PBS-treated groups (Table 3). The incidence in the HA230-treated group was also low, though not significantly so, compared with that in the PBS group. The data indicate that vacuolar degeneration was significantly suppressed in the lining cells of the HA84 group and moderately so in the HA230 groups.

**HA staining**

As shown in Figure 4a, HA staining was intense in synovial lining cells. Positive staining of HA was distinctly reduced in the cells after the ACLT operation (Fig. 4b,d), but was clearly demonstrated in the cells of the HA84-treated group (Fig. 4c), suggesting that HA staining was maintained in the synovial lining cells of the HA84-treated group (Table 3).

**Hsp72 expression**

As the degenerative changes in synovial lining cells were much milder in the HA-treated groups than in the PBS-treated group, we further examined im-
Fig. 7. Incidence (%) of Hsp72 positive cells in synovial lining cells (a) and relative optical intensity of immunostainability of Hsp72 in synovial lining layers (b). †: P<0.05 versus non-operated group, ‡: P<0.01 versus non-operated group, §§: P<0.01 versus PBS group.

Fig. 8. Fluorescein-labeled HA preparations in synovial tissues. No particles of the fluorescein are detected in the non-operated group (a). The fluorescein particles are scattered through the synovial tissues, positive in five cases out of six in the HA84-treated group (b-f). In the HA230-treated group, only a few particles of fluorescein are detected in the tissue, being positive in two cases out of 6 cases (g, h). ×120

Fig. 9. Fluorescein-labeled HA84 (FHA; green, a) and HA stained with HABP (Texas red; red, b) in the synovial tissue of the HA84-treated group. Most of fluorescein granules were stained with HABP (arrows). A few fluorescein granules, however, are not recognized by HABP (arrowheads). ×2,000
munoreactivity for Hsp72 in the synovium. As shown in Figure 5, immunoreactivity for Hsp72 appeared after the ACLT operation and was more intense in HA-treated groups than in PBS-treated groups. Double immunostaining of Hsp72 and cathepsin B demonstrated that Hsp72-immunopositive cells were not co-labeled with immunoreactivity for cathepsin B, indicating that Hsp72 was induced in fibroblast-like type B cells (Fig. 6).

The incidence of Hsp72-immunopositive cells was significantly higher in the ACLT-operated than in the non-operated groups, while it was higher in the HA-treated than the PBS-treated groups (Fig. 7a). The intensity of immunoreactivity for Hsp72 was high in the HA84-group, compared with the other groups (Fig. 7b). The results suggest that Hsp72 was intensely induced in fibroblast-like type B cells after treatment with HA, especially with HA84.

Distribution of injected HA84 and HA230
To examine the fate of injected HA preparations in the synovium, fluorescein-labeled HA84 and HA230 were injected into the joint cavity at the final trial. Fluorescein-labeled HA84 was scattered throughout the synovial tissue in five cases of the HA84-treated group, except for one case in which the fluorescent particles could hardly be detected in the tissue (Fig. 8b-f). In the HA230-treated group, only a few fluorescent granules were detectable in the synovium of two cases, but no positive particles were seen in the synovium of the other four cases (Fig. 8 g, h). Injected fluorescein was often observed as cell surface or intracellular particles (date not shown). When the sections of the synovium receiving fluorescein-labeled HA preparations were further stained by HABP with Texas red, most fluorescein granules were co-labeled with Texas red, indicating the presence of HA (Fig. 9). A few fluorescein granules, however, were not recognized as HA when HABP was applied (Fig. 9). The results indicate that HA84 penetrated into and was preserved in the synovium much more than HA230.

DISCUSSION
The present study confirmed that the canine arthritic model induced by the ACLT operation in the knee joint showing increases in synovial fluid volumes and PGE2, thickening of the synovial lining layers due to the proliferation of the lining cells, and vacuolar alterations in the lining cells, is a useful model of the early phase of OA and TA. Using this model, we demonstrated that the effects of HA preparations on the arthritic synovium differ between HA84 and HA230. This difference in the therapeutic effects corresponded well with differences in the inducibility of Hsp72 and the productibility of HA in the synovial lining cells between the two molecules of HA.

BALAZS and DELINGER (1993) have suggested that the intra-articular injection of HA restores the normal rheological environment in the joints, and that viscoelasticity is dependent on the MW of HA. According to YANAKI and YAMAGUCHI (1990), a distinct change in the MW-dependent network formation of HA occurs at a MW of 1x10^6. To examine the fate of HA molecules after they are injected into the synovial cavity, we used fluorescein-labeled preparations. The results showed that the labeled HA84 molecule was more detectable in the synovial lining layers than the labeled HA230. It has been shown that the decreased rigidity of the vitreous humour with age is associated with the decrease in molecular size of HA (SWANN, 1987), suggesting that larger molecules of HA may be diluted more than smaller molecules of HA by holding water, since HA has a high capacity for this (BALAZS, 1967). From the evidence, it seems likely that the difference in the behavior of the two injected molecules of HA in the synovium may be attributed to the difference in dilution.
into the synovial fluids between HA84 and HA230. In fact, in the HA230-treated group, the synovial fluid volume was not decreased compared with that in the HA84-treated group, suggesting that HA230 was more diluted in the synovial fluids than HA84.

Moreover, the fluorescein-labeled HA84 molecule showed that most fluorescein particles were colabeled with HABP, though some were not. HABP derived from cartilage proteoglycans does not react with the oligosaccharides of HA, whose molecular sizes are smaller than the decasaccharides of HA (HASCALL and HEINAGÅRD, 1974). These data indicate that HA84 was degraded into oligosaccharides in the synovial tissues.

It has been shown by an in vitro study that PGE₂—which is known to increase vascular permeability—is produced by synovial cells, and that its production is suppressed by HA in a MW-dependent manner (YASUI et al., 1992). The present study, however, showed that PGE₂ concentrations and volumes in synovial fluids, which were increased by the ACLT operation, were significantly suppressed in the HA84-treated group, but not in the HA230-treated group. As stated above, the HA84 molecule was more accessible to synovial lining cells than the HA230 molecule. This molecular characteristic may permit HA84 to suppress PGE₂ release from the cells more effectively than HA230 in vivo.

Hsps are essential to the ability of cells to survive environmental insult (BAKER et al., 1992). The present immunohistochemical study demonstrated that Hsp72 in synovial cells was slightly induced in the synovial lining cells after the ACLT operation, as these mostly underwent vacuolar alterations. The intra-articular injection of HA preparations greatly enhanced the expression levels of Hsp72 in the synovial lining cells. This enhanced expression level of Hsp72 was clearly associated with the suppression of the vacuolar degeneration in the lining cells. These results strongly indicate that HA preparations up-regulate Hsp72 expression levels, resulting in the suppression of degenerative changes in synovial lining cells. HA preparations are not considered to be a sort of stressor, but rather an up-regulator or enhancer of Hsp72. Moreover, by the measurement of immunostainability of Hsp72 in synovial lining cells, the immunoreactivity was greater in the HA84- than in the HA230-treated groups. This difference in immunoreactivity is also considered to be due to that in accessibility to the lining cells between HA84 and HA230, since the incidence of vacuolar cells in the synovial lining layers was significantly lower in the HA84- than in the HA230-treated groups.

The present electron microscopic study showed that vacuolar degeneration occurred in fibroblast-like synovial cells. Moreover, Hsp72 positive cells were not immunostained for cathepsin B, which is intensely positive in macrophage-like but not in fibroblast-like synovial cells. These data indicate that Hsp72 was up-regulated in fibroblast-like synovial cells.

In the HA84-treated group, HA staining was intense in the synovial layer, while the fluorescein-labeled HA preparations were scattered throughout the layer, indicating that the positive staining of HA by HABP is different from that of exogenously injected HA preparations, and results from the endogenous production of HA in the lining cells. In the present findings, it is interesting to note that HA staining was less intense in the PBS- and HA230-treated groups—in which vacuolar degeneration appeared in fibroblast-like cells—than in the HA84-treated group. In addition to the differences in induction of Hsp72 in synovial lining cells and PGE₂ concentrations in synovial fluids between the experimental groups, the difference in HA production may also explain how the higher incidence of vacuolar fibroblast-like cells is associated with smaller amounts of HA production in the PBS- and HA230-treated groups.

It has been shown that HA inhibits the proliferation of cultured fetal fibroblasts and synovial cells (GOLDBERG and TOOLE, 1987; BRUCE et al., 1993). The present ACLT model showed a thickening of synovial lining layers, in which mitotic figures were seen in the lining cells, indicating that this thickening of the layers was attributed to the proliferation of lining cells. In our present study, this proliferative change in the synovial lining cells was significantly suppressed by the treatment of HA preparations.

From the results mentioned above, it is inferred that therapeutic effects of HA preparations on synovial cell alterations, such as proliferation and vacuolization, and hydrarthrosis, differ depending on their molecular size and the smaller sized molecule, HA84, is more effective than the larger sized molecule, HA230. This difference may be attributed to that in the accessibility of HA molecules to the synovial lining cells as HA84 was more accessible to the cells than HA230. The accessibility of HA molecules to the lining cells may be responsible for the up-regulation of Hsp72 which may prevent degenerative changes in the cells and inhibit PGE₂ release from the cells (Fig. 10). In fact, the induction of Hsp72 corresponded well with the production of HA in the lining cells, which is important for the maintenance of synovial or joint functions. Further study will be required to determine the precise mechanism of the actions of HA molecules on the synovium.
REFERENCES


Akira ASARI, PhD
Tokyo Research Institute
Seikagaku Corporation
Tateno 3-1253, Higashiyamato
Tokyo, 207-0021 Japan

浅利晃
207-0021
東京都東大和市立野3-1253
生化学工業東京研究所