Inhibitory Effects of Hyaluronan on [^{14}C]Arachidonic Acid Release from Labeled Human Synovial Fibroblasts

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ABSTRACT — The effects of hyaluronan (HA) on the release of arachidonic acid (AA) from phospholipids induced by bradykinin in synovial fibroblasts of osteoarthritic patients were examined. HA inhibited [^{14}C]AA release from prelabeled synovial cells stimulated with and without bradykinin 1 hr after incubation with HA and thereafter. The inhibitory effects of HA on [^{14}C]AA release were dependent on the concentration and molecular weight of HA. However, inhibition of [^{14}C]AA release by HA was not merely due to the viscosity of HA. The [^{14}C]AA release induced by calcium-ionophore A23187 was also inhibited by HA with a high molecular weight. In addition, HA did not affect [^{14}C]AA uptake by the cells. Our results suggest that HA with a high molecular weight elicits anti-inflammatory effects, at least in part, by inhibiting AA release in inflamed joints.

Keywords: Hyaluronan, Arachidonic acid release, Synovial cell

Pain is a cardinal symptom of osteoarthritis. Current treatments for osteoarthritis using steroidal and non-steroidal anti-inflammatory agents focus on relieving pain arising from secondarily induced synovitis. Bradykinin is a potent pain-producing nonapeptide which is formed in damaged tissues (1). This peptide binds to receptors on the cell surface and triggers activations of both phospholipase-A_2 and -C simultaneously (2, 3). These phospholipases directly or indirectly generate free arachidonic acid (AA) by hydrolysis of phospholipids (4). Calcium-ionophore A23187 has also been known to activate phospholipase A_2 by directly increasing the intracellular calcium level (5). Bradykinin-induced pain is amplified by prostaglandin E_2, which sensitizes nociceptors in peripheral tissues (6). Since the formation of cyclooxygenase-derived products is dependent on prior release of AA (7, 8), inhibition of the AA release from phospholipids may result in the relief of pain.

Hyaluronan (HA), a major component of the joint synovial fluid, is a linear polysaccharide composed of alternating units of *N*-acetyl-D-glucosamine and D-glucuronic acid (9). In inflammatory arthritis, synovial HA is reduced in concentration, fragmented and depolymerized with a concomitant reduction in synovial fluid viscosity (10). In recent years, clinical trials of intra-articular administration of HA to patients with osteoarthritis have indicated its analgesic effects (11, 12). Thus, effects of HA with various molecular weights on the AA release from synovial fibroblasts of osteoarthritic patients were examined in this study to determine if this polymer actually inhibits the AA release.

MATERIALS AND METHODS

Hyaluronan

Four endotoxin-free HA preparations of different molecular weights (viscosity-average *Mr*: 2.0×10^6, 1.4×10^6, 9.8×10^5, and 2.8×10^5) were products previously purified (protein content less than 0.1%) from culture broths of *Streptococcus zooepidemicus* (13) (gifts from Shiseido Pharmaceutical Research Laboratories, Japan). HA preparations derived from pig skin (*Mr* $4.0 - 6.0 \times 10^4$, more than 95% pure) and human umbilical cord (*Mr* $0.8 - 1.2 \times 10^6$, protein content less than 0.1%) were products of Seikagaku Kogyo,

Japan.

Synovial cells

Synovium from the knee of osteoarthritic patients was isolated from surrounding tissues at the time of surgery for treating arthropathy and minced into 1–2-mm^2 fragments. Synovial fragments were explanted onto 60-mm diameter culture dishes containing Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, USA), 100 U/ml of penicillin (Meiji Seika, Japan) and 100 μg/ml of streptomycin (Meiji Seika, Japan). They were cultured at 37°C in a humidified 5% CO_2 atmosphere. Morphologically fibroblast-like cell out-growths from synovial fragments were trypsinized, seeded at a density of 4×10^4 cells/well in 24-well cell culture plates and allowed to grow to confluence.

Release of AA from synovial cells

Confluent synovial cells were prelabeled with [1-^{14}C]AA (1.4 kBq/0.5 ml/well, 1.9 GBq/mmole, New England Nuclear, USA) in DMEM containing 10% FBS for 24 hr at 37°C. After washing 3 times with serum-free DMEM, the labeled cells were exposed for 3 hr to 0.5 ml/well of DMEM containing 10% FBS with bradykinin (10 μM, Bachem, USA), calcium-ionophore A23187 (1 μM, Sigma, USA), or vehicle in the presence or absence of HA with different molecular weights at 0.5, 1.0 and 1.5 mg/ml. In place of HA treatment, 0.4, 1.5 or 4.0 mg/ml of methyl cellulose (MC; Tokyo Kasei, Japan) was added to other cultures to evaluate the effect of viscosity. At the end of the treatment period, the culture medium was collected and centrifuged at $2000 \times g$ for 15 min at room temperature to avoid any contamination of free cells or cell debris. The supernatant was isolated and its radioactivity was measured by a liquid scintillation counter.

Incorporation of AA in synovial cells

Confluent synovial cells were incubated with [^{14}C]AA (1.2 kBq/0.5 ml/well) in DMEM containing 10% FBS with or without HA (1.5 mg/ml, *Mr* 2.0×10^6) at 37°C for various intervals. At the end of incubation, the residual radioactivity in the medium was measured.

Analyses of lipids in cells and medium

Total lipid contents were extracted under HCl-acidified conditions by the method of Folch et al. (14). The resulting organic phase was analyzed by thinlayer chromatography with a silica gel 60-precoated plate (Merck, Germany). The solvent system used to separate phospholipids, AA, diacylglycerol and triacylglycerol in the cell extracts was petroleum ether / diethylether / acetic acid (50/50/1, v/v). Chloroform / methanol (93/7, v/v) was employed to analyze AA in the medium extract. Lipid standards were run as carriers with each sample. Lipid spots visualized with iodine vapor were removed, and the radioactivity was then determined.

Viscosity determinations

HA (1.5 mg/ml) and MC (0.4, 1.5 and 4.0 mg/ml) were prepared in DMEM containing 10% FBS. The viscosity (expressed in centipoise, cP) of the polysaccharide solution was measured by a calibrated rotational type viscometer (Tokyo Keiki, Japan) at 37°C. Bacterial HA preparations (1.5 mg/ml) of *Mr* 2.0×10^6, 1.4×10^6, 9.8×10^5 and 2.8×10^5 registered 14.5, 8.1, 3.8 and 1.8 cP, respectively. HA preparations derived from pig skin and human umbilical cord (1.5 mg/ml) had viscosities of 1.1 and 5.1 cP, respectively. The viscosity values of MC samples of 0.4, 1.5 and 4.0 mg/ml were 3.1, 7.6 and 12.9 cP, respectively. The viscosity of the medium was 0.6 cP.

Statistical analyses

Results were expressed as means ± S.D. The statistical significance of data was evaluated by the unpaired Student's *t*-test.

RESULTS

[^{14}C]AA metabolites in cells and medium

On labeling synovial cells with [^{14}C]AA (6.3×10^4 dpm) for 24 hr, 4.7×10^4 dpm of the radiolabeled compound were incorporated within the cells. Of the [^{14}C]AA incorporated in cells, 87% of the counts were found in [^{14}C]phospholipids (4.1×10^4 dpm) and 7.9%, in [^{14}C]triacylglycerol (3.7×10^3 dpm), whereas the amounts of [^{14}C]diacylglycerol and [^{14}C]AA were negligible (less than 1000 dpm in both). In labeled cells incubated for 3 hr in the absence (control) or presence of bradykinin (10 μM) or A23187 (1 μM), the highest release (11.3%) of incorporated radiolabeled ligand was induced by bradykinin, as compared to those of the control (5.5%) and A23187 (9.7%). When free [^{14}C]AA contents against the total releases in media incubated with bradykinin, A23187 or neither were expressed as a percentage, the values were 82.2%, 77.2% or 64.1%, respectively. Therefore, the release of [^{14}C]AA from cells was mainly thought to have been initiated by hydrolysis of phospholipids.

Effects of bradykinin and HA on [^{14}C]AA release

Prelabeled synovial cells were incubated with or without 10 μM bradykinin (within the concentration range

of 0.5 to 30 μM, 10 μM elicited the maximal [^{14}C]AA release from cells at 3 hr after the incubation in preliminary studies) in the presence or absence of HA (1.5 mg/ml, *Mr* 2.0 × 10^6). The kinetics of [^{14}C]AA release from prelabeled synovial cells were then examined for 6 hr (Fig. 1). The cells incubated without bradykinin spontaneously released [^{14}C]AA, and the [^{14}C]AA release at the 6-hr incubation exceeded that at the 2-hr incubation by 1.3-fold. A significant level of bradykinin-induced [^{14}C]AA release was observed 15 min after the incubation, and the amount of [^{14}C]AA released after a 3-hr incubation in bradykinin-stimulated cells was 2.2-fold that of the unstimulated cells. However, the cells treated with HA manifested a significant decrease in [^{14}C]AA release in the presence or absence of bradykinin after 1-hr incubation.

Dose- and molecular weight-dependent inhibitions of [^{14}C]AA release by HA

Prelabeled synovial cells were incubated for 3 hr with bradykinin (10 μM) in the presence of HA with different molecular weights (2.0 × 10^6, 1.4 × 10^6, 9.8 × 10^5, and 2.8 × 10^5) at various concentrations. HA dose-dependently inhibited the release of [^{14}C]AA from synovial cells within the concentration range of 0.5 to 1.5 mg/ml (Fig. 2). HA with a higher molecular weight manifested more potent inhibitory effects on [^{14}C]AA release than the same polymer with a lower molecular weight. Especially at a concentration of 1.5 mg/ml, HA had an inhibitory tendency consistent with the statistical significances achieved with different molecular weights (Fig. 2). When prelabeled synovial cells were stimulated for 3 hr with A23187 (1 μM), HA (*Mr* 2.0 × 10^6) also inhibited [^{14}C]AA release in a dose-dependent manner (Fig. 3).

Effects of viscosities of HA and MC on [^{14}C]AA release

Prelabeled synovial cells were incubated for 3 hr with or without bradykinin (10 μM) in the presence of HA (1.5 mg/ml) with different molecular weights and MC

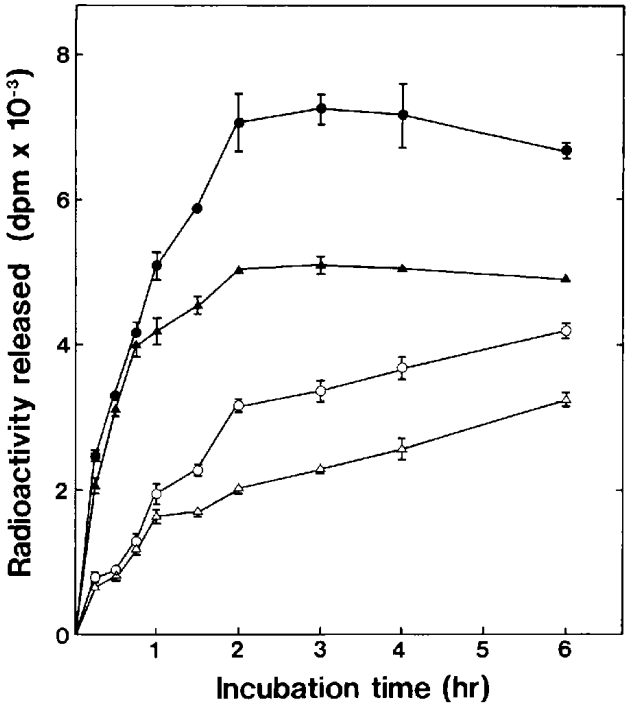

Fig. 1. Effects of bradykinin and hyaluronan (HA) on the kinetics of [^{14}C]arachidonic acid release. Prelabeled synovial cells were incubated with (closed symbols) or without (open symbols) bradykinin (10 μM) in the presence (triangles) or absence (circles) of 1.5 mg/ml HA (*Mr* 2.0 × 10^6) at 37°C. After subjecting the media to different incubation periods, radioactivity in the medium was determined. Each point represents the mean ± S.D. of three or four separate experiments.

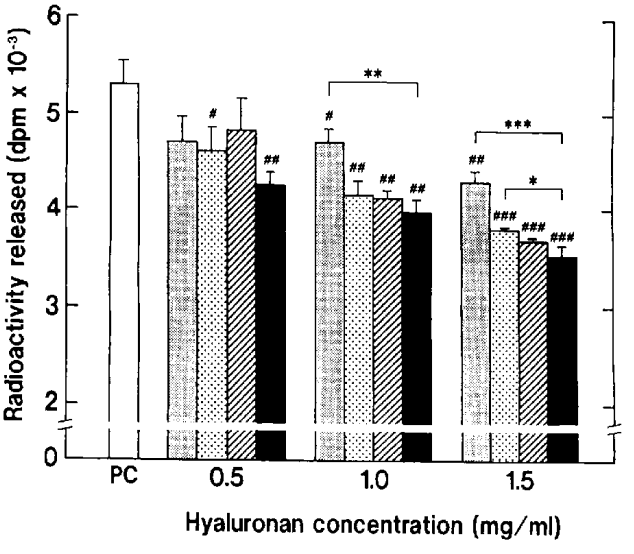

Fig. 2. Effects of varying the molecular weight of hyaluronan (HA) on bradykinin-induced [^{14}C]arachidonic acid (AA) release. Prelabeled synovial cells were exposed to bradykinin (10 μM) at 37°C for 3 hr in the absence (PC: positive control) or presence of HA of different molecular weights (gray column: 2.8 × 10^5, dotted column: 9.8 × 10^5, hatched column: 1.4 × 10^6, closed column: 2.0 × 10^6) at 0.5, 1.0, and 1.5 mg/ml. Each column represents the mean ± S.D. of three separate experiments. Significant differences of $P < 0.05$ ($^{\#}$, *); $P < 0.01$ ($^{\#\#}$, **); and $P < 0.001$ ($^{\#\#\#}$, ***) were values compared to either the positive control (represented by the symbol $^{\#}$) or the peak inhibition of [^{14}C]AA release at the highest molecular weight within the same concentrations (represented by the symbol *).

(0.4, 1.5, and 4.0 mg/ml). Effects of the polysaccharides on [^{14}C]AA release were then examined in media of different viscosities (Fig. 4). At viscosities less than 8.1 cP, the inhibitions of [^{14}C]AA release by HA and MC were significantly different, especially in the presence of bradykinin. At the concentrations of 0.4 mg/ml (3.1 cP) and 1.5 mg/ml (7.6 cP), MC did not elicit any significant inhibitions on [^{14}C]AA release, despite concomitant increases in viscosity of the preparations. In contrast, HA effectively suppressed [^{14}C]AA release. However, at viscosities exceeding 12.9 cP, there was little difference between the amount of released [^{14}C]AA induced by HA and that by MC.

Effect of HA on [^{14}C]AA incorporation

To investigate if HA had reduced the free [^{14}C]AA level in the medium through an enhancement in [^{14}C]AA uptake, the effect of HA on [^{14}C]AA incorporation in synovial cells was examined. Cells were incubated with 0.6 nmole of [^{14}C]AA (7×10^4 dpm), a concentration in excess of the level of [^{14}C]AA released from cells, in the presence or absence of HA (*Mr* 2.0 $\times 10^6$) at a concentration of 1.5 mg/ml (where the peak inhibition of bradykinin-induced [^{14}C]AA release was observed). HA did not alter the content of [^{14}C]AA incorporated into the cells for up to 22 hr (Table 1).

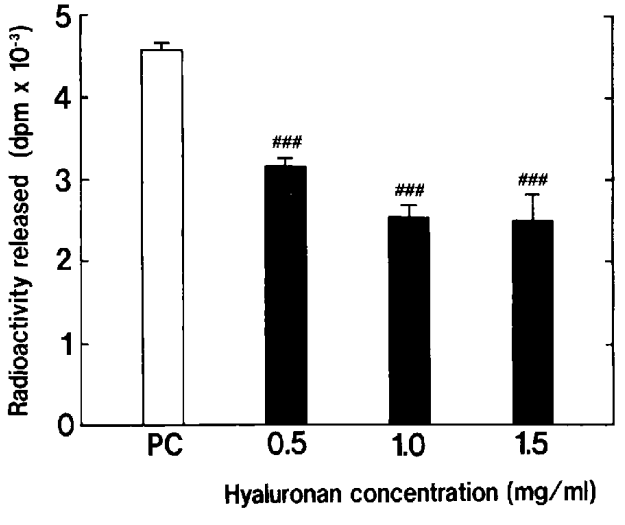

Fig. 3. Effects of hyaluronan (HA) on A23187-induced [^{14}C]-arachidonic acid release. Prelabeled synovial cells were exposed to A23187 (1 μM) at 37°C for 3 hr in the absence (PC: positive control) or presence of HA (*Mr* 2.0 $\times 10^6$) at 0.5, 1.0, and 1.5 mg/ml. Each column represents the mean ± S.D. of three separate experiments. Significant difference of $P < 0.001$ ($^{\#\#\#}$) was the value compared to the positive control.

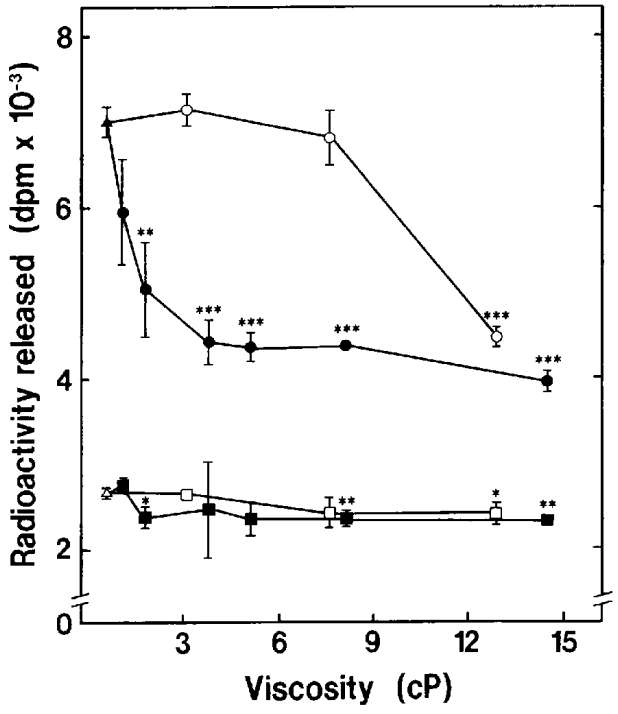

Fig. 4. Effects of varying the viscosity of hyaluronan (HA) and methyl cellulose (MC) samples on bradykinin-induced and spontaneous [^{14}C]arachidonic acid releases. Prelabeled synovial cells were incubated with (circles and closed triangle; positive control) or without (squares and open triangle; negative control) bradykinin (10 μM) at 37°C for 3 hr in the absence (triangles: controls) or presence of HA (closed symbols except triangle) of different molecular weights at 1.5 mg/ml or MC (open symbols except triangle) at 0.4, 1.5, and 4.0 mg/ml. Each point represents the mean ± S.D. of three separate experiments (except the positive control: n = 9). Significant differences of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were values compared to each control.

Table 1. Effects of hyaluronan (HA) on incorporation of labeled arachidonic acid (AA) in synovial cells

Time (hr) after incubation	Residual radioactivity in medium (dpm $\times 10^{-4}$)		
	Control (without HA)	Incubation with HA	n
0	6.95	6.94	2
2	5.78 ± 0.09	5.74 ± 0.03	3
6	4.29 ± 0.04	4.55 ± 0.09	3
22	2.59 ± 0.01	2.62 ± 0.01	3

Confluent synovial cells were incubated with [^{14}C]AA (7×10^4 dpm) in the presence (1.5 mg/ml) or absence (control) of HA (*Mr* 2.0×10^6). At the end of incubation, the residual radioactivity was measured. Each value represents the mean ± S.D. (except 0 hr).

DISCUSSION

In the present study, HA suppressed the quantitative [^{14}C]AA release from synovial cells incubated in the presence or absence of bradykinin. As bradykinin-induced [^{14}C]AA release exceeded that induced spontaneously without any bradykinin by 3.7-, 2.6- and 2.2-fold at 0.5, 1 and 3 hr after incubation, respectively, experiments were conducted with the use of bradykinin. In this way, experimental errors of HA effects on AA release would be minimized. In the case of the calcium-ionophore A23187, the compound not only induced a lesser amount of [^{14}C]AA release but it was also not an endogeneous substance.

It has been reported that intracellular AA levels are regulated by the equilibrium between AA release from and uptake in phospholipids (7). Thus, two possible mechanisms underlying the decrease in AA in the medium by HA are conceivable. First, HA may actually inhibit AA release from synovial cells. Secondly, HA may enhance AA uptake into synovial cells. To evaluate these possibilities, the effect of HA on [^{14}C]AA uptake in synovial cells was examined. It was found that HA (Mr 2.0×10^6) at 1.5 mg/ml did not alter the [^{14}C]AA uptake into the cells. This result suggests that the decreased rate of [^{14}C]AA release by HA is more likely due to the inhibition of [^{14}C]AA release.

The inhibitory effects of HA were dependent on both the concentration and molecular weight of the polymer. Because MC at a high concentration (4.0 mg/ml) also suppressed the bradykinin-induced [^{14}C]AA release from synovial cells, the inhibitory effects of HA with a high molecular weight are probably, at least in part, due to its viscosity, which may affect the mobility of AA molecules from the cell surface. However, unlike MC, HA manifested significant inhibitory effects on [^{14}C]AA release at lower viscosities. This result suggests that the inhibitory effects of HA on [^{14}C]AA release from cells are not merely a consequence of the increased viscosity of the medium, but also factors other than viscosity may be involved in the inhibitory effects of HA. HA had no significant effect on the quantitative [^{14}C]AA release stimulated in the presence or absence of bradykinin for an incubation period of up to 45 min. Because of its viscosity, HA may require the initial 1 hr or more to infiltrate the cell surface so as to elicit significant inhibitory effects. However, it remains unclear whether or not the time-lag in the inhibition of [^{14}C]AA release can be accounted for merely by the viscosity of HA.

While a plausible mechanism for the inhibition of [^{14}C]AA release by HA is not known at present, decreased AA levels by HA in the extracellular space may result in the diminished production of eicosanoids in joint synovial fluid, since AA is taken up by other cells such as activated leukocytes infiltrated in the joint space and is then metabolized to eicosanoids (15). We used cultured human synovial cells as a model to evaluate the in vitro effects of HA on articular tissues. Our results obtained from this model coincide well with a recent observation on the in vivo inhibitory action of HA on prostaglandin E_2 production in the synovial fluid of arthritic patients (16). As such, intra-articular administration of HA with a high molecular weight may be effective for treating inflammatory arthritis.

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REFERENCES

1 Miller, R.J.: Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. Trends Neurosci. **10**, 226–228 (1987)

2 Burch, R.M. and Axelrod, J.: Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A_2. Proc. Natl. Acad. Sci. U.S.A. **84**, 6374–6378 (1987)

3 Kaya, H., Patton, G.M. and Hong, S.L.: Bradykinin-induced activation of phospholipase A_2 is independent of the activation of polyphosphoinositide-hydrolyzing phospholipase C. J. Biol. Chem. **264**, 4972–4977 (1989)

4 Burgoyne, R.D. and Morgan, A.: The control of free arachidonic acid levels. Trends Biochem. Sci. **15**, 365–366 (1990)

5 Rittenhouse-Simmons, S.: Differential activation of platelet phospholipases by thrombin and ionophore A23187. J. Biol. Chem. **256**, 4153–4155 (1981)

6 Cohen, R.H. and Perl, E.R.: Contributions of arachidonic acid derivatives and substance P to the sensitization of cutaneous nociceptors. J. Neurophysiol. **64**, 457–464 (1990)

7 Irvine, R.F.: How is the level of free arachidonic acid controlled in mammalian cells. Biochem. J. **204**, 3–16 (1982)

8 Dennis, E.A.: Regulation of eicosanoid production: role of phospholipases and inhibitors. Biotechnology **5**, 1294–1300 (1987)

9 Laurent, T.C.: Biochemistry of hyaluronan. Acta Otolaryngol. **442**, 7–24 (1987)

10 Dahl, L.B., Dahl, I.M.S., Engström-Laurent, A. and Granath, K.: Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. Ann. Rheum. Dis. **44**, 817–822 (1985)

11 Dixon, A.S.J., Jacoby, R.K., Berry, H. and Hamilton, E.B.D.: Clinical trial of intra-articular injection of sodium hyaluronate in patients with osteoarthritis of the knee. Curr. Med. Res. Opin. **11**, 205–213 (1988)

12 Leardini, G., Mattara, L., Franceschini, M. and Perbellini, A.: Intra-articular treatment of knee osteoarthritis. A comparative study between hyaluronic acid and 6-methylprednisolone acetate. Clin. Exp. Rheum. **9**, 375–381 (1991)

13 Akasaka, H., Seto, S., Yanagi, M., Fukushima, S. and Mitsui, T.: Industrial production of hyaluronic acid by *Streptococcus zooepidemicus*. J. Soc. Cosmet. Chem. Japan **22**, 45–52 (1988) (Abs. in English)

14 Folch, J., Lees, M. and Stanley, G.H.S.: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509 (1957)

15 Flower, R.J. and Blackwell, G.J.: The importance of phospholipase-A_2 in prostaglandin biosynthesis. Biochem. Pharmacol. **25**, 285–291 (1976)

16 Punzi, L., Schiavon, F., Cavasin, F., Ramonda, R., Gambari, P.F. and Todesco, S.: The influence of intra-articular hyaluronic acid on PGE_2 and cAMP of synovial fluid. Clin. Exp. Rheumatol. **7**, 247–250 (1989)