The American Journal of Pathology, Vol. 171, No. 1, July 2007

DOI: 10.2353/ajpath.2007.060828

Tumorigenesis and Neoplastic Progression

Hyaluronan Oligosaccharides Inhibit Tumorigenicity of Osteosarcoma Cell Lines MG-63 and LM-8 *in Vitro* and *in Vivo* via Perturbation of Hyaluronan-Rich Pericellular Matrix of the Cells

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Numerous studies have demonstrated a correlation between hyaluronan expression and the malignant properties of various kinds of cancer, and inhibition of hyaluronan production causes decreased tumor growth. Hyaluronan oligosaccharides have been shown to inhibit several tumor cell types via disruption of receptor-hyaluronan interaction. However, few studies have addressed hyaluronan with respect to osteosarcoma. In this study, we examined the effects of exogenously added hyaluronan oligosaccharides on tumorigenicity of murine osteosarcoma cells, LM-8, and human osteoblastic osteosarcoma cells, MG-63. Moreover, the critical size of oligomers needed to inhibit malignant properties was defined. Fluorescent hyaluronan oligosaccharides accumulated both on the surface of cells and in the cytoplasm, and this retention was blocked by pretreatment with an anti-CD44 monoclonal antibody. Hyaluronan octasaccharides significantly inhibited cell viability and induced apoptosis as defined by cell proliferation and terminal deoxynucleotidyl transferase dUTP nick-end labeling assays, respectively. Octasaccharides also abrogated functional cell-associated matrices and significantly reduced the retention of endogenous hyaluronan. Further, octasaccharide treatment affected an inhibition of cell motility as well as cell invasiveness. Pretreatment of the cells with anti-CD44 antibody reduced the antitumor effect of the octasaccharides. *In vivo*, intratumoral injection of hyaluronan octasaccharides reduced the hyaluronan accumulation in local tumors, resulting in significant suppression of the formation of distant lung metastasis. Together these data suggest that hyaluronan oligosaccharides have potent antitumor effects functioning in part by the abrogation of hyaluronan-rich cell-associated matrices. *(Am J Pathol 2007, 171:274–286; DOI: 10.2353/ajpath.2007.060828)*

Hyaluronan (HA) is a high-molecular-weight linear glycosaminoglycan comprised of repeating disaccharide units, glucuronic acid, and *N*-acetylglucosamine. HA is an abundant component of the extracellular matrix and plays a role in regulating matrix assembly, cell migration, differentiation, and proliferation.[1–3] HA expression increases during active tissue remodeling, eg, during morphogenesis and wound healing.[4] Furthermore, HA binding proteins also contribute to these cellular behaviors through interactions with HA and assembly of pericellular coats.[5] Increased HA levels are also observed in malignant tumors, including gastric cancer, colorectal cancer, breast cancer, glioma, lung cancer, and ovarian cancer.[6–14] *In vitro* studies have demonstrated that the HA levels correlate with the invasive and metastatic capacity of tumor cells.[15,16] Increased HA-rich matrix deposition may help invasion by providing a suitable environment for cancer cells,[6] stimulating cell motility via interactions with cell surface receptors of HA,[17] and forming a barrier for cancer cells against host immunocompetent cells.[18] Perturbations of these endogenous HA-HA receptor interactions often inhibit tumor growth, invasion, or metastasis in

Supported in part by grant-in-aid 16591483 for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Aichi D.R.G. Foundation.

Accepted for publication March 30, 2007.

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select kinds of cancer.[19–24] HA oligosaccharides also have inhibitory effects on tumor growth[25] via competing for endogenous polymeric HA, replacing high-affinity, multivalent receptor interactions with low-affinity, low-valency interactions.[26,27]

Osteosarcoma is the most frequent primary malignant bone tumor in children and adolescents.[28,29] The term osteosarcoma is used to describe a heterogeneous group of lesions with diverse morphology and clinical behavior. Understanding the basic biology of heterogeneous osteosarcoma may provide insight for a novel tool for treatment. The prognosis of osteosarcoma has been improved with the introduction of chemotherapy; however, it is difficult to improve current response rates even with progressive dose escalation. Therefore, there is a clear need to develop newer and alternative agents for the treatment of patients with osteosarcoma.

Little has been reported on the association of HA and osteosarcoma tumorigenicity. The selective inhibition of HA synthase-2 (HAS-2) mRNA in osteoblastic osteosarcoma cells, MG-63, by antisense phosphorothioate oligonucleotides reduces HA accumulation and diminishes cell-associated matrix formation by these cells. These changes subsequently affect a substantial decrease in cell proliferation, a decrease in cell motility, and a decrease in cell invasiveness.[30] MG-63 cells also have an abundant HA-rich cell-associated matrix, leading to the hypothesis that inhibition of this HA-rich matrix retention at the cell surface might have effects on the tumorigenicity of these cells as well. However, the use of HAS-2 antisense oligonucleotides to inhibit HA synthesis has limited clinical practicality at present. Recently, investigators have used small oligosaccharides of HA to deplete HA-rich matrices from cells.[31,32] The proposed mechanism is that these small oligosaccharides compete with the binding of high-molecular-mass HA with cell surface receptors such as CD44. Given their size, purity, and ease of permeability into tissues, the use of such small oligosaccharides may have suitable clinical applicability.

In this study, we determined the critical size of HA oligosaccharides necessary to inhibit cell surface retention of HA in the osteoblastic osteosarcoma cell line MG-63 and to provide for antitumor effects. In addition, the effects of HA oligosaccharide application to osteosarcoma tumors *in vivo* were analyzed.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), trypsin ethylenediamine tetraacetic acid, and TRIzol reagent for RNA isolation were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). High-molecular-weight hyaluronan (HMWHA; 600 to 1200 kd) was purchased from Seikagaku Co. (Tokyo, Japan). Anti-CD44 monoclonal antibodies, Hermes-1, homing-associated cell adhesion molecule (HCAM), and IM7 were purchased from Pierce Biotechnology, Inc. (Rockford, IL), BioVision Research Products (Mountain View, CA), and StemCell Technologies (Seattle, WA), respectively. A GeneAmp RNA PCR kit for reverse transcription-polymerase chain reaction was purchased from Perkin-Elmer (Norwalk, CT). Specific primers for HAS-1, HAS-2, HAS-3, CD44, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were custom made by Integrated DNA Technologies (Coralville, IA). SYBR Green I gel stain was purchased from Molecular Probes (Eugene, OR). Biotinylated hyaluronic acid binding protein was purchased from Seikagaku America (Falmouth, MA). Human sCD44std enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bender MedSystems (Vienna, Austria). Streptavidin peroxidase (component of the Vectastain ABC kit) was purchased from Vector Laboratories (Burlingame, CA). Agarose was from FMC BioProducts (Rockland, ME). Cell Proliferation Kit I was purchased from Roche (Mannheim, Germany). Transwell inserts with 12.0-μm pore size were purchased from Corning Inc. (Corning, NY). Matrigel was purchased from BD Biosciences (Bedford, MA). All other enzymes and chemicals, molecular biology grade or reagent grade materials, and CelLytic-M were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

MG-63 cells (human osteoblastic osteosarcoma cell line) from the American Type Culture Collection (Manassas, VA) and LM-8 (murine osteosarcoma cells; a kind gift from Dr. A. Uchida, University of Mie, Tsu, Japan) were maintained at 37°C in an atmosphere with 5% CO_2 with DMEM, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were cultured in the presence or absence of HMWHA or various-sized HA oligosaccharides (HA oligos) in chamber slides at a concentration of 1×10^4 cells/well for Fl-HA, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and HA staining. The cells were cultured in 12-well plates as monolayer cultures at a concentration of 1×10^4 cells/dish for particle exclusion assay. For cell proliferation assays, the cells were cultured in 96-well plates at a concentration of 10^4 cells/well and in Transwells with 12.0-μm pore size at a concentration of 5×10^5 cells/well for cell invasion or motility assays.

Hyaluronan Oligosaccharides

HA oligos and fluorescein isothiocyanate-conjugated hyaluronan oligosaccharides (FITC-oligos) were prepared by Seikagaku Co. as described previously.[33] In brief, HA was depolymerized by partial digestion with testicular hyaluronidase and separated into size-uniform HA oligos by anion exchange chromatography after removal of the hyaluronidase. FITC-conjugated HA oligosaccharides of 34 monosaccharides in length (FITC-HA oligos; 34 mers) were obtained from Seikagaku Co. The purity and size of each HA oligo was confirmed by using

high-performance liquid chromatography analyses. Endotoxins, proteins, and DNA were absent in these oligosaccharide preparations.

Distribution of Exogenous FITC-HA Oligosaccharides

The retention of exogenously added FITC-HA oligos was investigated using MG-63 and LM-8 cells cultured on chamber slides at a density of 5.0×10^4 cells/well for 2 hours, treated with 250 μg/ml FITC-HA oligos for 24 hours, washed three times with phosphate-buffered saline (PBS), and then observed using a fluorescent microscope (Olympus CKX-FL-1; Tokyo, Japan) equipped with a digital camera (Olympus Camedia C-5050). To evaluate the specific binding of FITC-HA oligos to CD44, a cell surface receptor of HA, cells were pretreated with an anti-CD44 neutralizing antibody, Hermes-1 (0 to 10 μg/ml) or HCAM (0 to 10 μg/ml), for 2 hours before the addition of FITC-HA oligos for 24 hours at 37°C. MG-63 cells were pretreated with Hermes-1, and LM-8 cells were pretreated with the HCAM antibody. After washing three times with PBS, the retention of the FITC-HA oligos was evaluated by fluorescent microscopy. Fluorescence intensity of cells was semiquantified with ATTO Image Analysis software, CS Analyzer version 2.00a (ATTO Corp., Tokyo, Japan).

Cell Viability Assay

Given that the length of FITC-HA oligos might be crucial for antitumor effects, we performed a cell viability assay with treatment of HA tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, and dodecasaccharides (HA4, HA6, HA8, HA10, HA12) as well as HMWHA. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay 48 hours after treatment with or without 250 μg/ml HA4, HA6, HA8, HA10, HA12, or HMWHA. Preliminary studies demonstrated that at 48 hours, the cultures remained subconfluent and generated values within the linear optimal ranges of the MTT assay. Microscopic inspection of the wells confirmed that decreased absorbance values correlated with decreased cell number. Color intensity was determined on a microculture plate reader at 550 nm. The data presented are the means $\pm$ SD from triplicate wells per microtiter plate with three replicate microtiter plates per experiment. Based on the results of cell proliferation assays, HA4, HA8, and HMWHA were used for all of the following experiments. To evaluate whether the antitumor effects of HA8 were mediated via CD44, MG-63 cells were pretreated with 10 μg/ml anti-CD44 antibodies, either Hermes-1 or IM7, for 2 hours at 4°C, followed by HA8 for 48 hours and then assayed for cell viability using the MTT assay.

TUNEL Staining

TUNEL staining was used to evaluate the apoptotic effect of HA oligos on MG-63 or LM-8 cells. The cells were seeded into chamber slides (Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to adhere to the bottom of the slides for 12 hours. The cells were incubated with or without 250 μg/ml HA4, HA8, or HMWHA. After 72 hours of culture, the cells were fixed with paraformaldehyde and evaluated by TUNEL staining using an In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH). The cells were counterstained with hematoxylin-eosin. Cells with brown-stained nuclei in 20 different fields were counted under a light microscope at a magnification of 400×, and the percentage of positive-staining cells treated with HA oligomers was compared with positive-staining cells within the untreated control group.

Staining for HA

The cells were seeded into chamber slides (Becton Dickinson Labware) at a density of 10^4 cells/well, allowed to adhere to the bottom of the slides for 12 hours, and then treated with or without 250 μg/ml HA4, HA8, or HMWHA for 72 hours. Cultured MG-63 cells were fixed with 2% paraformaldehyde buffered with PBS at room temperature for 2 hours. The cells were treated with 0.3% H_2O_2 in 30% methanol for 30 minutes at room temperature to block the internal peroxidase activity, followed by incubation with 1% bovine serum albumin in PBS for 1 hour at room temperature. Cells were then incubated with 2.0 μg/ml biotinylated HA-binding protein (b-HABP) probe for 2 hours at room temperature. This b-HABP binds to HA with high affinity and specificity (similar to an antibody) with a minimum binding site of ~41 HA monosaccharides. Bound b-HABP was detected by the addition of streptavidin-peroxidase reagents (Vectastain kit) and diaminobenzidine-containing substrate solution (Sigma Fast DAB). As a control, cells were pretreated with 5 units/ml *Streptomyces* hyaluronidase for 1 hour at 60°C before incubation with b-HABP.

Particle Exclusion Assay

Cell-associated pericellular matrices were visualized using a particle exclusion assay.[34] Briefly, following the 72-hour treatment of monolayer cultures of MG-63 cells in 35-mm diameter dishes, with or without HA4, HA8, or HMWHA, the culture medium was removed and replaced with a 0.75-ml suspension of formalin-fixed erythrocytes (10^8 per ml) in PBS containing 0.1% bovine serum albumin. The particles were allowed to settle for 15 minutes. The cells were observed and photographed with an inverted phase-contrast microscope. Morphometric analysis for b-HABP stainable areas and functional cell-associated matrix areas of randomly selected cells captured as digital images were analyzed using NIH Image software (Bethesda, MD). A matrix area was defined by the area delineated by the cell-associated matrix minus the

area delineated by the plasma membrane (defined as cell area). In the absence of detectable matrix, the matrix/cell ratio would be 0.0.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from MG-63 cells that had been treated with or without 250 μg/ml HA4, HA8, or HMWHA for 7 days. The cells were treated with TRIzol (Life Technologies, Grand Island, NY) following the manufacturer's instructions. The purified RNA was dissolved in diethyl pyrocarbonate-treated H_2O, and the concentration of RNA in the samples was measured by its absorbance at 260 nm. In some experiments, total cytoplasmic RNA extracted from MG-63 cells with TRIzol reagents was subjected to reverse transcription and quantitative competitive PCR. Briefly, 0.125 μg of total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase in the presence of 0.15 μmol/L HAS-2- or CD44-specific downstream primers (HAS-2: 5′-TTTCTTTATGTGACTCATCTGTCTCACCGG-3′; CD44: 5′-AACCGCGAGAATCAAAGCCAAGGCC-3′). DNA fragments that share the same primer template sequence with the target cDNA but contain a completely different, smaller or larger intervening sequence, were prepared and used as DNA internal standards (ie, mimics).[35–37] Aliquots of sample cDNA mixed together with serial dilutions of DNA mimics were coamplified as templates in the presence of downstream primers and 0.15 μmol/L upstream primers for HAS-2 and CD44 (HAS-2 upstream: 5′-ATTGTTGGCTACCAGTTTATCCAAACGG-3′; CD44 upstream: 5′-GATCCACCCCAATTCCATCTGTAC-3′) in a PCR mixture consisting of 2 mmol/L magnesium chloride, 200 μmol/L of each deoxyribonucleotide, and 2.5 units of AmpliTaq DNA polymerase. The DNA was denatured by heating at 95°C for 2 minutes, followed by 23 cycles of 1 minute at 95°C, annealing at 60°C, and extension at 72°C for 1 minute (Perkin-Elmer thermocycler). This reaction was followed by a final elongation step that lasted 5 minutes at 72°C. The amplified products were analyzed by electrophoresis on 1.5% agarose gels followed by staining with SYBR Green I. The stained products were scanned and quantified using a fluoroimaging system (Molecular Dynamics, Sunnyvale, CA).

In other experiments, HAS-1 and HAS-3 expression was determined using conventional RT-PCR analysis. Following reverse transcription of total RNA into cDNA as above, the samples were PCR-amplified for 35 cycles in the presence of 0.15 μmol/L HAS-1 and -3 specific primers (HAS-1 upstream: 5′-GGACTACGTGCAGGTCTGTGACTC-3′; downstream: 5′-ACTTGGTAGCATAACCCATGCTGAG-3′; HAS-3 upstream: 5′-AGAGACCCCCACTAAGTACCTCCG-3′; downstream: 5′-CAGAAGGCTGGACATATAGAGGAGGG-3′) as well as GAPDH primer pairs. As a positive control for HAS-1 mRNA expression, total RNA isolated from human synovial cells was used.

To determine that all samples contained equivalent amounts of RNA (or to normalize results due to small differences), in a separate set of reactions total RNA from samples were coamplified in the presence of serial dilutions of an RNA internal standard (mimic) prepared for GAPDH. The GAPDH RNA mimic shares the same primer template sequence but contains a smaller intervening sequence. Samples containing 0.125 μg of sample total RNA were co-reverse-transcribed with twofold serial dilutions of GAPDH RNA mimic in the presence of 0.15 μmol/L GAPDH-specific downstream primer (5′-TTACTCCTTGGAGGCCATGTGGGCC-3′). The sample and mimic cDNA products were then co-amplified in the presence of the GAPDH-specific downstream primer together with 0.15 μmol/L upstream primer (5′-ACTGCCACCCAGAAGACTGTGGATGG-3′) using PCR conditions as described for HAS-2 amplification.

ELISA Analysis for Protein Expression of CD44

Changes in CD44 protein expression in MG-63 cells with or without exposure to HA4, HA8, or HMWHA for 7 days were quantified using a human sCD44std ELISA kit. Cell proteins were extracted using CelLytic-M according to the manufacturer's instructions. Concentration of the reagent including extracted protein of each sample was measured by bicinchoninic acid protein assay. One hundred microliters (1.6 mg/ml) of each sample was subjected to the ELISA analysis. Color intensity was quantified on a microtiter plate reader at 450 nm. The data presented are the means ± SD from triplicate wells per treatment.

Motility and Matrigel Invasion Assays

Chemotactic motility of MG-63 cells was investigated using 12-well cell culture chambers containing inserts with 12-μm pores. Invasion of MG-63 or LM-8 cells was assayed in the same chambers that also contained a reconstituted extracellular matrix supported on the membrane (Matrigel layered on a 12-μm pore membrane). Cells were added to the upper chamber (5×10^5 cells/500 μl of DMEM/well) in the presence or absence of HA4, HA8, and HMWHA, and 1500 μl of chemotaxis buffer including 10 μg/ml fibronectin was placed in the lower chamber. After incubation for 24 hours at 37°C and 5% CO_2, cells on the upper surface were wiped off with a cotton swab. Migrating and invading cells on the lower surface of the membrane were stained with hematoxylin. Cells from 20 different fields were counted under the light microscope using a magnification of ×200.

Effects of Hyaluronan Oligosaccharides on Tumor Growth and Lung Metastasis in Vivo

LM-8 cells were used in this study, since they are known to be tumorigenic when injected subcutaneously into syngeneic hosts, consistently forming local tumor masses as

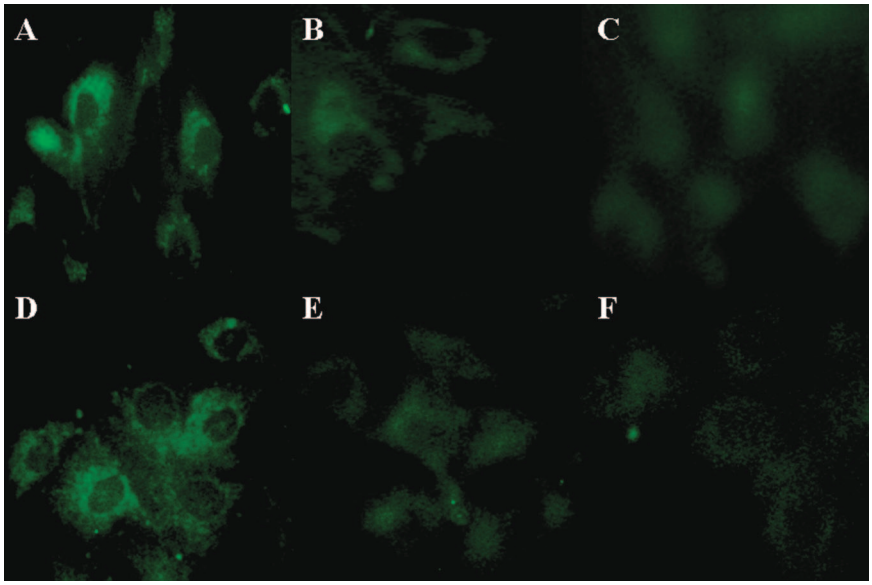


Figure 1. Retention of FITC-HA oligos is mediated by CD44. MG-63 cells at a density of 5.0×10^4 cells/well were pretreated with 0, 5, or 10 μg/ml anti-CD44 monoclonal antibody (Hermes-1) for 2 hours at 4°C (**A–C**, respectively). LM-8 cells at the same density were also pretreated with 0, 5, and 10 μg/ml anti-CD44 monoclonal antibody (HCAM) for 2 hours at 4°C (**D–F**, respectively). After washes, the cells were next incubated with 250 μg/ml FITC-conjugated HA oligos for 24 hours at 37°C. After washing with PBS three times, the cultures were examined by fluorescent microscopy and imaged using a digital camera (Olympus Camedia C-5050) (original magnification, ×400). Fluorescence of the representative single cell was semiquantified with ATTO Image Analysis software, CS Analyzer version 2.00a.

well as distant lung metastases.[38] LM-8 cells (2×10^6) suspended in 200 μl of serum-free DMEM were implanted into the dorsal flank of 5-week-old C3H/He mice and allowed to grow *in vivo* for a period of 14 days, at which time small tumors were identified. The mice were randomly divided into four groups ($n = 5$ mice/group), a control group and groups treated with HA4, HA8, or HMWHA at a concentration of 250 μg/ml. One hundred microliters of DMEM containing HA oligos or HMWHA were injected daily directly into the primary tumor mass from day 14 to day 23. The same volume of DMEM was injected into the control mice. At day 28, all of the mice were sacrificed and their local tumors and lungs excised and subjected to the analysis for tumor wet weight and the number of metastatic colonies to the lung, respectively. The excised lungs were fixed in 5% buffered formalin and stained with hematoxylin. The number of metastatic lesions in the lungs was counted under a microscope at the coronal-midline section.

Effects of HA Oligosaccharides on HA and CD44 Expression in Local Tumors

Primary local tumors were established in the dorsal flank of the mice and allowed to grow for 14 days. The subcutaneous tumors were then treated without or with daily single injections of 250 μg/ml HA oligomers for 10 days. The tumors were then excised, fixed with 10% formalin, and embedded in paraffin. Sections of 6 μm were subjected to HA histochemistry using b-HABP and CD44 immunohistochemistry using the anti-CD44 monoclonal antibody HCAM.

Statistical Analysis

All of the quantitative experiments were performed more than three times. Data were statistically analyzed using analysis of variance and the post hoc test (Bonferroni-Dunn). *P* values of less than 0.05 were considered statistically significant.

Results

Retention and Localization of Exogenous FITC-Conjugated HA Oligos

Because the smaller-sized HA oligos such as HA4 and HA8 could not be conjugated with FITC, 34-mers of HA oligos were conjugated with FITC and used in a cellular localization assay. As shown in Figure 1, A and D, FITC-conjugated HA 34-mers accumulated at the cell surface and in the cytoplasm of MG-63 and LM-8 cells, respectively. The cell surface retention of HA oligos was specifically blocked in a dose-dependent manner using neutralizing anti-CD44 monoclonal antibodies, Hermes-1 for MG-63 and HCAM for LM-8 cells (Figure 1, B and C, and E and F, respectively), suggesting the binding of HA oligos to the cell surface was mediated by CD44. The relative fluorescence intensities of representative MG-63 cells, determined by ATTO Image Analysis software, were 101.0, 35.6, and 18.1 following pretreatment of 0, 5, and 10 μg/ml Hermes-1. Changes in the relative fluorescence intensity of representative LM-8 cells were 132, 52.2, and 32.2 following pretreatment of 0, 5, and 10 μg/ml HCAM, respectively.

Cell Viability Assay

The effect of various sizes of HA oligos on cell viability was examined. Previous studies on chondrocytes had demonstrated that an effective concentration of 250 μg/ml of exogenous HA oligos was necessary to affect cell behavior.[31] As shown in Figure 2A, both HA oligos as

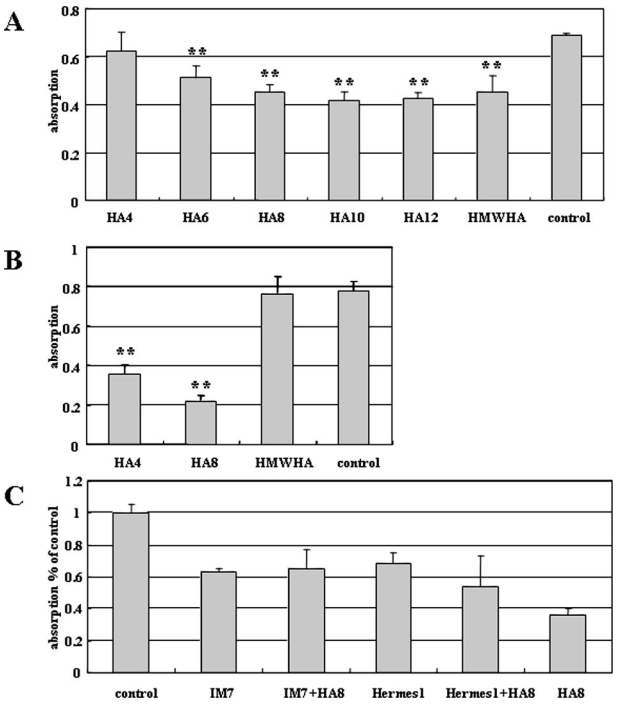


Figure 2. Effect of HA oligos on MG-63 and LM-8 cell viability. MG-63 (**A**) and LM-8 (**B**) cells were treated with various HA oligos or HMWHA for 48 hours and then assayed for cell viability using a MTT assay. MG-63 cells were pretreated with 10 μg/ml anti-CD44 monoclonal antibodies (IM-7 or Hermes-1) for 2 hours at 4°C, incubated with or without HA8 for 48 hours, and then assayed for cell viability using a MTT assay. Values represent the average ± SD compared with control absorbance (control = 1.0) derived from experiments performed in triplicate (**C**). Values represent the average ± SD of absorbance readings at 550 nm derived from experiments performed in triplicate (**$P < 0.01$).

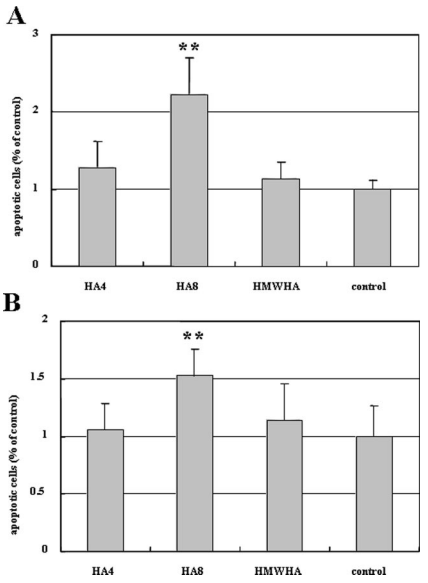


Figure 3. Effects of HA oligos on apoptotic activity in osteosarcoma cells. MG-63 (**A**) and LM-8 (**B**) cells in monolayer were cultured with or without 250 μg/ml HA4, HA8, HMWHA for 72 hours and then analyzed by TUNEL staining using an In Situ Cell Death Detection Kit. The percentage of positive cells with brown-stained nuclei was calculated in 20 different fields using light microscopy at a magnification of ×400. Percentage of apoptotic cells with treatment of HMWHA or HA oligos was compared with control (control, 1.0) and indicated as the *y* axis. Bars represent means ± SD. **Statistically significant difference from control group ($P < 0.01$).

well as HMWHA inhibited the growth of the MG-63 cells at this concentration. The HA10 and HA12 oligos exhibited the greatest effect of 40% inhibition following 48 hours of treatment ($P < 0.0001$ and $P < 0.0001$, respectively, compared with control), followed in activity by a 35% reduction following treatment with the HA8 oligos ($P < 0.0001$). Less inhibition was observed following treatment of the cells with HA4, HA6, or HWMHA ($P = 0.1044$, $P = 0.001$, and $P < 0.0001$, respectively). HA8 also inhibited the growth of the LM-8 cells significantly ($P < 0.0001$). To maximize differences between groups, all subsequent experiments were performed with HA4, HA8, and HMWHA for comparison.

To evaluate whether the antigrowth effects of HA8 were mediated via CD44, the MG-63 cells were pretreated with the blocking anti-CD44 antibodies, either IM7 or Hermes-1. As shown in Figure 2C, compared with control cells, the HA8 exhibited potent antiproliferative activity (63% decrease, $P < 0.05$). Pretreatment with IM-7 or Hermes-1 also resulted in a modest 30 to 35% decrease in cell number. However, pretreatment with either IM-7 or Hermes-1 before HA8 addition reduced the antiproliferative effects of HA8 (Figure 2C) back to levels observed with IM-7 or Hermes-1 alone. These data suggest that the cellular effects of HA8 are mediated through interactions with CD44.

Effects of HA Oligos on Apoptotic Activity

To determine the mechanism of the growth inhibitory effects of HA oligos on MG-63 and LM-8 cells, the number of apoptotic cells was determined by TUNEL staining. As shown in Figure 3A, the average percentage (mean ± SD) of apoptotic MG-63 cells' exposure to HA8 was significantly increased (2.2-fold stimulatory effect) as compared with the control ($P < 0.0001$), whereas there was no significant increase in apoptotic activity observed on exposure to HA4 or HMWHA ($P = 01415$ and $P = 03321$, respectively). Apoptotic activity was also significantly stimulated in LM-8 cells (1.5-fold) following treatment with HA8 ($P < 0.0001$, Figure 3B).

Effects of HA Oligos on Functional Cell-Associated Matrix of MG-63 Cells

Biotinylated HABP (b-HABP) was used to visualize endogenous HMWHA retained at the cell surface of MG-63 cells. As shown in Figure 4D, control MG-63 cells exhibited prominent b-HABP staining (arrows) indicative of HA-rich cell-associated matrix. Because the cells were permeabilized in the preparation, intracellular granules containing HA could also be detected (Figure 4D, arrowheads). Differences in b-HABP-stainable areas were determined by morphometric analysis (Figure 4E). Treatment of cells with HA4 (Figure 4A) or HMWHA (Figure 4C) did not result in significant changes in b-HABP staining as compared with control culture ($P = 0.89$ and $P = 0.152$, respectively). However, cells treated with HA8

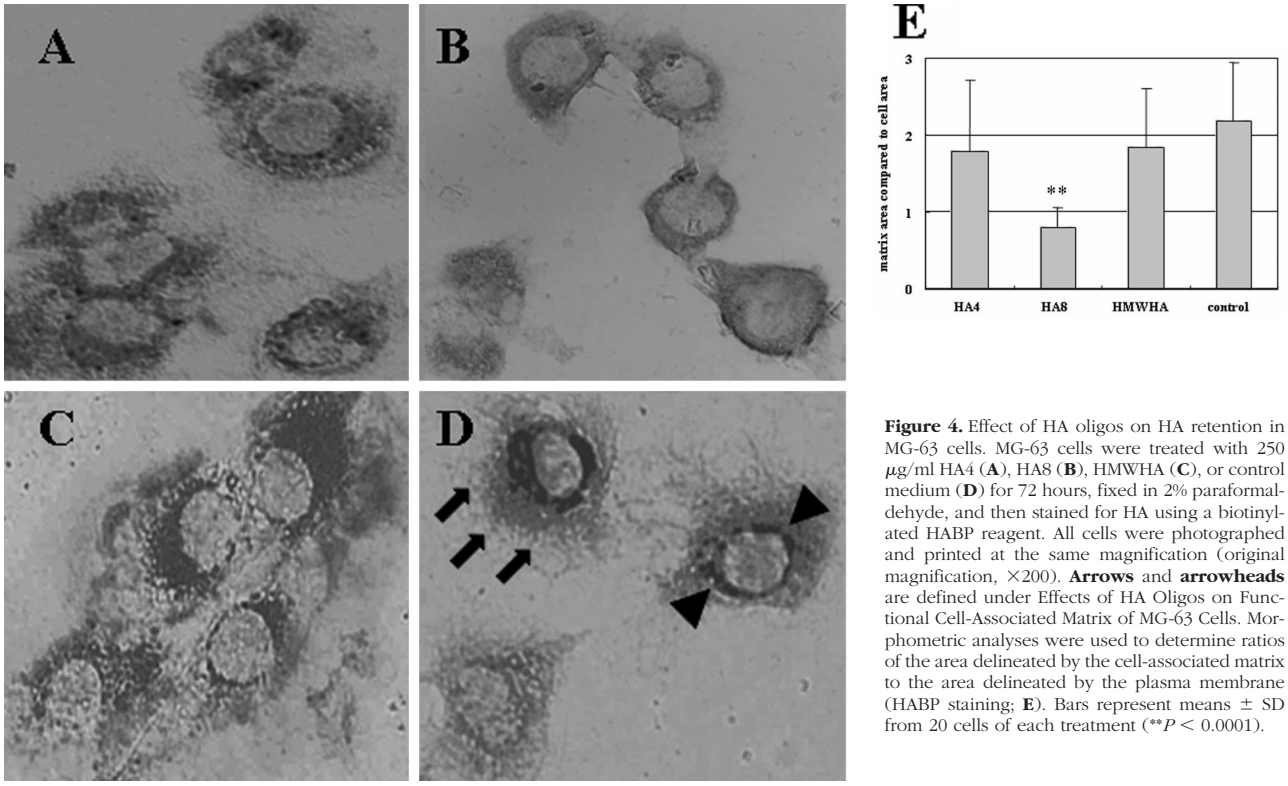


Figure 4. Effect of HA oligos on HA retention in MG-63 cells. MG-63 cells were treated with 250 μg/ml HA4 (**A**), HA8 (**B**), HMWHA (**C**), or control medium (**D**) for 72 hours, fixed in 2% paraformaldehyde, and then stained for HA using a biotinylated HABP reagent. All cells were photographed and printed at the same magnification (original magnification, ×200). **Arrows** and **arrowheads** are defined under Effects of HA Oligos on Functional Cell-Associated Matrix of MG-63 Cells. Morphometric analyses were used to determine ratios of the area delineated by the cell-associated matrix to the area delineated by the plasma membrane (HABP staining; **E**). Bars represent means ± SD from 20 cells of each treatment (**$P < 0.0001$).

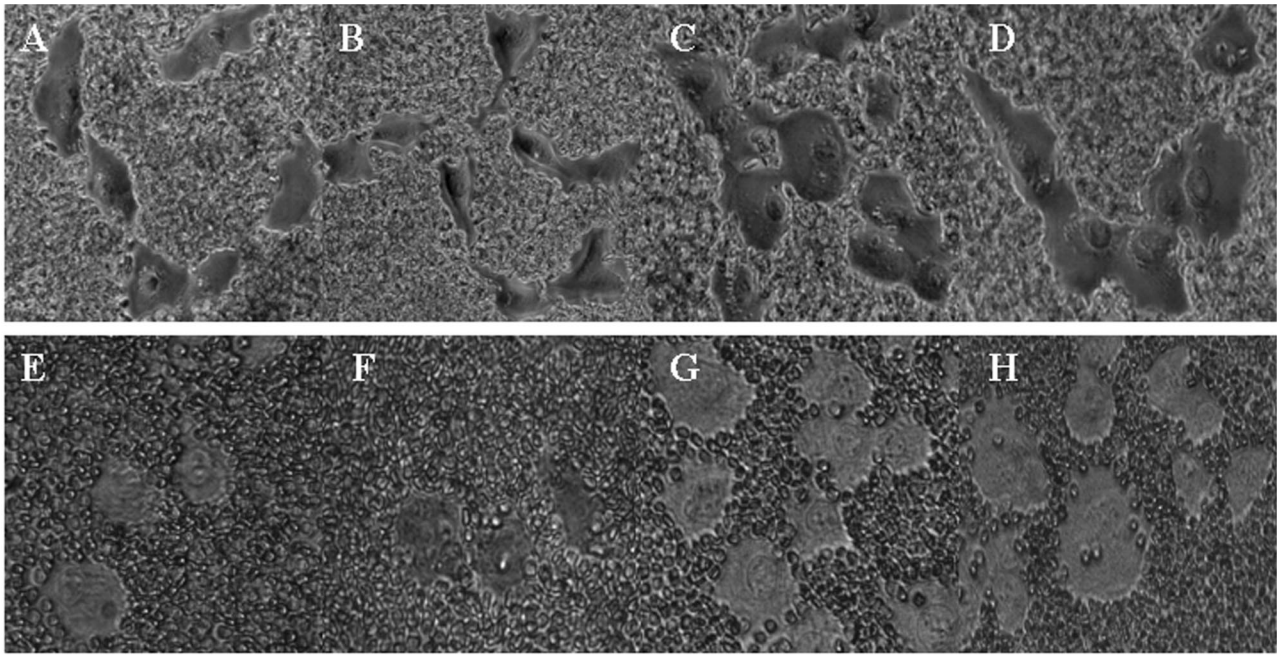
displayed a 64% decrease in staining for HA within cell-associated matrix (Figure 4B) compared with control ($P < 00001$). Thus, only the HA8 oligosaccharides affected the retention of endogenous HMWHA to MG-63 cells.

One of the functions of surface-retained HA is to serve as a scaffold for the assembly of a cell-associated matrix.[5,34,39] On tumor cells, such matrices may favor tumor growth and survival by inhibiting apoptosis or shielding the tumor cells from immunocompetent cells.[18] Cell-associated matrices can be visualized around live MG-63 cells by use of a particle exclusion assay (Figure 5D). These cell-associated matrices could be abrogated by treating tumor cells with HA8 (Figure 5B). Morphometric analysis indicated the 70% decrease in functional cell-associated matrix with treatment of HA8 as compared with control (Figure 5I, $P < 0.0001$). HMWHA treatment (Figure 5C) also resulted in a substantial decrease in the diameter of cell-associated matrices (Figure 5I, $P = 0.041$), although the effect of HA8 was more prominent. Similar inhibitory effects of HA8 were observed on LM-8 cells. HA8 treatment resulted in a statistically significant 50% decrease of functional cell-associated matrix (Figure 5F) compared with control (Figure 5H, $P < 0.0001$).

Changes in HAS and CD44 mRNA Levels after the Treatment of HA Oligos

HAS-2 has been demonstrated to be a main enzyme involved in the synthesis of HA by MG-63 cells.[30] Furthermore, the results of FITC-HA 34-mers binding (Figure 1) and displacement of endogenous HA by HA8 (Figure 4B) suggest that CD44 is the principal cell-surface receptor of HA in MG-63 cells. To determine whether HA oligos affect the expression of either HAS-2 or CD44 mRNA in MG-63 cells, the cells treated with HA oligos were analyzed by quantitative competitive RT-PCR (Figure 6, A and B). HAS-2 mRNA copy number was normalized by GAPDH copy numbers. As shown in Figure 6C, treatment of MG-63 cells with HA8 resulted in a 19% up-regulation of HAS-2 mRNA expression after 7 days after treatment, compared with the control ($P = 0.1488$). No significant effects on HAS-2 were observed following treatment with HA4 oligos or HMWHA (Figure 6C). There was 139% increase of CD44 mRNA levels following treatment of the cells with HA8 compared with control (Figure 6D, $P = 0.0009$). HA4 oligos also affected an increase in CD44, but the increase was not as prominent as following HA8 treatment. HMWHA had no effect on CD44 expression (Figure 5D). Stimulated expression of CD44 mRNA following treatment with HA8 (Figure 6D) was consistent with the CD44 protein expression determined by ELISA analysis; CD44 in HA8-treated cells was significantly higher compared with control (Figure 6G, $P = 0.0004$).

To clarify whether compensatory expression of other HAS isoforms had occurred, primer sets for HAS-1 and HAS-3 were tested using conventional RT-PCR amplification. As shown in Figure 6F, the expression of HAS-3 mRNA was also up-regulated with treatment of HA8 compared with control, but no changes in GAPDH mRNA were detected (Figure 6E). Although HAS-1 mRNA was detected using total RNA isolated from the synovia of



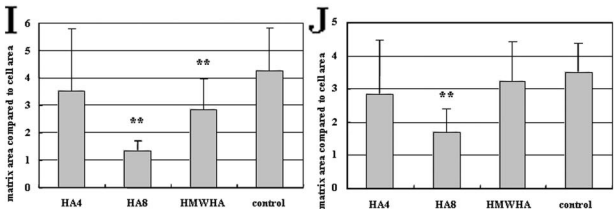


Figure 5. Effect of HA oligos on assembly of cell-associated matrices in MG-63 and LM-8 cells. To visualize pericellular matrices, inert particles (glutaraldehyde-fixed erythrocytes) were applied to cultures of MG-63 cells following 72 hours of treatment with 250 μg/ml HA4 (**A**), HA8 (**B**), HMWHA (**C**), or control medium (**D**), as well as LM-8 cells incubated with HA4 (**E**), HA8 (**F**), HMWHA (**G**), or control medium (**H**). All cells were photographed and printed at the same magnification (original magnification, ×200). Morphometric analyses were used to determine ratios of the area delineated by the cell-associated matrix to the area delineated by the plasma membrane (**I**, MG-63; **J**, LM-8). Bars represent means ± SD from 10 cells of each condition (**$P < 0.0001$).

patients with rheumatoid arthritis, HAS-1 mRNA could not be detected in any of the samples derived from MG-63 cells (data not shown).

Effect of HA Oligos on Cell Migration and Invasiveness

As shown in Figure 7A, HA8-treated MG-63 cells exhibited a statistically significant reduction (50%) in migratory activity at the 24-hour time point compared with control cells ($P < 0.0001$). In the invasion assay, the HA8-treated MG-63 cells also exhibited a significantly lower capacity (30%) to pass through the Matrigel-coated filters as compared with control cells (Figure 7B, $P < 0.0001$). A slight inhibition (30%) was observed in cells treated with HMWHA but no change in cells treated with HA4. Invasiveness was also inhibited by 41% in LM-8 cells with treatment of HA8 compared with control (Figure 7C, $P < 0.0001$). No significant inhibitory effects were exhibited following treatment with HA4 or HMWHA.

Effects of HA Oligos on LM-8 Growth in Vivo *and Lung Metastasis*

LM-8 cells are highly metastatic in mice and thus useful for *in vivo* investigations into the efficacy of HA oligos on tumor growth and metastasis. Like the MG-63 cells, the LM-8 cells have abundant cell-associated matrix and display similar sensitivity to HA oligos with regards to cell viability, invasiveness, and apoptosis *in vitro* (Figures 2, 3, and 7). *In vivo*, daily application of HA8 showed a trend toward inhibition of LM-8 tumor growth (Figure 8E) as measured by tumor mass. However, given the deviation between tumors, the values did not reach significance. On the other hand, treatment of the primary tumor with HA8 did result in a significant 82% reduction in the number of metastatic lung lesions ($P = 0.0136$, Figure 8F). These results can be compared visually, for example, the lung metastasis in HA8-treated tumors (Figure 8B, arrows) versus control, untreated tumor-bearing animals (Figure 8D) or animals treated with HA4 or HMWHA (Figure 8, A and C, respectively). No additional complications of HA oligo treatment on skin or body weight were observed.

Effects of HA Oligos on CD44 and Hyaluronan Expression in LM-8 Local Tumors

The inhibitory effects of HA8 treatment on lung metastasis formation might be due to the abrogation of HA retention by tumor cell CD44. Analysis for HA by b-HABP staining and CD44 expression by immunohistochemistry revealed that HA8 reduced the HA retention in LM-8 local tumors (Figure 9B) compared with control

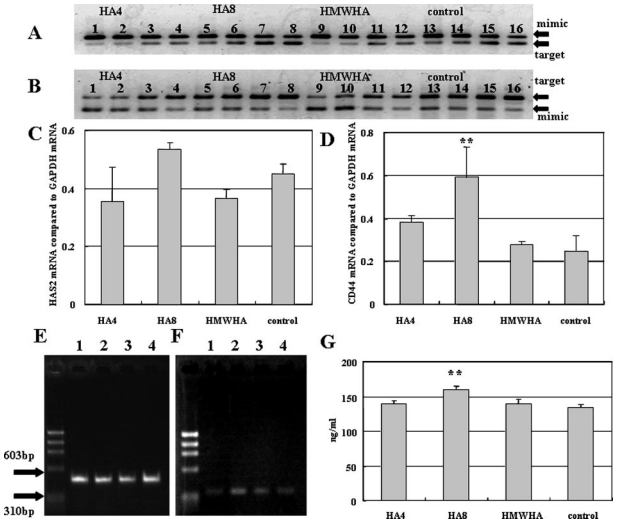


Figure 6. Effect of HA oligo treatment on HAS and CD44 expression in MG-63 cells. Aliquots of total RNA (0.125 μg) derived from MG-63 cells cultures treated with 250 μg/ml HA4, HA8, HMWHA, or control medium for 7 days were reverse-transcribed and PCR-amplified for 23 cycles in the presence of human HAS-2- or CD44-specific primers. The products were separated on 1.5% agarose gels and visualized by SYBR Green I staining. The gels were scanned and quantified using a fluoroimaging system (Molecular Dynamics). RT-PCR amplification of HAS-2 is shown in **A**, CD44 in **B**. **Lanes 1–4** represent in each panel amplification of RNA derived from HA4 oligo-treated cultures; **lanes 5–8**, HA8-treated cultures; **lanes 9–12**, HMWHA-treated cultures; **lanes 13–16**, control untreated cultures. In **A**, reverse-transcribed cDNA samples were coamplified with 30, 15, 7.5, and 3.75 attomoles of HAS-2 DNA mimic (**lanes 1–4**, **5–8**, **9–12**, and **13–16**, respectively). In **B**, reverse-transcribed cDNA samples were coamplified with 4, 2, 1, and 0.5 attomoles of CD44 DNA mimic (**lanes 1–4**, **5–8**, **9–12**, and **13–16**, respectively). HAS-2 target product size is 409 bp; mimic size is 523 bp. CD44 target size is 587 bp; mimic size is 379 bp. After normalization of competitive RT-PCR reactions with respect to a GAPDH RNA mimic, the ratio of mRNA copy numbers was determined. The comparative copy number of HAS-2 (**C**) and CD44 (**D**) mRNA derived from cultures treated with HA4, HA8, HMWHA, or untreated controls are shown. Bars represent means ± SD from three different cultures ($^*P < 0.001$). Aliquots of total RNA derived from MG-63 cells cultures treated with 250 μg/ml HA4, HA8, HMWHA, or control medium for 7 days were also subjected to conventional RT-PCR for 35 cycles in the presence of human HAS-3 or GAPDH-specific primers. Total RNA derived from synovial cells of patients with rheumatoid arthritis was used as positive control for the HAS-1 and -3 mRNA expression. The products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. **E** and **F** depict the amplification of GAPDH and HAS-3, respectively, with HA4 (**lane 1**), HA8 (**lane 2**), HMWHA (**lane 3**), or untreated controls (**lane 4**). A human sCD44std ELISA kit was used to determine changes in the expression levels of CD44 protein. MG-63 cells with treatment of HA4, HA8, HMWHA, or control medium for 7 days were lysed using CelLytic-M and subjected to sCD44std ELISA. Color intensity was determined on a microtiter plate reader at 450 nm. The data presented are the means ± SD from triplicate wells per treatment ($^{**}P < 0.001$).

(Figure 9D), whereas the same treatment up-regulated CD44 expression in local tumors (Figure 9F) compared with control (Figure 9H).

Discussion

Previous studies have shown that HA plays a crucial role in the progression of several cancers.[40] Inhibition of endogenous HA-tumor cell interaction results in the suppression of growth and metastasis of the tumor cells.[40] Reduction of anchorage-independent growth by this inhibition is due to suppression of the phosphoinositide 3-kinase/Akt pathway.[40,41] In several types of tumor cells, glioma[42] and melanoma[25] cells, antitumor effects have

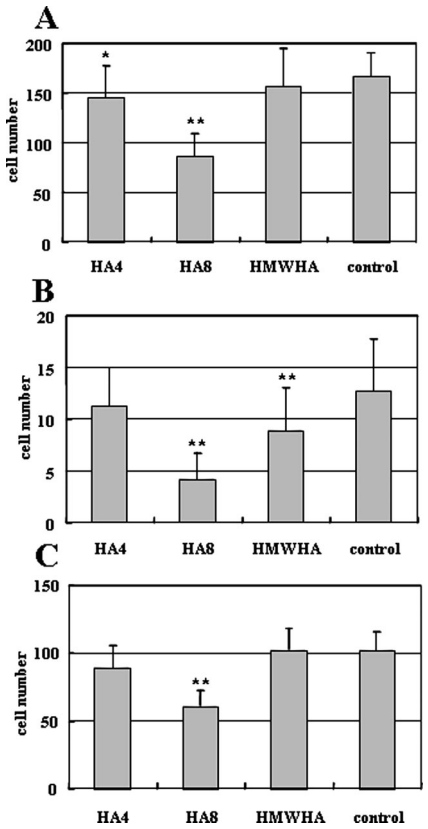


Figure 7. The effect of HA oligos on MG-63 or LM-8 cell motility and invasiveness. MG-63 cells were assayed for changes in cell motility (**A**) and invasiveness (**B**) in Boyden chambers in the absence or presence of HA4, HA8, or HMWHA. LM-8 cells were also subjected to the assay for invasiveness (**C**). Following 24 hours of incubation, the cells were fixed with 2% paraformaldehyde and visualized by hematoxylin staining. The number of cells on lower surface of the membrane was counted in 20 randomly selected high-power fields. The data are presented as the average ± SD for cell motility (**A**) and cell invasion (**B** and **C**). **Statistically significant difference from control group ($P < 0.0001$).

been demonstrated with treatment of HA oligos. However, these studies did not analyze the effects of HA oligosaccharides on the formation of cell-associated matrix or the critical size of HA oligomers to have antitumor effects. In this study, we showed that treatment of MG-63 human osteoblastic osteosarcoma cells, which have abundant HA-rich cell-associated matrix,[30] as well as LM-8 murine osteosarcoma cells, with HA oligos inhibited the formation of HA-rich cell-associated matrix, resulting in the inhibition of growth, motility, and invasiveness and induction of apoptotic activity *in vitro*. Antitumor effects were also observed *in vivo*.

CD44 is the primary cell surface receptor for the HA in many cell types.[26,43] Several lines of evidence were used in this study to implicate the participation of CD44 in the antitumor effects elicited by the HA oligos. First, anti-CD44 blocking antibodies prevented the binding of FITC-conjugated HA 34-mers to the cell surface (or accumulation within the cytoplasm) of MG-63 and LM-8 cells. Although such an approach can be used to demonstrate specific binding of HA oligos to CD44, a blocking antibody approach is more difficult to use to verify the participation of CD44 in HA oligo-initiated antitumor effects.

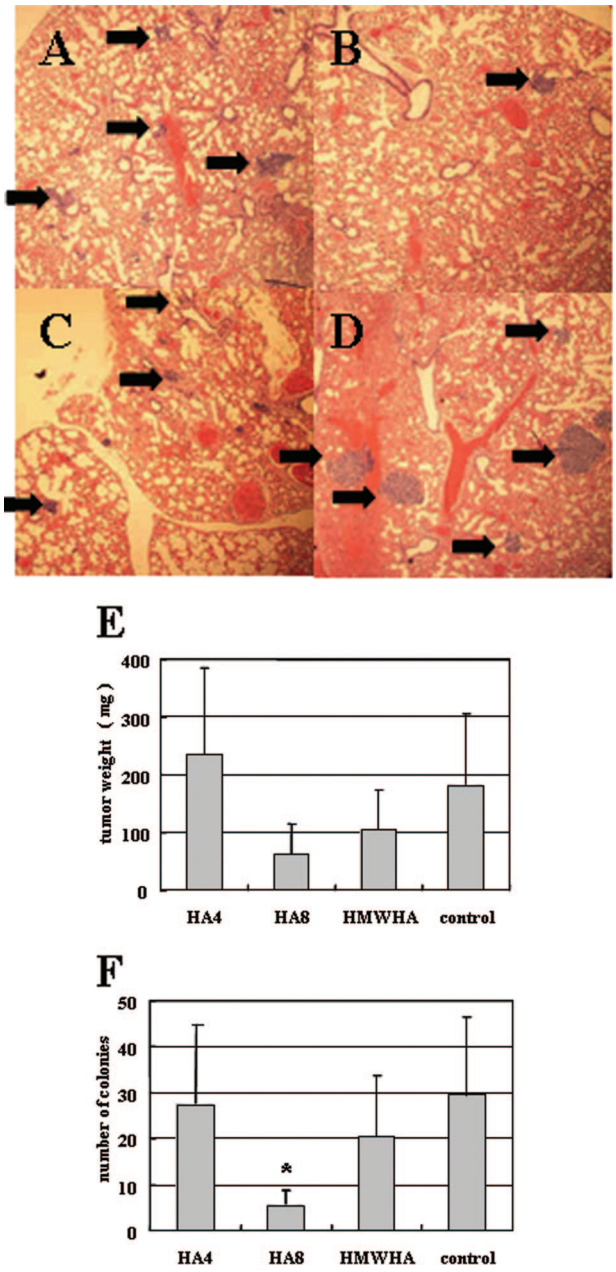


Figure 8. The effect of HA oligos on tumor mass and lung metastasis of LM-8 cells. Mouse LM8 osteosarcoma cells were transplanted subcutaneously into C3H/He mice. From day 14 to day 23, 250 μg/ml HA4, HA8, HMWHA, or control medium was injected daily, directly into the tumor mass. The mice were sacrificed at day 28, the subcutaneous tumors excised, and their blotted wet weight measured (**E**). In addition, numbers of lung metastases at the coronal midline section were counted in sections of hematoxylin-stained lung tissue (**F**). **A** to **D** depict representative midcoronal sections of lung treated with HA4, HA8, HMWHA, and control, respectively. Original magnification, ×400. *Statistically significant difference from control group ($P < 0.05$).

CD44-blocking antibodies, like the HA oligos, also interfere with HMWHA binding to the cell surface and thus initiate the same antitumor effects.[44–46] For example, the invasion of metastatic colon carcinoma cells into Matrigel was inhibited by 95% following preincubation with the anti-CD44 antibody IM7.[44] In this study, both IM7 and Hermes-1 antibodies also independently affected the proliferation of osteosarcoma cells by 30 to 35%. However, by pretreatment with these anti-CD44 antibodies, the antiproliferative activity the effects of HA8 (63% reduction in cell proliferation) were nullified.

The third line of evidence implicating CD44 in our study is ligand size specificity. HA tetrasaccharides (HA4) do not compete with HA binding to CD44.[47,48] Lesley et al[27] found that only HA oligos between 6 and 18 monosaccharides exhibited monovalent binding to CD44. In this study we demonstrate that, although HA8 oligos have potent antitumor effects, HA4 oligos exhibit no stimulatory effect on apoptosis or inhibitory effects on cellular retention of HA, HAS-2 mRNA expression, migratory activity, or lung metastasis. Thus, the absence or presence of antitumor responses because of HA4 or HA8 is consistent with the ligand size specificity of CD44. Nonetheless, although it remains possible that HA oligos interact or interfere with another glycan-binding protein, the data are again consistent with mediation via CD44.

That the mechanism for the antitumor responses involves the displacement of HMWHA is derived from our previous study that suppression of cell-associated matrix in MG-63 cells by antisense oligonucleotide inhibition of HAS-2 gene expression results in the inhibition of cell proliferation, motility, and invasiveness.[30] Indeed, MG-63 cells in logarithmic phase of proliferation show prominent b-HABP-positive staining in the cytoplasm for HA.[30] Interestingly, cells in control culture show dark b-HABP-positive staining in cytoplasm, whereas cells incubated with HA8 show light staining, suggesting that treatment of HA8 inhibits the endogenous HA deposition in cell-associated matrix together with the suppression of internalization of endogenous extracellular HA through the cell surface receptor CD44.[49]

A limitation of this study is the lack of analysis of potential signaling pathways. Several cell surface HA receptors including CD44 and RHAMM influence cell proliferation, survival, and motility. Further, Toll-like receptor 4 (TLR-4) is reported to be the receptor for HA degradation products in dendritic cells.[50] Constitutive interactions between HA and CD44 resist growth arrest and apoptosis by cancer cells.[22,41] HA has also been demonstrated to activate the phosphatidylinositol 3 kinase-AKT signaling pathway and the phosphorylation of focal adhesion kinase and BAD.[51–53] Inhibition of the interaction between constitutive HA and CD44 reverses these effects.[41,53] In accordance with these results, our data demonstrate that HA8, an oligosaccharide that interferes with the constitutive interaction between endogenous HA and CD44, affects a stimulation of apoptotic activity, whereas HA4, an oligosaccharide that does not inhibit this interaction, does not induce apoptotic activity. Transcriptional analysis reveals the up-regulation of HAS-2 and CD44 mRNA levels with treatment of HA8, which will also need further investigation to reveal the regulatory signaling pathway. Significant increases of HAS-2 and CD44 mRNA levels by HA8 might result from positive feedback for reduction of cell-associated matrix formation by osteosarcoma cells. After removal of HA8, the cell-associated matrices of MG-63 and articular chondrocytes were recovered.[32] Recovery of LM-8 cell matrices following washout were also observed (data not

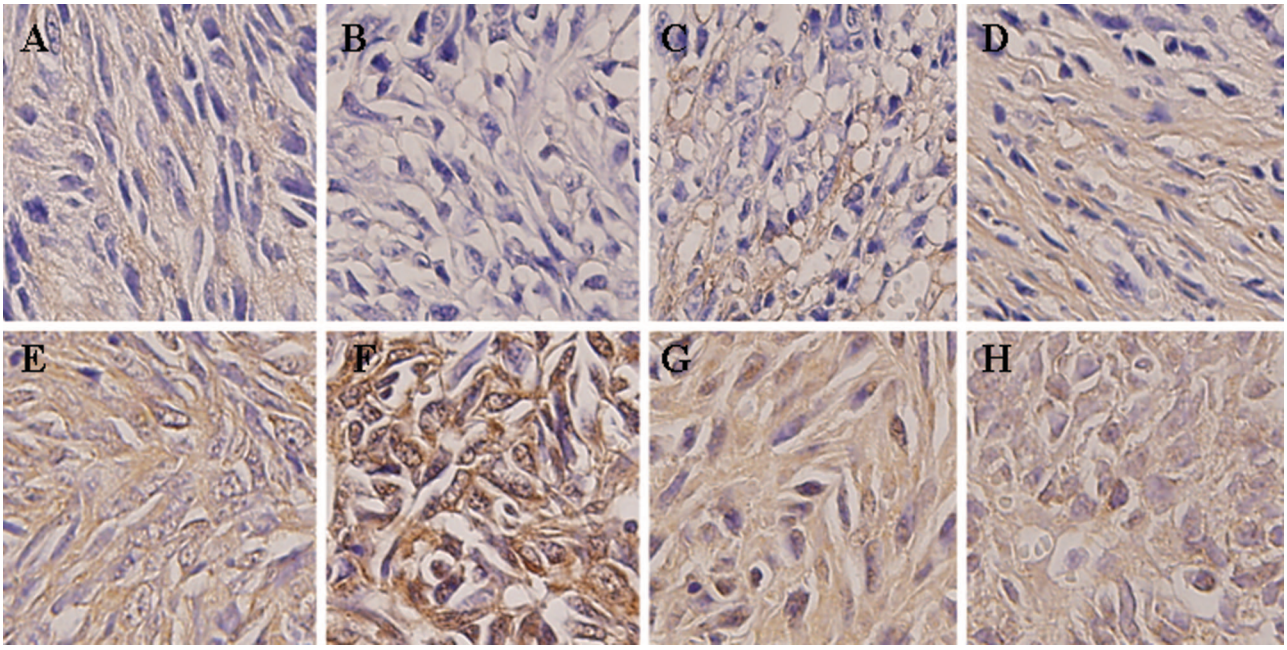


Figure 9. The effect of HA oligos on HA accumulation and CD44 expression in local subcutaneous LM-8 cells tumors. LM-8 tumors were grown in the dorsal flank of C3H/He mice. The tumors were then given daily, single subcutaneous injections of 250 μg/ml HA4 (**A** and **E**), HA8 (**B** and **F**), HMWHA (**C** and **G**), or control medium (**D** and **H**) for 10 successive days. The tumor masses were then excised, fixed in 10% formalin, and embedded in paraffin. Sections were subjected to HA staining with b-HABP (**A–D**) or CD44 immunohistochemistry (**E–H**).

shown). However, direct effects of HA oligos to cell signaling via some cell surface receptors remain to be elucidated.

Antitumor effects with treatment of HA8 are more prominent for lung metastasis *in vivo* than *in vitro* in the current study. Recent studies have demonstrated that adhesion of metastatic prostate carcinoma cells can be mediated by the pericellular HA surrounding the metastatic cells, whereas nonmetastatic cells having pericellular HA do not adhere to endothelial cells.[54] One explanation for the significant reduction of the lung metastasis by osteosarcoma cells in our studies might be due to the inhibition of pericellular HA with treatment of locally injected HA8. The tendency of suppression in local tumor growth may be that abrogation of cell-associated matrix facilitates the attack by host immunological system, together with the direct inhibitory effects of HA8 on cell proliferation.

Although survival for patients with localized osteosarcoma approaches 70%, the prognosis of the cases arising from pelvis or recurrent disease is dismal[55,56] because of the unresectability of the tumor. Considering that a positive margin results in the poorer prognosis for patients, cases in which there is difficulty to obtain complete tumor removal need additional chemotherapy, radiotherapy, or investigational agents. Some groups have reported using radiotherapy for unresectable patients, but the efficacy was limited.[57,58] New effective agents against such resistant osteosarcoma should be introduced, based on the understanding of the biology for osteosarcoma.

In summary, the present study reveals the inhibitory effects of HA oligosaccharides on cell proliferation, motility, and invasiveness in MG-63 human osteoblastic osteosarcoma cells as well as LM-8 murine osteosarcoma cells via suppression of cell-associated matrix formation and the critical length of HA for these effects. Considering that osteosarcoma is a group of heterogeneous lesions, size of the cell-associated matrix, expression of cell surface receptors for HA, and signaling pathways might diverse among cases. Accumulation of data from various types of osteosarcoma may be necessary to shed light on the utility of HA oligosaccharides as novel therapeutic tools for uncontrollable osteosarcoma.

Acknowledgments

We thank Seikagaku Co. for kindly providing HA oligosaccharides, Dr. Mitsutoshi Uchibori for technical assistance, and Eri Ishihara for secretarial assistance.

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