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Suppressive Effects of Hyaluronic Acid on Elastase Release from Rat Peritoneal Leucocytes

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Abstract—Effects of hyaluronic acid on the release of elastase from rat peritoneal leucocytes were studied by measuring the leucocyte elastase activity using a synthetic peptide substrate. Leucocyte elastase release was induced by opsonized zymosan, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine combined with cytochalasin B. Calcium ionophore A23187 potentiated the action of TPA on leucocyte elastase release, whereas hyaluronic acid inhibited leucocyte elastase release regardless of the method of stimulation. Inhibitory effects of hyaluronic acid were dependent on its concentration and molecular weight. Hyaluronic acid of the highest molecular weight ($2{\cdot}0 \times 10^6$) indicated a potent inhibitory effect on elastase release. Our present findings suggest that hyaluronic acid may elicit an anti-inflammatory effect by inhibiting leucocyte elastase-dependent pathological processes.

Proteolytic degradation of articular cartilage, a common feature of joint destruction in rheumatoid arthritis and osteoarthritis, may involve the action of enzymes derived from synovial cells (Murphy et al 1981), chondrocytes (Nojima et al 1986) and neutrophils (Sandy et al 1981). Polymorphonuclear leucocytes have been indicated as a major component of the cellular infiltrate in joint cavities during the early stage of inflammation (Santer et al 1983). The cells inflict cartilage damage, which is mediated by certain proteolytic enzymes secreted from their own granule fractions (Janoff et al 1976). The major proteolytic enzymes are leucocyte elastase (Barrett 1981; Adeyemi et al 1986), cathepsin G (Velvart & Fehr 1987) and collagenase (Murphy et al 1980). Of these proteolytic enzymes, leucocyte elastase has been reported to degrade most of the matrix proteins such as collagen (Gadher et al 1988), fibronectin (McDonald & Kelley 1980) and proteoglycan (Schalkwijk et al 1988). Degradation of these proteins significantly reduces the mechanical strength of cartilage (Bader et al 1981; Baici et al 1982). We therefore believe that inhibition of leucocyte elastase release promises therapeutic benefits for patients suffering from inflammatory arthritis.

Hyaluronic acid, a linear polysaccharide composed of alternating units of *N*-acetyl-D-glucosamine and D-glucuronic acid, is a major component of the joint synovial fluid (Laurent 1987). In inflammatory arthritis, the synovial hyaluronic acid is reduced in concentration and mol. wt (Jessar 1972; Dahl et al 1985). It has been demonstrated that hyaluronic acid modulates leucocyte functions such as phagocytosis (Brandt 1974; Forrester & Balazs 1980), adherence (Forrester & Lackie 1981) and chemotaxis (Forrester & Wilkinson 1981) in a dose- and mol. wt-dependent manner. Hitherto, effects of hyaluronic acid on leucocyte elastase release induced by various stimuli have not yet been investigated. In this study, we examined the effects of hyaluronic acid on leucocyte elastase release from rat peritoneal leucocytes in-vitro.

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Materials and Methods

Chemicals

N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), calcium ionophore A23187, cytochalasin B (Sigma Chemical Co., USA), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Nacalai Tesque, Japan) and zymosan (Schwarz/Mann Biotech, USA) were used as the stimuli to induce leucocyte elastase release from leucocytes. Opsonized zymosan was prepared by incubating 50 mg zymosan with 10 mL foetal bovine serum (Hyclone Co. Ltd, USA) for 30 min at 37°C, followed by washing twice with physiological saline. Diisopropyl fluorophosphate (DFP), methoxysuccinyl-alanine-alanine-proline-valine-chloromethyl ketone (MAAPV-CMK), superoxide dismutase (SOD, 3570 units mg^{-1}), α_1-protease inhibitor (α_1-PI) (Sigma Chemical Co., USA), *o*-phenanthroline (Nacalai Tesque, Japan), catalase (65 000 units mg^{-1}) (Boehringer Mannheim, Germany), *p*-chloromercuribenzoic acid (PCMB) and ethylenediaminetetraacetic acid (EDTA) (Wako Pure Chemical Co., Japan) were employed as protease inhibitors. Bovine serum albumin (Sigma Chemical Co., USA), penicillin and streptomycin (Meiji Seika Pharm. Co., Japan) were added to RPMI1640 (ICN Biomedicals Inc., Japan) throughout the culture period at 1 mg mL^{-1}, 100 units mL^{-1} and 100 $\mu g\ mL^{-1}$, respectively. Stock solutions of FMLP (10 mM), cytochalasin B (2 mg mL^{-1}), TPA (2 mg mL^{-1}) and A23187 (2 mM) were dissolved in dimethylsulphoxide (DMSO) and stored at −20°C before use.

Hyaluronic acid

Endotoxin-free hyaluronic acid samples (gifts from Shiseido Pharmaceutical Research Laboratories, Japan) of various mol. wts (viscosity-average mol. wt $2{\cdot}0 \times 10^6$, $9{\cdot}5 \times 10^5$, $2{\cdot}8 \times 10^5$) containing $<0{\cdot}1\%$ protein were previously purified from culture broths of *Streptococcus zooepidemicus* (Akasaka et al 1988).

Preparation of leucocytes

Male Wistar rats (Kari Co., Japan), 250–300 g, were injected intraperitoneally with 7 mL 3% sodium caseinate (Wako

Pure Chemical Co., Japan) dissolved in isotonic saline. The animals were killed after 16 h and their abdomens shaved. After surgically exposing the abdominal cavity along the midline, the exudate was removed from the peritoneal cavity by lavaging with 6 × 5 mL portions of ice-cold phosphate-buffered saline. The exudate was collected with siliconized glass pipettes and centrifuged at 300 *g* for 5 min at 4°C before discarding the supernatant fluid. Cells were then allowed to stand for 30 min at room temperature in 20 mL of 2·47% gelatin-containing saline to precipitate tissue debris. After centrifugation at 300 *g* for 5 min at room temperature, the cells were collected. Remaining erythrocytes were hypotonically lysed with chilled 20 mL 0·2% NaCl by mixing gently for 30 s before adding an equal volume of 1·6% NaCl to the cell suspension. Cells were washed twice with treated RPMI1640 (see Chemicals) and resuspended in the same medium. Cells were counted in a Neubauer chamber and viability was assessed by trypan blue exclusion. Cell viability was routinely greater than 82%. Based on Percol gradient centrifugation (Kudo et al 1985), more than 80% of the cells are neutrophils. Leucocytes thus prepared were used in these experiments.

Leucocyte elastase assay

Samples of 100 μL (total volume) containing each of the respective stimulating compounds in the absence or presence of hyaluronic acid with different mol. wts were dispersed in round-bottom 96-well microplates (Corning, USA) followed by incubation at 37°C for 10 min. Leucocytes suspended in the (previously described) treated RPMI1640 (volume equivalent to the sample) were dispersed in the microplate (final concentration 2×10^7 cells mL^{-1}) and gently agitated in a horizontal direction before incubating the mixtures at 37°C for up to 360 min. Hyaluronic acid with different mol. wts was prepared to final concentrations of 0·5, 1·0 and 2·0 mg mL^{-1}. Final concentrations of stimuli such as opsonized zymosan, FMLP, cytochalasin B, TPA, and calcium ionophore A23187 were 1·5 mg mL^{-1}, 1 μM, 5 μg mL^{-1}, 250 ng mL^{-1} and 1 μM, respectively. At the end of incubation, the cells were isolated from the medium by centrifugation at 1500 *g* for 10 min at 4°C.

As the positive control for leucocyte elastase release, hyaluronic acid-free reaction mixtures with each of the respective stimuli were used. As the negative control, a reaction mixture devoid of both stimuli and hyaluronic acid was employed.

Briefly, leucocyte elastase was assayed (Visser & Blout 1972; Starkey & Barrett 1976) by determining the enzyme activity in the supernatant fluid spectrophotometrically using a synthetic peptide substrate *N-t*-BOC-L-alanine-*p*-nitrophenyl ester (Sigma Chemical Co., USA). A sample (50 μL) of supernatant fluid was added to 140 μL 0·1 M Tris-HCl (pH 7·8) in a flat-bottom 96-well microplate (Corning, USA) before incubating the mixture at 25°C for 10 min. A volume of 10 μL of the substrate (4 mM, in DMSO) was added to the mixture. Quantitative release of *p*-nitrophenol from the substrate was measured by the increase in absorbance at 405 nm (*p*-nitrophenol having an extinction coefficient at this wavelength of $1{\cdot}6 \times 10^4$ M^{-1} cm^{-1}). A unit of leucocyte elastase activity was defined as 1 nmol *p*-nitrophenol released min^{-1} under the above conditions.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test.

Inhibition studies

Leucocytes (2×10^7 cells mL^{-1}) were treated with 1 μM FMLP combined with 5 μg mL^{-1} cytochalasin B (FMLP/cytochalasin B) for 15 min at 37°C. After centrifugation at 1500 *g* for 10 min at 4°C, the supernatant was incubated with the following protease inhibitors at 37°C for 30 min (final concentrations are indicated below within parentheses); DFP (250 μM), MAAPV-CMK (50 μM), α_1-PI (125 μg mL^{-1}), PCMB (25 μM), SOD (25 units mL^{-1}), catalase (750 units mL^{-1}), *o*-phenanthroline (1·25 mM), and EDTA (1·25 mM). The enzyme (elastase) activity of the mixture was assayed using the similar substrate *N-t*-BOC-L-alanine-*p*-nitrophenyl ester (see above).

Effects of hyaluronic acid on secreted leucocyte elastase activity

To determine if hyaluronic acid has a direct effect on the enzyme activity, leucocytes (2×10^7 cells mL^{-1}) were stimulated with FMLP/cytochalasin B for 15 min at 37°C. After centrifugation at 1500 *g* for 10 min at 4°C, supernatant was incubated with hyaluronic acid of different mol. wts ($2{\cdot}0 \times 10^6$, $9{\cdot}5 \times 10^5$, $2{\cdot}8 \times 10^5$) at 37°C for 30 min (final concentration was 2·0 mg mL^{-1}). The mixture was assayed for enzyme activity (see above).

Results

Identification of peptidase activity secreted from leucocytes

Table 1 shows the effects of protease inhibitors on the peptidase activity of elastase secreted from leucocytes. DFP, α_1-PI and MAAPV-CMK markedly inhibited the enzyme activity. However, EDTA, *o*-phenanthroline, PCMB, SOD and catalase did not indicate any significant suppressive activities.

Table 1. Effects of protease inhibitors on elastase activity of rat peritoneal leucocytes.

Inhibitor	Concn	Activity (units mL^{-1})	Inhibition (%)
None	—	28·02 ± 0·68	—
DFP	250 μM	1·70 ± 0·17	93·9
MAAPV-CMK	50 μM	3·56 ± 0·11	87·3
α_1-PI	125 μg mL^{-1}	3·95 ± 1·31	85·9
PCMB	25 μM	28·92 ± 0·50	NE
o-Phenanthroline	1·25 mM	28·94 ± 0·43	NE
EDTA	1·25 mM	30·47 ± 0·45	NE
SOD	25 units mL^{-1}	28·52 ± 0·26	NE
Catalase	750 units mL^{-1}	28·38 ± 0·61	NE

Values are expressed as the means ± s.d. of eight experiments. NE denotes insignificant or no effect. Protease inhibitors used were diisopropyl fluorophosphate (DFP), methoxysuccinyl-alanine-alanine-proline-valine-chloromethyl ketone (MAAPV-CMK), α_1-protease inhibitor (α_1-PI), *p*-chloromercuribenzoic acid (PCMB), ethylenediamine tetraacetic acid (EDTA), superoxide dismutase (SOD) and other tabulated agents.

Direct effects of hyaluronic acid on peptidase activity secreted from leucocytes

The leucocyte elastase activity in a conditioned medium of leucocytes stimulated with FMLP/cytochalasin B was $29{\cdot}62 \pm 1{\cdot}03$ units mL^{-1}. When hyaluronic acids of different mol. wts, $2{\cdot}0 \times 10^6$, $9{\cdot}5 \times 10^5$, and $2{\cdot}8 \times 10^5$ were added to the medium separately, leucocyte elastase activities were $31{\cdot}00 \pm 1{\cdot}34$, $29{\cdot}93 \pm 1{\cdot}15$ and $31{\cdot}22 \pm 1{\cdot}13$ units mL^{-1}, respectively. Hyaluronic acid itself did not influence the leucocyte elastase activity in this assay system.

Effects of hyaluronic acid on leucocyte elastase release from leucocytes

The kinetics of leucocyte elastase release from leucocytes were examined with various stimuli (Table 2). Increase in leucocyte elastase release was time-dependent when leucocytes were stimulated by opsonized zymosan or TPA with or without A23187. The effects of opsonized zymosan and TPA on elastase release began to plateau at 180 min. The combination of A23187 and TPA increased the release within 15 min compared with TPA alone. Neither FMLP nor cytochalasin B indicated any significant stimulatory effects when used alone. However, a combination of both agents (FMLP/cytochalasin B) manifested a marked release of leucocyte elastase within 15 min of incubation. The basal release of leucocyte elastase in non-treated leucocytes did not show any increases for up to 360 min.

When leucocytes were incubated at 37°C for 180 min with hyaluronic acid of different mol. wts at the concentration range of 0·5–2·0 mg mL^{-1} in the presence of either opsonized zymosan or TPA, leucocyte elastase release was inhibited by hyaluronic acid in a dose- and mol. wt-dependent manner (Table 3). Similar effects of hyaluronic acid on leucocyte elastase release were observed when leucocytes were treated with TPA plus A23187 or FMLP/cytochalasin B for 15 min at 37°C. In all these cases, hyaluronic acid with the highest mol. wt ($2{\cdot}0 \times 10^6$) elicited the most potent inhibition compared with effects of the same polymer of lower mol. wts ($9{\cdot}5 \times 10^5$, $2{\cdot}8 \times 10^5$).

Discussion

Extracellular release of leucocyte elastase from leucocytes can occur by either incomplete closure of the phagosome

Table 2. Effects of various stimuli on leucocyte elastase release from rat peritoneal leucocytes.

	Leucocyte elastase release (units mL^{-1}) Incubation time (min)				
Stimulus	15	30	60	180	360
Control	1·20 ± 0·04	1·23 ± 0·21	1·81 ± 0·13	1·71 ± 0·18	1·92 ± 0·75
Opsonized zymosan (1·5 mg mL^{-1})	1·80 ± 0·43	3·65 ± 0·30***	5·79 ± 0·16***	14·44 ± 0·97***	15·50 ± 0·40***
Control	1·91 ± 0·12	1·85 ± 0·15	1·88 ± 0·27	2·11 ± 0·04	2·58 ± 0·17
TPA (250 ng mL^{-1})	3·79 ± 0·13***	4·85 ± 0·22***	7·30 ± 0·21***	21·18 ± 0·41***	27·00 ± 0·88***
A23187 (1 μM)	1·96 ± 0·09	2·32 ± 0·11**	2·58 ± 0·15**	2·83 ± 0·10***	3·97 ± 0·36***
TPA (250 ng mL^{-1}) + A23187 (1 μM)	15·20 ± 0·44***	17·80 ± 0·75***	21·88 ± 1·29***	34·38 ± 1·11***	34·56 ± 1·66***
Control	2·12 ± 0·10	2·10 ± 0·21	2·12 ± 0·20	2·07 ± 0·09	2·70 ± 0·04
FMLP (1 μM)	4·63 ± 0·07***	4·62 ± 0·07***	4·73 ± 0·25***	4·46 ± 0·16***	4·47 ± 0·16***
Cytochalasin B (5 μg mL^{-1})	2·49 ± 0·20*	2·74 ± 0·08**	2·76 ± 0·11**	2·60 ± 0·13***	3·33 ± 0·35*
FMLP (1 μM) + cytochalasin B (5 μg mL^{-1})	32·88 ± 0·65***	31·69 ± 1·88***	33·83 ± 0·35***	35·30 ± 1·97***	32·22 ± 0·71***

Values are expressed as the means ± s.d. of four individual wells of a microplate culture. Significant differences of $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$ compared with the respective controls. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA) with and without the calcium ionophore A23187, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) with and without cytochalasin B, and opsonized zymosan were the stimuli used.

Table 3. Effects of different molecular weights and concentrations of hyaluronic acid on leucocyte elastase release from rat peritoneal leucocytes induced by various stimuli.

Hyaluronic acid mol. wt	Hyaluronic acid (mg mL^{-1})	Leucocyte elastase release (units mL^{-1}) Opsonized zymosan[a]	TPA[a]	TPA + A23187[b]	FMLP + cytochalasin B[b]
—	—	14·44 ± 0·97	21·18 ± 0·41	15·20 ± 0·44	32·88 ± 0·65
	0·5	11·83 ± 0·63**	19·20 ± 0·72**	13·50 ± 0·70**	32·06 ± 0·76
$2{\cdot}8 \times 10^5$	1·0	10·67 ± 0·90**	19·93 ± 0·37**	14·77 ± 1·49	31·04 ± 2·11
	2·0	8·09 ± 0·93***###	22·40 ± 0·58###	15·30 ± 1·17###	27·71 ± 1·81***###
	0·5	9·74 ± 0·27***	18·69 ± 0·56***	13·16 ± 0·72**	27·71 ± 1·54***
$9{\cdot}5 \times 10^5$	1·0	7·42 ± 0·41***	15·89 ± 1·41***	12·50 ± 1·94	27·02 ± 0·84***
	2·0	6·24 ± 0·57***#	17·09 ± 0·98***###	9·69 ± 1·10######	21·98 ± 0·35***###
	0·5	6·17 ± 0·22***	13·56 ± 0·91***	8·32 ± 0·22***	26·73 ± 1·72***
$2{\cdot}0 \times 10^6$	1·0	4·93 ± 0·61***	12·33 ± 0·50***	5·54 ± 0·85***	18·86 ± 0·86***
	2·0	4·64 ± 1·11***	10·01 ± 1·37***	3·69 ± 0·61***	14·64 ± 2·34***

Incubation intervals were 180 min[a] and 15 min[b]. Values are expressed as the means ± s.d. of four individual wells of a microplate culture. Significant differences of $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$ were compared with the value in the absence of hyaluronic acid (unpaired Student's *t*-test). Statistical differences where hyaluronic acid treatments with the highest mol. wt ($2{\cdot}0 \times 10^6$) were compared with those of lower mol. wts ($9{\cdot}5 \times 10^5$, $2{\cdot}8 \times 10^5$) at 2·0 mg mL^{-1} are indicated as follows: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

during phagocytosis or direct active secretion (Stein et al 1985). Such a release can be stimulated by opsonized zymosan (Schettler et al 1991), TPA with or without A23187 (Wright et al 1977; Kajikawa et al 1983), and FMLP/cytochalasin B (Niessen et al 1991). Addition of similar stimuli to leucocytes in our experiments induced leucocyte elastase release, and A23187 enhanced the effect of TPA-induced release (Table 2). This observation is consistent with a previous demonstration (Kajikawa et al 1983). The enzyme activity of elastate secreted from leucocytes was inhibited by DFP, α_1-PI (serine-protease inhibitors), and MAAPV-CMK (elastase-specific inhibitor), indicating that the enzymatic cleavage of a peptide substrate used in this study was mainly due to serine-proteases, especially elastase (Table 1).

Hyaluronic acid indiscriminately inhibited leucocyte elastase release induced by opsonized zymosan, TPA with or without A23187, and FMLP/cytochalasin B (Table 3). Although the underlying mechanism cannot be clarified in our present experiment, the suppressive effects of hyaluronic acid on opsonized zymosan-induced leucocyte elastase release may be due to inhibition of phagocytosis attributed to steric hindrance of the continuous polymeric network of this polymer. The possibility of steric hindrance was advocated by Forrester & Balazs (1980), who demonstrated that phagocytosis induced by polystyrene latex spheres was inhibited by hyaluronic acid in a dose- and mol. wt-dependent manner. Moreover, Forrester & Wilkinson (1981) have shown that hyaluronic acid inhibits the binding of the chemotactic factor to the leucocyte surface. Although it is not clear from our experiments, the accessibility of not only particulates like zymosan, but also soluble stimuli to the leucocyte surface was limited by the steric hindrance from hyaluronic acid. Besides, the mobility of soluble and insoluble stimuli may be suppressed in a dose- and mol. wt-dependent manner due to increased viscosity of the medium by addition of hyaluronic acid.

Leucocyte elastase has been identified as one of the factors causing enzymatic cartilage destruction in inflammatory arthritis (Velvart & Fehr 1987). It has been demonstrated that leucocyte elastase is inactivated by an antiprotease, α_1-PI, present in the synovial fluid (Kleesiek et al 1982). However, Schalkwijk et al (1987) have illustrated that leucocyte elastase shed by leucocytes in close contact with the cartilage escapes from complexing with α_1-PI and consequently inflicts degradation of the cartilage matrix in the presence of antiprotease. From our results, hyaluronic acid could be useful in such a case, as the release of leucocyte elastase from leucocytes was directly inhibited by this polymer. In fact, intra-articular injections of hyaluronic acid have been used effectively in the treatment of various arthropathies (Punzi et al 1988; Leardini et al 1991). From this in-vitro study, our observations suggest that hyaluronic acid may elicit an anti-inflammatory effect by inhibiting leucocyte elastase-dependent pathological processes.

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