Effect of Hyaluronan Oligosaccharides on the Expression of Heat Shock Protein 72*

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We have previously shown that intraarticular treatment with a hyaluronan (HA) preparation (840 kDa), HA84, up-regulates heat shock protein 72 (Hsp72) expression and suppresses degeneration of synovial cells in an arthritis model. In that study, the HA84 administered was degraded into HA oligosaccharides in the synovial tissue, suggesting that HA84 or degradation products of HA may up-regulate Hsp72 expression. Thus, in the present study, we examined the effects of HA of various molecular sizes on Hsp72 expression and cell death in stressed cells. Western blotting analysis showed that treatment of K562 cells with HA tetrasaccharides up-regulated Hsp72 expression after exposure to hyperthermia. On the other hand, treatment of the cells with HA of other sizes (di-, hexa-, deca-, dodecasaccharides), HA84, or tetrasaccharides of keratan sulfate did not elicit any change in expression of the Hsp72 protein. Treatment of the cells with tetrasaccharides of HA up-regulated not only expression of the Hsp72 protein but also Hsp72 mRNA expression and enhanced activation of HSF1, a transcription factor controlling Hsp72 expression, after exposure to hyperthermia. Because the level of Hsp72 protein was not affected by tetrasaccharides of HA when the K562 cells were kept at 37 °C without any stress, it is evident that tetrasaccharides of HA did not act as a stress factor. In addition, tetrasaccharides of HA suppressed cell death in the case of K562 cells exposed to hyperthermia and of PC12 cells under serum deprivation. These results suggest that a certain size of oligosaccharides, i.e. the tetrasaccharides of HA, up-regulates Hsp72 expression by enhancing the activation of HSF1 under stress conditions and suppresses cell death.

Heat shock proteins (Hsps)* are induced to suppress cell damage when cells are exposed to environmental insult (1, 2).

Hsp70 suppresses apoptosis by preventing processing of caspase 3 (3, 4). It is well known that brief ischemia induces tolerance to subsequent ischemia in hippocampal neurons as a result of the induction of Hsp70 expression (5). We have previously shown that intraarticular treatment with hyaluronan (HA) preparation (840 kDa), HA84, suppresses degeneration of synovial cells in a canine arthritis model and up-regulates Hsp72 expression (6, 7). In that study, we also injected fluorescent-labeled HA84 in synovial tissues and found that some labeled HA particles could not be detected by means of an HA-binding protein that binds specifically to HA molecules larger than decasaccharides (7). These observations suggested that HA oligosaccharides formed through degradation of HA84 in the tissue might suppress cell damage by up-regulating Hsp72 expression. In the present study, we prepared HA oligosaccharides of various molecular sizes and treated cultured cells with them under stress conditions in an effort to understand the appropriate size of HA oligosaccharides required to up-regulate Hsp72 expression or to suppress cell death. Effects of HA molecules on Hsp72 were investigated by examining both Hsp72 protein levels and Hsp72 mRNA levels, and the activation of heat shock factor 1 (HSF1), a transcription factor controlling Hsps expression, in K562 cells exposed to the stress of hyperthermia. HSF1 is known to be transferred to the nucleus from the cytoplasm, and it binds to a heat shock element (HSE) in the DNA (8, 9). Moreover, HSF1 is phosphorylated, and its molecular weight thereby increases when activated soon after heat shock treatment (9). In addition to Hsp72 expression and HSF1 activation, the effects of HA molecules on cell death were investigated using K562 cells exposed to hyperthermia and PC12 cells under conditions of serum deprivation in the present study.

It has been reported that low molecular weight fragments of HA induce angiogenesis (10) and/or induce the expression of genes involved in the inflammatory response, e.g. genes for chemokines and cytokines (11). In addition to these activities, we show here a novel activity of HA oligosaccharides, the acceleration of Hsp72 expression through activation of HSF1 under stress conditions and its suppressive effect on cell death.

EXPERIMENTAL PROCEDURES

Materials—HA, chondroitin sulfate C type, keratan sulfate, chondroitinase ACI, and chondroitinase ACII were obtained from Seikagaku Corporation (Tokyo, Japan). Other reagents and chemicals were obtained from commercial sources.

Preparation of Oligosaccharides—Glycosaminoglycan oligosaccharides were prepared by the modified method of Inoue and Nagasawa (12). Saturated HA tetra-(HA4), hexa-(HA6), octa-(HA8), deca-(HA10), and dodeca-(HA12) saccharides were prepared from the degradation products generated by treatment of HA with testicular hyaluronidase (Biozyme Laboratory, Gwent, UK) in 0.1 m sodium phosphate buffer, pH 5.3, containing 150 mM NaCl at 37 °C. Saturated saccharides of HA (HA84) were prepared from the degradation products generated by treat-
ment of HA with dimethyl sulfoxide containing 10% of 0.1 M HCl for 16 h at 95 °C. Unsatuated HA di- (ΔHA2), tetra- (ΔHA4), and hexa-
(ΔHA6) saccharides were generated by enzymatic digestion of HA with chondroitinase ACI in 0.1 M acetate buffer, pH 6.0, at 30 °C and sepa-
rated by the same chromatography method as mentioned above. Chon-
droitin tetrasaccharides (Ch6) were generated by treatment of chon-
droitin sulfate C type with the dimethyl sulfoxide containing HCl.
Chondroitin sulfate C type tetrasaccharides (Ch6S) were prepared by testicular hyaluronidase (Biozyme) digestion of chondroitin sulfate C type. Keratan sulfate oligosaccharides, Gal(SO3)2-GlcNAc(SO3)2-L4 and Gal(SO3)2-GlcNAc(SO3)2-Gal(SO3)2-GlcNAc(SO3)2 (L4L4), were prepared from a keratanase II (Seikagaku Corporation) digest of keratan sulfate (shark fin, Seikagaku Corporation) through sequential steps of gel
filtration and anion exchange adsorption column chromatography. The degraded oligosaccharides were divided into fractions of each size by sequential steps of anion exchange chromatography.

All oligosaccharides were checked by Limulus amebocyte lysate as-
says using Toxicolor LS Set (Seikagaku Corporation). HA contains 0.03 pg/mg endotoxins, and similar results were obtained in other oligosaccharides.

Sizes and purity of HA oligosaccharides were determined by HPLC,
fluorophore-assisted carbohydrate electrophoresis, and mass spectromet-
ry. Assignments of 1H and 13C NMR spectroscopy and of element
analysis were obtained for each HA oligosaccharide. Oligosaccharides
of chondroitin, chondroitin sulfate C type, and keratan sulfate were
analyzed by HPLC and/or capillary electrophoresis (data not shown). In
addition, keratan sulfate oligosaccharides have been analyzed by mass
spectrometry (13).

Culture of K562 Cells for Detection of Hsp72 and HSF1—Western
blotting was done with K562 cells that were incubated in the presence
of 0, 1, 10, or 100 ng/ml HA or HA4, HA6, HA8, HA84, or L4L4 at 43 °C for 20 min followed by further incubation at 37 °C for 2 h. K562
cells incubated at 37 °C for 2 h and 20 min without any treatment were
used as the “no heat shock” normal control. Moreover, K562 cells
incubated in the presence of ΔHA4 at 37 °C for 2 h and 20 min were
examined by Western blotting to investigate whether Hsp72 expression
is induced by ΔHA4 under non-stress conditions. For the culture of
K562 protein expression by flow cytometry, K562 cells were incubated
in the presence or absence of 1 ng/ml ΔHA4 at 43 °C for 20 min followed
by further incubation at 37 °C for 2 or 4 h.

To detect Hsp72 mRNA expression by Northern blotting, K562 cells
were incubated in the presence or absence of 1 ng/ml ΔHA4 at 43 °C for
20 min with or without further incubation at 37 °C for 30 min, 1 h, or
2 h. To evaluate HSF1 activation by Western blotting, K562 cells were
incubated with 0, 1, 10, or 100 ng/ml ΔHA6, HA6, HA8, HA84, or HA84.
The K562 cells were stressed at 42 or 43 °C for 20 min. K562 cells

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presence or absence of 1 ng/ml ΔHA4 incubated at 37 °C for 2 h and 20 min (left column), at 43 °C for 20 min (center column), and at 43 °C for 20 min followed by incubation at 37 °C for 2 h (right column). The treatment with ΔHA4 up-regulated and down-regulated the level of nuclear HSF1 in the K562 cells immediately and 2 h after the exposure to hyperthermia, respectively. **, $p < 0.01$; *, $p < 0.05$ (Student’s t test).

Antibodies Used in Immunostaining for Hsp72 and HSF1—For the detection of Hsp72, monoclonal anti-Hsp72 antibody (Amersham Biosciences) was used as the first antibody, and horseradish peroxidase- or FITC-conjugated goat anti-mouse IgG (Jackson Laboratory, West Grove, PA) was used as the second antibody. For the detection of HSF1, rabbit anti-HSF1 polyclonal antibody (Stressgene, Victoria, British Columbia, Canada) was used as the first antibody, and horseradish peroxidase- or FITC-conjugated goat anti-rabbit IgG (Jackson Laboratory) was used as the second antibody.

Western Blotting Analysis of Hsp72 and HSF1—K562 cells were surveyed by antibodies against Hsp72 or HSF1 described above. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane. To reduce nonspecific interactions, the membrane was blocked by incubation with 0.3% skim milk in Tris-buffered saline (TBS) at 37 °C for 1 h. Following incubation with the first antibodies at 4 °C overnight, the membrane was washed three times with 0.1% Tween 20 in TBS and incubated with the secondary antibodies described above at 37 °C for 1 h. Color development was performed with 0.05% diaminobenzidine solution in TBS containing 0.01% H$_2$O$_2$.

Northern Blotting Analysis of Hsp72—Total RNA was prepared from control K562 cells and each culture of ΔHA4-treated cells.
Effects of HA Oligosaccharides on Hsp72 Expression—Hsp72 protein expression was detected even in non-treated K562 cells not exposed to hyperthermia (Fig. 1, A and B). The results showed that treatment of the K562 cells with HA4 up-regulated Hsp72 expression 2 h after exposure to hyperthermia (Fig. 1A). The same result was obtained in the case of HA4 treated cells (data not shown). The Hsp72 protein level was not changed by treatment with HA2, HA6, HA84, L4L4 (Fig. 1A), HA4 (data not shown), or HA6 (data not shown). The expression of Hsp72 in the case of K562 cells exposed to hyperthermia. Hsp72 expression was not affected by HA4 treatment in the case of cells not exposed to hyperthermia (Fig. 1B). Flow cytometry showed that treatment with 1 ng/ml ΔHA4 up-regulated and down-regulated HA4 expression 2 and 4 h after exposure to hyperthermia, respec-

FIG. 9. HPLC analyses of the digestion product of HA4. A, intact HA4. B, HA4 treated with boiled chondroitinase ACII. C, HA4 treated with chondroitinase ACII. HA4 was degraded into HA2 plus ΔHA4. D, mixture of HA oligosaccharides before isolating into each size of HA oligosaccharides.

FIG. 10. Effects of digestion product of HA4 on cell death on the survival rates of PC12 cells under conditions of serum deprivation. The apoptosis of PC12 cells was not prevented by the treatment with HA4 digestion, that is HA4 treated with chondroitinase ACII, i.e. HA4 plus ΔHA4. HA4 boil, HA4 treated with boiled chondroitinase ACII. NGF, nerve growth factor.

at 4 °C followed by FITC-conjugated goat anti-rabbit IgG (1:100) for 1 h at room temperature.

After immunostaining for HSF1, Annexin V staining, the cells were analyzed by flow cytometry (FACScan, Becton-Dickinson) using an instrument equipped with a 15-mA ion laser and with filter settings for FITC. Ten thousand cells from each sample were computed in list mode, and data analysis was done with a commercial software program (CELLQuest, Becton-Dickinson). Analysis gates were set on leukocyte, according to forward and side scatter properties.

Detection of Cell Death—To evaluate the effect of ΔHA4 on cell death induced by hyperthermia, K562 cells were incubated with 1 ng/ml ΔHA4 for 20 min at 43 °C followed by incubation for 2 or 4 h at 37 °C. K562 cells incubated for 4 h at 37 °C without any treatment were used as a no heat shock normal control. Then these cells were incubated with Annexin V (Bender Medsystems, Vienna, Austria), which binds to phosphatidylserine exposed on the outer surface of the cell membrane of dead cells, just after cell culture as described above. Cell death was analyzed by flow cytometry (FACScan, Becton-Dickinson).

It has been reported that serum deprivation induces apoptosis in PC12 cells (15). PC12 cells were cultured under conditions of serum deprivation in the presence of HA oligosaccharides, HA84, L4, L4L4, Ch08, and Ch58 at 100 ng/ml. The cell death assay was done by the trypan blue exclusion method, 24 h after the start of culture. The survival rate of cells cultured in the absence of serum but in the presence of 100 ng/ml nerve growth factor was taken to be 100%.

Detection of Hsp72—One mg of HA4 was digested with 0.01 units of chondroitinase ACII in 0.1 M sodium acetate buffer, pH 6.0, at 37 °C for 20 h. The reaction was stopped by boiling 3 min. Boiled chondroitin ACII was added to HA4 as a negative control. These samples were ultracentrifugated with Centrifuge Plus-20 10K (Millipore Co., Bedford, MA) to remove endotoxins and separated by anion exchange chromatography. The separated oligosaccharides were identified by HPLC. These products of HA4 were applied in the cell death assay using PC12 cells described above.

HPLC Procedures—Normal phase HPLC was performed using YMC-pack NH2 (4.0 × 250 mm, YM, Kyoto, Japan). Oligosaccharides were eluted with a linear gradient of 2–100% 0.8 M NaH2PO4 at a flow rate of 1.0 ml/min at 40 °C. Absorbance was monitored at 210 nm.

Statistical Analysis—Comparisons were analyzed by using the unpaired Student’s t test or Dunnet multiple comparison test.

RESULTS

Effects of HA Oligosaccharides on Hsp72 Expression—Hsp72 protein expression was detected even in non-treated K562 cells not exposed to hyperthermia (Fig. 1, A and B). The results showed that treatment of the K562 cells with ΔHA4 up-regulated Hsp72 expression 2 h after exposure to hyperthermia (Fig. 1A). The same result was obtained in the case of HA4 treated cells (data not shown). The Hsp72 protein level was not changed by treatment with HA2, HA6, HA84, L4L4 (Fig. 1A), HA4 (data not shown), or HA6 (data not shown). The expression of Hsp72 in the case of K562 cells exposed to hyperthermia. Hsp72 expression was not affected by ΔHA4 treatment in the case of cells not exposed to hyperthermia (Fig. 1B). Flow cytometry showed that treatment with 1 ng/ml ΔHA4 up-regulated and down-regulated HA4 expression 2 and 4 h after exposure to hyperthermia, respec-
The level of HSF1 retained in the nucleus was even more immediately after and 2 h after exposure to hyperthermia (Fig. 5). The level of non-phosphorylated HSF1 (Fig. 4B). Activation of HSF1 was little influenced by HA4 (Fig. 4B) or HA84 (data not shown). Treatment with HA84 up-regulated the level of non-phosphorylated HSF1 but not of phosphorylated HSF1 (Fig. 4B).

The treatment with HA4 did not alter the retention of HSF1 in the nucleus of cells cultured at 37 °C (Fig. 5). The level of HSF1 retained in the nucleus was found to be elevated immediately after and 2 h after exposure to hyperthermia (Fig. 5). In addition, HA4 increased the levels of both phosphorylated and non-phosphorylated HSF1 when the cells were exposed to hyperthermia at 43 °C (Fig. 4B). In the case of PC12 cells, the treatment did not alter the size of CD44-positive granules (Fig. 4B).

The treatment with HA4 did not alter the retention of HSF1 in the nucleus of cells cultured at 37 °C (Fig. 5). The level of HSF1 retained in the nucleus was found to be elevated immediately after and 2 h after exposure to hyperthermia (Fig. 5). The level of HSF1 retained in the nucleus was even more elevated in the presence of HA4 immediately after exposure to hyperthermia (Fig. 5). However, in cells treated with HA4, the level of HSF1 retained in the nucleus was slightly diminished 2 h after exposure to hyperthermia (Fig. 5).

Immunodeposits of HSF1 were detected in HA4-treated (Fig. 6B) as well as non-treated (Fig. 6A) K562 cells not exposed to hyperthermia. Following exposure to hyperthermia, immunodeposits of HSF1 were detected as aggregated granular structures in the K562 cells incubated in the absence of HA4 (Fig. 6, C and E). The HSF1-positive granules in the cells incubated at 37 °C for a further 2 h (Fig. 6E) were slightly larger in size than those observed immediately after exposure to hyperthermia (Fig. 6C). The immunodeposits of HSF1 in the K562 cells incubated in the presence of HA4 (Fig. 6, D and F) were finer than those in the cells incubated in the absence of HA4 (Fig. 6, C and E) after exposure to hyperthermia.

Effects of Tetrascaricases of HA on Cell Death—Treatment with HA4 suppressed cell death in the case of K562 cells as determined 2 and 4 h after exposure to hyperthermia (Fig. 7). The treatment with tetrascaricases of HA up-regulates the activation of HSF1 to enhance the level of Hsp72 expression and suppression of cell death.

The treatment with tetrasaccharides of HA up-regulates the activation of HSF1 to enhance the level of Hsp72 expression and suppression of cell death. The treatment with tetrasaccharides of HA up-regulates the activation of HSF1 to enhance the level of Hsp72 expression and suppression of cell death.

Fig. 11. Proposed mechanism of the effect of HA4 on Hsp72 expression and cell death. The difference in thickness of arrows between A and B indicates intensities of activation of HSF1, transcription and translation of Hsp72, and suppression of cell death. The treatment with tetrasaccharides of HA up-regulates the activation of HSF1 to enhance the level of Hsp72 expression and suppression of cell death.

Fig. 12. Cell biological activities of hyaluronan depending on its molecular sizes. See “Discussion” for details.
ment does not induce Hsp72 expression under non-stress conditions but up-regulates it under stress conditions.

Sistonen et al. have shown that HSF1 is transferred from the cytoplasm to the nuclei in K562 cells after exposure to hyperthermia as demonstrated by a biochemical method examining nuclear and cytoplasmic fractions (20). It has been reported that there is a significant decrease in the level of HSF in the nuclear fraction prepared from unshocked cells, whereas nuclei from heat-shocked cells retain a high level of HSF (14). Because activated HSF1 binds to HSE in the nuclei of heat shocked cells, the activated HSF1 is retained in the nucleus even after fractionation (14). Activation of HSF1, which is reflected by cells, the activated HSF1 is retained in the nucleus even after exposure to hyperthermia in the present study. The suppression of cell death observed 4 h after exposure to hyperthermia may be due to the prior up-regulation of Hsp72 expression in cells treated with HA because the Hsp72 level was lower in the HA4-treated cells than non-treated cells 4 h after exposure to hyperthermia (Fig. 11).

PC12 cells undergo apoptosis when cultured under conditions of serum deprivation (15, 25). To confirm the effect of HA oligosaccharides on cell death in a cell type except for K562 cells under stress conditions other than hyperthermia, we treated PC12 cells with HA oligosaccharides under conditions of serum deprivation in the present study. In this experiment, tetrasaccharides of HA were found to be more effective in suppressing cell death than the other HA oligosaccharides tested. Further experiments are required to elucidate the relationship between cell death and Hsp72 expression in PC12 cells under conditions of serum deprivation. After digestion of HA4 by chondroitinase ACII, the product did not suppress cell death of PC12 cells, confirming the specificity of the suppressive effect of HA4 on the cell death.

Hyaluronidase activity is known to be elevated in tumors (26) and inflammatory tissues (27) and to depolymerize HA. Free radicals also depolymerize HA in inflammatory tissues (28). It has been reported that hyaluronic acid with a molecular mass about 1.2 MDa inhibits the advanced glycation end-products-induced activation of the transcription factor nuclear factor-xB (NF-xB) and the NF-xB-regulated cytokines interleukin-1α, interleukin-6, and tumor necrosis factor-α (29). In addition to it, expression of interleukin-1β mRNA in synovium has been suppressed in the mild grades of osteoarthritis by the intraarticular treatment with high molecular mass HA (about 1.0 MDa) (30). Moreover, high molecular mass HA is known to suppress the proliferation of endothelial cells (31). On the contrary, it is well known that low molecular weight HA induces angiogenesis (10) and inflammation (11). When HA is natively depolymerized by hyaluronidases or radicals in vivo, HA may acquire novel functions. One of them is the up-regulation of Hsp72 expression (Fig. 12).

In conclusion, our results show that tetrasaccharides of HA up regulate Hsp72 expression by enhancing the activation of HSF1 under stress conditions and suppress cell death (Fig. 12).

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Hyaluronan Oligosaccharides on a Heat Shock Protein

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