Effects of the molecular weight of hyaluronic acid and its action mechanisms on experimental joint pain in rats

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Abstract

***Objectives*—It has been shown previously that hyaluronic acid (HA) has an analgesic action on bradykinin induced pain in the knee joints of rats. This study further clarifies the effects of the molecular weight of HA and its mechanism of action in the same model using HA of molecular weight 800 to $2{\cdot}3 \times 10^6$ daltons and a bradykinin antagonist.**

***Methods*—Bradykinin and the test HA preparations were given to rats by intra-articular injection, and the severity of pain was evaluated by a change in the walking behaviour.**

***Results*—HA with a molecular weight greater than 40 kilodaltons produces analgesic effects with a simultaneous or earlier injection. The ID_{50} values of HA with molecular weight 40, 310, 860, and 2300 kilodaltons were greater than 2·5, 0·6, 0·07, and 0·06 mg/joint respectively. The duration of the analgesic effect of 860 and 2300 kilodalton HA was 72 hours at 10 mg/ml, whereas that of 310 kilodalton HA was short, being undetectable after 24 hours. The analgesic action of HA of 860 kilodaltons was not changed by pretreatment with four saccharide HA and inhibited by pretreatment with HA larger than six to eight saccharides, capable of binding to HA receptors. Further, HA did not interfere with the analgesic action of the bradykinin antagonist, indicating that HA does not directly bind with bradykinin receptors.**

***Conclusions*—HA with a molecular weight of greater than 40 kilodaltons produced an analgesic effect, and HA of 860 and 2300 kilodaltons produced high and long-lasting analgesia. These effects of HA appear to be caused by the interaction between HA and HA receptors.**

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The main clinical symptom of osteoarthritis is joint pain, which makes daily life unpleasant. The pain may also promote progress of the disease process,[1-3] and result in decreased mobility of the joint due to lack of use. Consequently, reducing pain is important for the maintenance of joint activity and quality of life.

Hyaluronic acid (HA) is the main component of synovial fluid. High molecular weight HA with a random coil configuration has viscoelasticity and occupies a large amount of hydrodynamic space in solution.[4] Its functions in the joint are considered to be as a lubricant,[5 6] a protector of cartilage,[7-9] and an effector of the metabolism of synovial tissue.[10]

Intra-articular HA treatment of the knee of patients with osteoarthritis reduces painful symptoms and improves general activities of daily living and joint mobility.[11-13] The mechanism of joint pain is not clear, so the role of HA in pain suppression requires further study. In our previous study using the bradykinin induced joint pain model in rats, we showed that HA acting in a joint can induce analgesia.[14]

In this study we investigated the effects of the molecular weight of HA on analgesia and further attempted to clarify its mode of action using HA of molecular weight 800 to $2{\cdot}3 \times 10^6$ daltons in the same model.

Materials and methods

MATERIALS

HA preparations were extracted and highly purified from rooster combs; their average molecular weights were 6·8 (HA 6·8), 40 (HA 40), 310 (HA 310), 860 (HA 860), and 2300 kilodaltons (HA 2300). The molecular weight was calculated from the intrinsic viscosity according to the formula of Laurent *et al.*[15] HA of four saccharide units (4S HA) and a mixture of six and eight saccharide HA (8S HA) were prepared from HA 6·8 as follows. HA 6·8 was digested by partially purified bovine testicular hyaluronidase (Seikagaku, Tokyo, Japan) at 50°C for 96 hours. After digestion, degraded HA was fractionated by ethanol precipitation, followed by gel filtration chromatography with Cellulofine-GCL 90 m (Seikagaku). Each oligosaccharide fraction was desalted, lyophilised and analysed by high performance liquid chromatography (Hitachi Model 638-30) according to the method of Yoshida *et al.*[16] Bradykinin and bradykinin specific inhibitor (bradykinin antagonist), Des-Arg^9-(Leu^8)-bradykinin, were purchased from the Peptide Institute (Osaka, Japan). All preparations were dissolved in phosphate buffered saline (pH 7·4) or saline.

Table 1 Changes in walking behaviour caused by intra-articular injection of bradykinin

Walking behaviour	*Grade*	*Indication*
Normal		No change in walking
Lameness	Lam-I	Lameness alone for 20 seconds or less
	Lam-II	Lameness alone for more than 20 seconds
Pulling	Pul-I	Only transient pulling up of the injected leg (five seconds or less)
	Pul-II	Pulling up of the injected leg, followed by lameness
Three legs	Thr-I	Transient (for five seconds or less) walking on three legs, followed by lameness
	Thr-II	Walking on three legs for more than five seconds, followed by lameness

INDUCTION OF KNEE PAIN AND EVALUATION SYSTEM

Male SD rats, bred by Japan SLC (Shizuoka, Japan), aged 5–6 weeks, were used. The animals were previously placed for more than five minutes in long steel cages, allowing clear observation of their feet, and acclimatised to the new environment. Each injection was carried out with 0·05 ml/joint a time. A control group was set up in every experiment to check the reproducibility. Tests were performed by three workers; one operator prepared and injected the test solutions and the rest of the workers observed the animals for two minutes and evaluated the pain reaction blindly. Intra-articularly injected bradykinin causes short knee pain reactions from five to 10 seconds to about two minutes after the injection. A low dose of bradykinin causes transient lameness, which is extended by slightly increasing the amount of bradykinin. If the dose is increased further, pain symptoms include pulling up of the injected leg, and subsequently walking on the three uninjected legs. The last two were followed by lameness in most instances. The changes were differentiated and evaluated as shown in tables 1 and 2.

STATISTICS

Results are shown as the mean (SE) of several experiments. Differences among the groups were evaluated by one way analysis of variance followed by Newman-Keuls's test for multiple comparisons.

Results

BRADYKININ INDUCED KNEE JOINT PAIN RESPONSE AND ITS REPRODUCIBILITY

Bradykinin caused dose dependent pain reactions in rats when 0·05 ml was injected at a concentration of 0·56–45 μg/ml (fig 1). The analgesic score was 3 at a bradykinin concentration of 5 μg/ml. A score of 3 means that the rats pulled up the injected leg, followed by lameness, and the rating of score 3 is easy. Unless noted otherwise, 5 μg/ml bradykinin was used to induce pain in the subsequent examinations. There was no difference in the pain level among experimental days when 5 μg/ml bradykinin or bradykinin 30 minutes after a saline injection was given (fig 2).

Table 2 Behaviour grading criteria for evaluating pain reaction

Grade of algesia	*Walking behaviour*	*Evaluation score*
None	Normal walking	0
Slight	Lam-I	1
Mild	Lam-II or Pul-I	2
Moderate	Pul-II or Thr-I	3
Severe	Thr-II	4

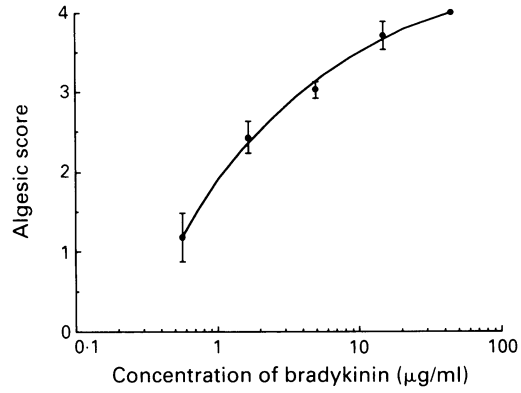

Figure 1 Dose-response curve of pain response induced by intra-articular injection of bradykinin 0·05 ml in rats. Results are shown as mean (SE) values (n = 6–38).

EFFECTS OF MOLECULAR WEIGHT OF HA INJECTED WITH BRADYKININ ON PAIN RESPONSE

HA preparations of different molecular weights of various concentrations were injected with bradykinin simultaneously. HA 40, 310, 860, and 2300 inhibited the pain at concentrations of ≥50, ≥10, ≥2·5, and ≥1·25 mg/ml respectively. HA 6·8 had no effect, even at 100 mg/ml (fig 3).

The percentage inhibition was obtained with respect to the control. The ID_{50} values of HA 40, 310, 860, and 2300 were >50, 12, 1·4, and 1·2 mg/ml as concentration, and >2·5, 0·6, 0·07, and 0·06 mg/joint as dose respectively (fig 4). The ID_{50} value decreased with increased molecular weight of HA, but no difference between the ID_{50} values of HA 860 and HA 2300 was noted.

DURATION OF ANALGESIA

A pain reaction was induced 24, 48, 72, and 96 hours after administration of HA 310, 860, and 2300. HA 860 and 2300 inhibited pain at ≥5 mg/ml after 24 hours, at 10 mg/ml after 48 and 72 hours, and had no effect after 96 hours. HA 310 had no effect at any of these times (fig 5).

EFFECTS OF HA OLIGOSACCHARIDE ON ANALGESIA OF HA 860

This test was conducted to examine whether the analgesic action of HA is produced by the interaction of HA with HA receptors. It is well

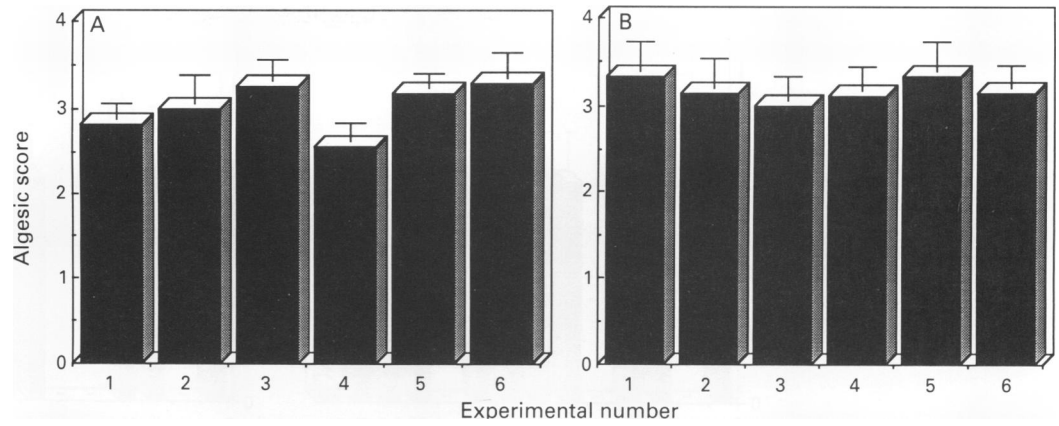

Figure 2 Reproducibility of bradykinin induced knee pain response. (A) Bradykinin injection of 5 μg/ml (n = 4–8); (B) Bradykinin of 5 μg/ml at 30 minutes after saline injection (n = 3–10).

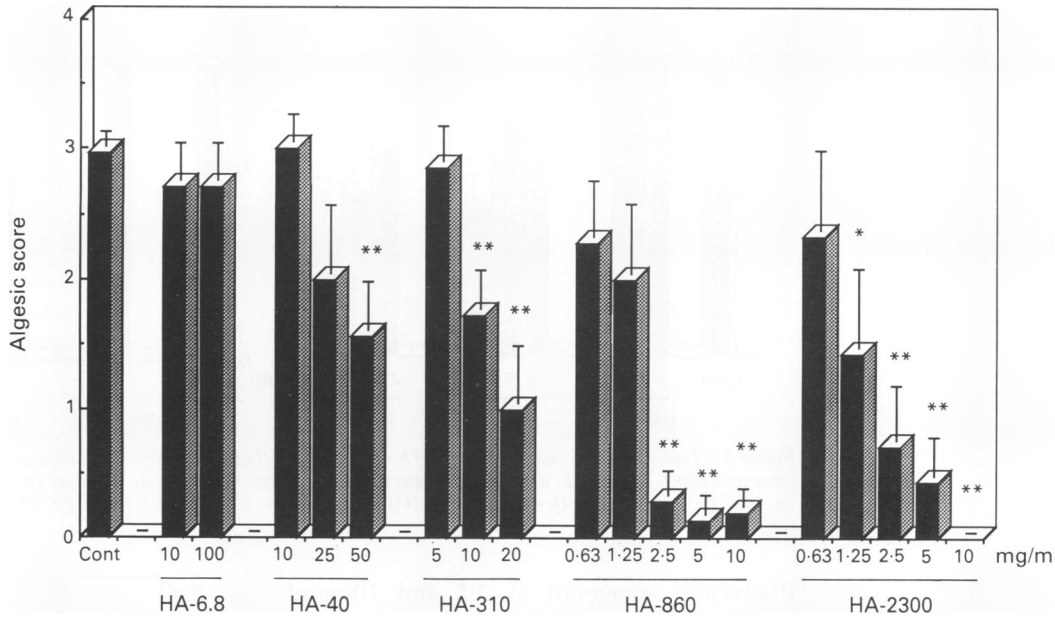

*Figure 3 Effects of hyaluronic acid (HA) with different molecular weights on bradykinin induced joint pain in rats. Each HA was injected into a joint simultaneously with bradykinin (n = 6–31). *p < 0·05; **p < 0·01. Molecular weight of HA-6·8, HA-40, HA-310, HA-860, and HA-2300 are 6·8, 40, 310, 860, and 2300 kilodaltons.*

known that four saccharide HA does not bind to the HA receptors on the cell surface, and HA with six or eight saccharide units is the minimum size for binding.[17 18] Thus we examined whether the analgesic effect of HA 860 was suppressed by pretreatment with

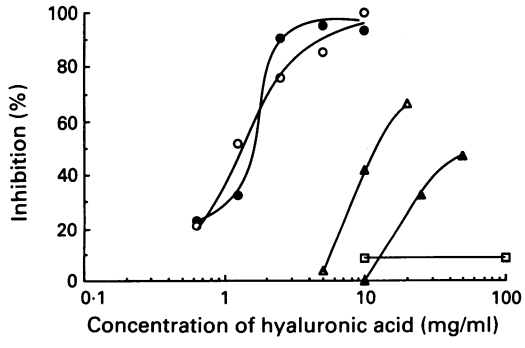

Figure 4 Dose-response curves of hyaluronic acid (HA) with different molecular weights. Each percentage inhibition of pain response was obtained with respect to the control using the data of fig 3. (□) HA-6·8; (▲) HA-40; (△) HA-310; (●) HA-860; and (○) HA-2300. The abbreviations are the same as those in fig 3.

4S HA, 8S HA, and HA 6·8. The pain reaction was induced by the injection of bradykinin alone and by the simultaneous injection of bradykinin and HA 860 of 10 mg/ml 30 minutes after administration of 4S HA, 8S HA, and HA 6·8. HA 860 inhibited the pain in knees with preinjected saline, 4S HA of 10 mg/ml, and 8S HA of 2·5 mg/ml, but did not inhibit it in knees injected with 10 mg/ml 8S HA or ≥2·5 mg/ml HA 6·8. The results indicated that pretreatment with 8S HA and HA 6·8 suppressed the analgesic effect of HA 860, and HA 6·8 greatly decreased it, whereas 4S HA did not (fig 6). These findings suggest that the analgesia of HA is produced by the interaction between HA and HA receptors.

EFFECT OF LOW MOLECULAR WEIGHT HA ON ANALGESIA OF BRADYKININ ANTAGONIST

To examine whether HA binds directly to bradykinin receptors and competes against bradykinin antagonist activity, a test was conducted with the combined use of HA.

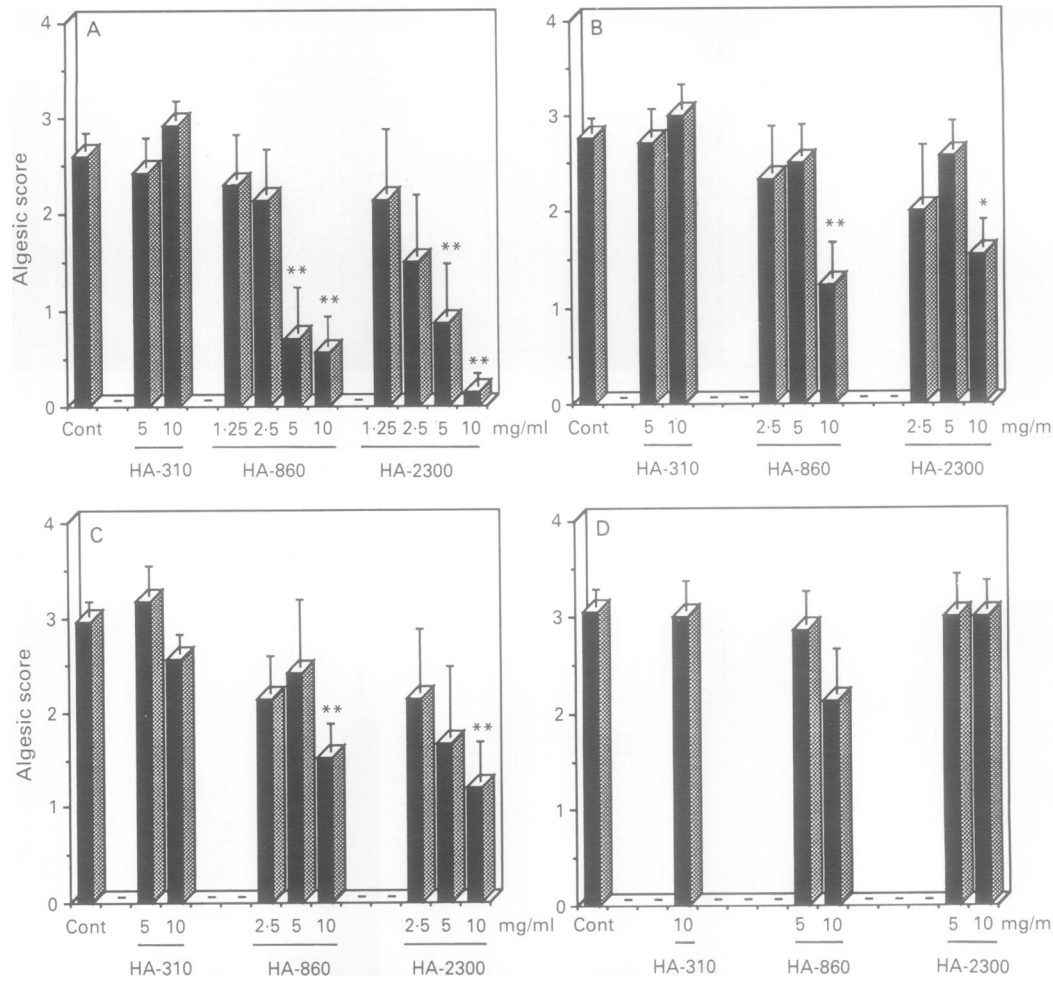

*Figure 5 Time courses of analgesic effects of hyaluronic acid (HA) with different molecular weights. HA was administered intra-articularly 24, 48, 72, and 96 hours before the bradykinin injection. (A) 24 hours (n = 6–28); (B) 48 hours (n = 6–21); (C) 72 hours (n = 6–27); and (D) 96 hours (n = 7–19). *p < 0·05; **p < 0·01. The abbreviations are the same as those in fig 3.*

Bradykinin antagonist at 2·5 and 10μg/ml inhibited pain, depending on the dose, when injected simultaneously with 5 μg/ml bradykinin (fig 7). As the bradykinin antagonist has a short life in vivo, bradykinin was injected five minutes after administration of the bradykinin antagonist. This short interval between injections enhanced the pain. Thus in this test a bradykinin concentration of 2·5 μg/ml was chosen to obtain an analgesic score of 3 in the control group for easy rating. HA 6·8 was selected as it binds to HA receptors but shows no analgesia, and will suppress bradykinin antagonist activity if it binds to bradykinin

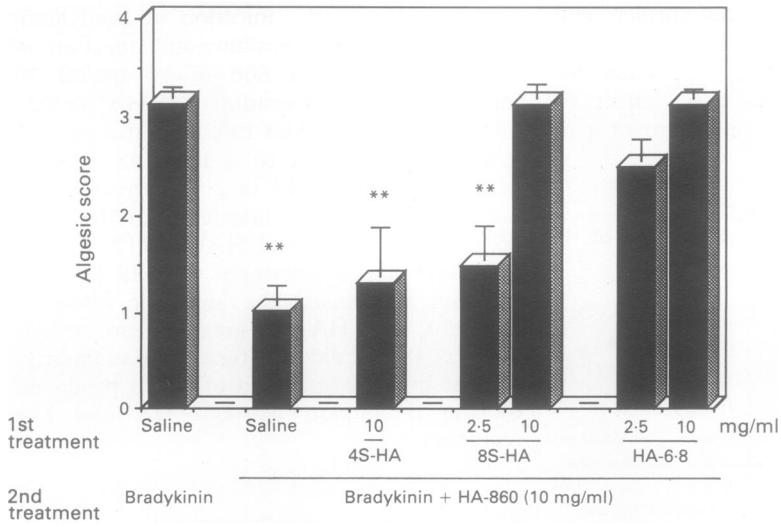

*Figure 6 Antagonistic effects of hyaluronic acid (HA) oligosaccharide on analgesia of HA with high molecular weight. Saline and each oligosaccharide were administered intra-articularly 30 minutes before bradykinin or injection of a combination of bradykinin and high molecular weight HA (n = 6–36). **p < 0·01. (4S-HA) four saccharide HA, (8S-HA) mixture of six and eight saccharide HA. The abbreviations are the same as those in fig 3.*

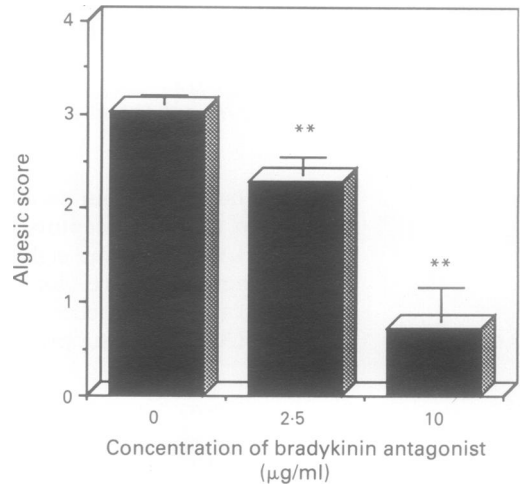

*Figure 7 Antagonistic effect of Des-Arg^9-(Leu^8)-bradykinin (bradykinin antagonist) on bradykinin induced pain response. Drug was injected simultaneously with bradykinin (n = 7–38). **p < 0·01. Concentration of bradykinin, 5 μg/ml.*

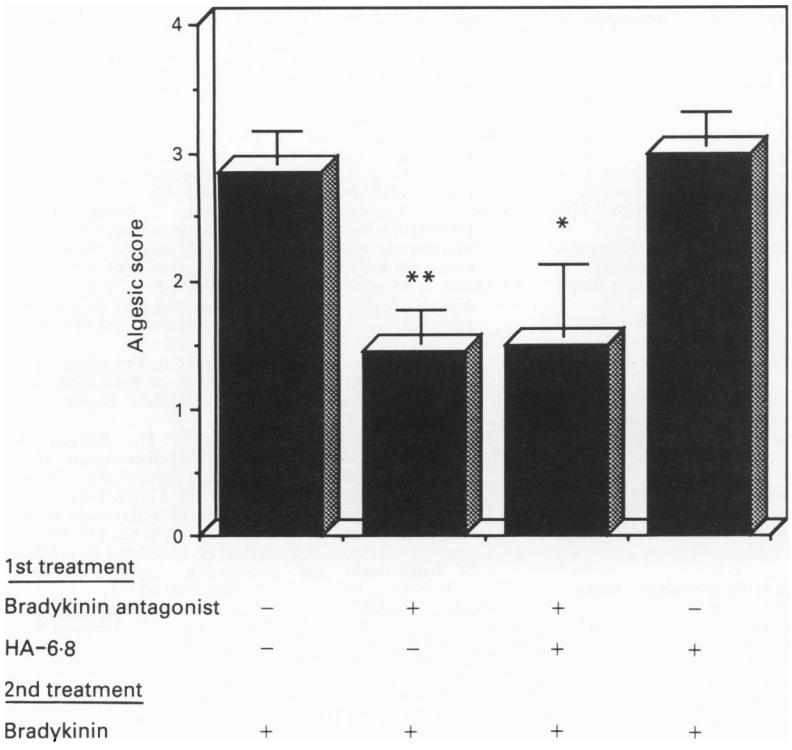

*Figure 8 Effect of low molecular weight hyaluronic acid (HA) on analgesic effect of bradykinin antagonist. Drugs were given intra-articularly five minutes before bradykinin injection (n = 7–38). *p < 0·05; **p < 0·01. Concentrations of bradykinin, bradykinin antagonist and low molecular weight HA (HA-6·8) were 2·5, 10, and 10 mg/ml respectively. The abbreviations are the same as those in figs 3 and 7.*

receptors. A pain reaction was induced by injecting 2·5 μg/ml bradykinin five minutes after administration of bradykinin antagonist (10 μg/ml), HA 6·8 (10 mg/ml), and a mixture of the two. Bradykinin antagonist and the mixture with HA 6·8 inhibited the pain equally, but HA 6·8 did not. This indicates that HA had no effect on the interaction between bradykinin and bradykinin receptors (fig 8).

Discussion

Although many investigations have suggested that HA plays an important part in various biological activities, the precise function of HA in these events is not yet clear. Nevertheless, it is likely that the influence of HA depends on (*a*) its remarkable hydrodynamic properties and (*b*) its interaction with the cell surface.[19]

We hypothesised that information on the effects of the molecular weight of HA might be the key for revealing the mechanisms of the analgesia of HA. In this study using HA with a molecular weight of 800 to $2·3 \times 10^6$ daltons, the results indicated that HA with a molecular weight range of 40 to 860 kilodaltons produced an analgesic action dependent on molecular weight and concentration. HA 860 and HA 2300 showed almost the same ID_{50} value at 1·4 and 1·2 mg/ml (0·07 and 0·06 mg/joint) on simultaneous injection with bradykinin, and the same duration of analgesia (72 hours). HA 310 showed a short term effect, suggesting that a relatively high molecular weight HA is needed to produce longlasting analgesia. HA 860 and HA 2300 show almost the same activity, however, in spite of the fact that HA 2300 has a higher viscosity. A previous study suggested that the analgesic effect of HA was obtained by covering the tissue and cell surface pain receptors, and not by viscosity.[14] From these findings, we have concluded that the analgesic effect of HA is due to its affinity to cells rather than to the direct hydrodynamic physical properties and viscosity corresponding to molecular weight.

The interaction of HA with HA binding receptors on the cell surface is considered to be an important factor in determining cell behaviour. Underhill and Toole[17] and Laurent *et al*[18] investigated the effect of the molecular weight of HA on the binding ability to the receptor in cultured cells in vitro and found that binding of HA to its receptors occurred when the saccharide number of HA was larger than six or eight. The larger the molecule of HA, the higher the affinity; when the hydrodynamic size of exogenous HA was very large, the number of binding molecules that could occupy the available cell surface receptors was reduced.

This led us to investigate the role of HA receptors on the cell surface in the analgesic action of HA. Among 4S HA, 8S HA, and HA 6·8, 8S HA and HA 6·8, capable of binding to receptors, suppressed the biological activity of HA 860, and the degree of inhibition depended on the concentration and saccharide chain length of HA. On the other hand, the action of the bradykinin antagonist was not affected by HA 6·8, indicating that HA molecules do not appear to bind directly to bradykinin receptors. Consequently, the effect of HA appears to be brought on by the interaction between HA and HA receptors, and not by the interaction between HA and bradykinin receptors.

We had previously proposed another possible explanation for the analgesic action. HA has an expanded random coil configuration which causes entanglement between neighbouring molecules. We had thought that HA in the synovial fluid may trap bradykinin in its molecule by the ion bonds formed between the carboxyl base in HA with the negative charge and bradykinin with the cation charge, followed by maintaining an entangled coil configuration. This is not feasible, however, because analgesia by HA 860 no longer occurred after HA oligosaccharide pretreatment, as shown in fig 5, and when the bradykinin solution was dialysed with the filter membrane, the dialysis rate of bradykinin was not changed by adding HA in solution (data not shown).

The results of this study indicate that (*a*) HA with a molecular weight greater than 40 kilodaltons produces analgesic effect, (*b*) high and longlasting analgesia is induced by HA with molecular weights of 860 and 2300 kilodaltons equally, and (*c*) that analgesia was inhibited by oligosaccharides of HA. Moreover, HA did not directly bind to bradykinin receptors, indicating that analgesia of HA appears to be brought on by the interaction of HA and HA receptors on or surrounding the free nerve endings that detect pain in the joint tissue.

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