Hyaluronan (HA), one of the major components of the extracellular matrix, is a high molecular weight linear glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, whose molecular size can reach 10^7 kDa (1, 2). Despite its simple composition, HA possesses a number of functions. It influences the hydration and physical properties of tissues (1, 3), interacts with the cell-surface receptors for HA (6), and promotes cell motility (7). Hyaluronan (HA), an extracellular matrix glycosaminoglycan that interacts with cell-surface receptors, including CD44. Although HA usually exists as a high molecular mass polymer, HA of a much lower molecular mass that shows a variety of biological activities can be detected under certain pathological conditions, particularly in tumors. We previously reported that low molecular weight HAs (LMW-HAs) of a certain size range induce the proteolytic cleavage of CD44 from the surface of tumor cells and promote tumor cell migration in a CD44-dependent manner. Here, we show that MIA PaCa-2, a human pancreatic carcinoma cell line, secreted hyaluronidases abundantly and generated readily detectable levels of LMW-HAs ranging from ~10- to 40-mers. This occurred in the absence of any exogenous stimulation. The tumor-derived HA oligosaccharides were able to enhance CD44 cleavage and tumor cell motility. Inhibition of the CD44-HA interaction resulted in the complete abrogation of these cellular events. These results are consistent with the concept that tumor cells generate HA oligosaccharides that bind to tumor cell CD44 through the expression of their own constitutive hyaluronidases. This enhances their own CD44 cleavage and cell motility, which would subsequently promote tumor progression. Such an autocrine/paracrine-like process may represent a novel activation mechanism that would facilitate and promote the malignant potential of tumor cells.

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Hyaluronidases play critical roles in HA metabolism (15). Six human hyaluronidase-like sequences are present in the human genome. These are HYAL1, HYAL2, HYAL3, HYAL4, SPAM1, and PHYAL1, which, respectively, encode Hyal-1, Hyal-2, Hyal-3, Hyal-4, PH-20, and a pseudogene that is transcribed but not translated (16). Of these hyaluronidases, only Hyal-1 and Hyal-2 have been characterized well. Hyal-1 is an acid-active lysosomal enzyme that can utilize HA of any size as a substrate, generating predominantly tetrasaccharides (15). Hyal-2 is an acid-active enzyme anchored to the plasma membrane by a glycosylphosphatidylinositol linkage and digests high molecular mass HA to a limit product of ~20 kDa, or about 50 disaccharide units (15). These two hyaluronidases appear to co-operate in HA degradation and both have been implicated in tumor invasion and metastasis. Hyal-1 is highly expressed in an active form in the tissue and urine specimens of patients with bladder (17) or prostate (18) cancers. It is associated with the appearance of detectable levels of low molecular weight HAs (LMW-HAs) (17, 19), whereas Hyal-2 is expressed in mammary tumors (20).

Accumulating evidence indicates that LMW-HAs have distinct functions from high molecular weight-HAs, and that HA molecules of different sizes have different functions (21). For instance, HA 4–16-mers induce cytokine gene expression in dendritic cells (22) and promote their maturation (23), HA 6–20-mers induce chemokine gene expression in murine alveolar endothelial cells (24), and HA 8–50-mers induce angiogenesis (25). In addition, LMW-HAs have been suggested to play a role in tumor biology. High levels of LMW-HAs have been found in several human tumors, such as bladder (19) and prostate (17) cancers, Wilms tumor (26), and mesothelioma (27). HA 36-mers activate tumor cell integrins, resulting in the up-regulation of cell binding to intercellular adhesion molecule 1 (ICAM-1) (28). HA 6–36-mers enhance CD44 cleavage and promote the migration of tumor cells in a CD44-dependent manner (29), in accordance with the idea that LMW-HAs can activate tumor cells via the cell-surface receptor CD44.

CD44 is a widely distributed cell-adhesion molecule that serves as one of the cell-surface receptors for HA (6). CD44 participates in a number of biological events, such as tumor cell invasion and migration (30). CD44 is proteolytically cleaved at the extracellular domain by metalloproteases such as MT1-MMP (31), ADAM-10, and ADAM-17 (32), followed by further cleavage at its intracellular domain that may lead to the up-regulation of CD44 expression (33). Enhanced CD44 cleavage
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has been reported in various human tumors, including glioma, breast carcinoma, non-small cell lung carcinoma, colon carcinoma, and ovarian carcinoma (34), suggesting that this cleavage plays an important role in tumor progression.

Based on these observations, we hypothesized that tumor cells themselves may generate LMW-HAs of a certain size range, and that the tumor-related LMW-HAs may in turn activate the tumor cells by interacting with CD44. Here, we demonstrate that LMW-HAs ranging from ~10- to 40-mers are generated by the action of hyaluronidas in a human pancreatic carcinoma cell line, MIA PaCa-2, and that the generated LMW-HAs activate the cells by enhancing CD44 cleavage and cell motility in a CD44-dependent manner. Our results provide evidence for an autocrine/paracrine process that represents a novel activation mechanism that leads to the promotion and maintenance of the malignant properties of tumor cells.

MATERIALS AND METHODS

Reagents—A rabbit polyclonal antibody (pAb), anti-CD44cyto pAb, which is directed against the cytoplasmic domain of CD44 was kindly provided by Dr. Hideyuki Saya (Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan) (35). An anti-human CD44 monoclonal antibody (mAb), BRIC225, was purchased from the International Blood Group Reference Laboratory (Bristol, UK), and the Fab fragment of BRIC225 was prepared as described previously (29). An anti-β-tubulin mAb and mouse IgG were purchased from Calbiochem (Cambridge, MA) and Sigma, respectively. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from American Qualex (San Clemente, CA). HA oligosaccharides (HA 10-mer and HA 36-mer) were generously provided by Seikagaku Kogyo Co. (Tokyo, Japan) (36). Human umbilical cord HA, mainly consisting of 1,000-kDa HA, was purchased from Sigma. pAbs against hyaluronidases (anti-Hyal-1 and anti-Hyal-2) were generated by one of us consisting of 1,000-kDa HA, was purchased from Sigma. pAbs against hyaluronidases (anti-Hyal-1 and anti-Hyal-2) were generated by one of us (37). Sheep testicular hyaluronidase and hyaluronidase from Streplococcus dysgalactiae were purchased from Sigma and Seikagaku Kogyo Co., respectively. Carboxbenzoxyl-leucinyl-leucinyl-leucinal (MG132) was from the Peptide Institute (Osaka, Japan).

Cell Culture—The human pancreatic carcinoma cell line MIA PaCa-2 (29) was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum, 1% (v/v) 100 µM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin, at 37 °C in an atmosphere containing 5% CO₂.

Preparation of MIA PaCa-2 Culture Supernatant—MIA PaCa-2 cells were seeded in a 75-cm² flask (3 × 10⁵ cells/flask), and incubated overnight at 37 °C. After being washed 3 times with serum-free RPMI medium, the cells were further incubated in the RPMI medium for 3 days. The culture medium was collected and cleared by centrifugation (1,200 × g, 4 °C, 5 min). The preparation (M-sup/conc) used in the measurement of hyaluronidase activities and the detection of hyaluronidase proteins was concentrated 50-fold using Centricon (cut-off size: 30 kDa) (Millipore, Bedford, MA) before being filtered through a 0.22-µm pore filter (Millipore), and the preparation (M-sup) used in other assays was not concentrated but filtered through a 0.22-µm pore filter.

Reverse Transcriptase-PCR—For the detection of HYAL-1 and HYAL-2 transcript expression, RNA isolated from MIA PaCa-2 cells by TRIzol (Invitrogen) and RNeasy (Qiagen, Hilden, Germany) was reverse transcribed using an OmniScript RT kit (Qiagen). PCR was conducted using primers for HYAL-1 (TCAGCCCCAAGGTTGCTCGACCA, CTGCCAGCCAGGTTAGCATCGACAT), HYAL-2 (CTTCTACCGCGACCCTGCA, GCTCTGACATAACGCAGTG), and β-actin (GGATGTTGCTCCTCTCGAGC, GACCTTACGGTTCAGGTTT), and Z-Taq polymerase (Takara, Shiga, Japan) by 40 cycles of denaturation (98 °C, 5 s), annealing (57 °C, 5 s), and extension (72 °C, 20 s), followed by a further extension at 72 °C for 5 min. The PCR products (1,300 bp for HYAL-1, 359 bp for HYAL-2, and 308 bp for β-actin) were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. As controls, reverse transcriptase-PCR was carried out without the OmniScript RT kit reagents or in the absence of template in the PCR mixture.

Measurement of Hyaluronidase Activities (Enzyme-linked Immunosorbent (ELISA)-like Assay)—Hyaluronidase levels present in the samples were measured using an ELISA-like assay (17, 38, 39). The wells in a 96-well microtiter plate were coated with 200 µg/ml 1,000-kDa HA. The HA-coated wells were incubated with various amounts of standard sheep testis hyaluronidase in the culture supernatant of tumor cells for 16 h at 37 °C in hyaluronidase assay buffer (0.1 M sodium formate, 0.15 M NaCl, 0.2 mg/ml bovine serum albumin) ranging from pH 3.5 to 4.4. After being washed with PBS containing 0.1% Tween 20, the wells were incubated with 5 µg/ml biotinylated HA-binding protein (HABP). The HABP bound to the wells was then analyzed quantitatively using an avidin-biotin detection system. A standard curve was drawn by plotting the absorbance at 490 nm against the concentration of the residual 1,000-kDa HA that was not digested by the hyaluronidas, and using this curve, the hyaluronidase activities in each sample were calculated. In some assays, the samples were boiled for 5 min or combined with 5 µg/ml of apigenin (Sigma) before being applied to the wells.

Detection of Hyaluronidase Proteins (SDS-PAGE and Western Blotting)—MIA PaCa-2 cells were seeded into 10-cm dishes at 1 × 10⁵ cells per dish, cultured overnight at 37 °C, and then lysed with 50 mM Tris-HCl (pH 7.5) containing 140 mM NaCl, 1.6 mM MgCl₂, 1% Nonidet P-40, 5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonfluoride. Samples extracted from equal numbers of cells were separated by electrophoresis on a SDS-polyacrylamide gel under non-reducing conditions, and transferred to a polyvinylidene difluoride filter. In some assays, 15 µL of M-sup/conc or fresh RPMI was applied instead of cell lysate. The filter was blocked in PBS containing 3% bovine serum albumin and then incubated with anti-Hyal-1 pAb, anti-Hyal-2 pAb, or anti-β-tubulin mAb. The filters were then incubated with HRP-conjugated anti-rabbit IgG to detect the anti-Hyal-1 and anti-Hyal-2 pAbs, or with HRP-conjugated anti-mouse IgG to detect the anti-β-tubulin mAb. The secondary Abs were detected using ECL Western blotting detection reagents (Amersham Biosciences).

Measurement of HA Levels (ELISA)—The HA concentrations in the tumor cell culture supernatants and their gel-filtrated samples were determined by an ELISA that is based on a competitive binding principle (39, 40). The HA present in the samples competes with the microtiter wells to bind biotinylated HABP (39, 40). Ninety-six-well microtiter plates coated with 25 µg/ml 1,000-kDa HA were incubated with serial dilutions of the culture supernatant of tumor cells, the gel-filtrated samples of the culture supernatants, or 1,000-kDa HA and 1 µg/ml biotinylated HABP. Following incubation at 37 °C for 4 h, the wells were washed in PBS containing 0.1% Tween 20. The HABP bound to the wells was then analyzed quantitatively using an avidin-biotin detection system. A standard curve was plotted by plotting the absorbance at 490 nm against the concentration of 1,000-kDa HA, and using this curve, the HA concentration in each sample was calculated.
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Size Profiling and Purification of MIA PaCa-2-related HA Oligosaccharides (Gel Filtration Chromatography) —Size profiles of the HA species present in the tumor cell culture supernatant were examined by gel filtration chromatography, in which the sizes of the HA molecules were determined by calibrating the column with 1,000-kDa HA, HA 36-mers (6.9-kDa HA), and HA 10-mers. The tumor cell culture supernatants were applied to a Sepharose CL-6B column (1.5 x 120 cm, Amersham Biosciences) equilibrated with PBS. The column was eluted in PBS at 0.875 ml/min, and 3.5-ml fractions were collected. Subsequently, the fractions were assayed for HA by ELISA as described above. Fractions containing HA oligosaccharides ranging from ~10- to 40-mers were collected and designated as MIA PaCa-2-derived HA oligosaccharides (M-HA).

HA Degradation (Hyaluronidase SD Treatment) —HA fragments contained in the gel-filtrated samples of the tumor cell culture supernatant were digested with 0.05 units of hyaluronidase SD at 37 °C for 4 h, and then passed through a 0.22-μm pore filter. The samples were dialyzed against PBS, and assayed for residual HA by ELISA as described above. In some experiments, hyaluronidase SD was heat-inactivated by boiling before use.

CD44 Cleavage Assay —The CD44 cleavage assay was performed as reported previously (29). In brief, MIA PaCa-2 cells (5 x 10⁵ cells per well) were seeded into 24-well plates, cultured overnight at 37 °C, and then incubated with 10 μM MG132 for 30 min at 37 °C to inhibit the secondary cleavage of the CD44 intracellular domain (33). The cells were then incubated with various samples for 1 h at 37 °C in the presence of 10 μM MG132. The cells were lysed with SDS sample buffer (2% SDS, 10% glycerol, 0.1 M dithiothreitol, 120 mM Tris-HCl, pH 6.8, 0.02% bromphenol blue) and boiled for 5 min. Samples extracted from equal numbers of cells were separated by electrophoresis on an SDS-polyacrylamide gel under reducing conditions and transferred to a polyvinylidene difluoride filter. The filter was blocked in PBS containing 3% bovine serum albumin and then incubated with anti-CD44cyto pAb or with anti-β-tubulin mAb. The filter was then incubated with HRP-conjugated anti-rabbit IgG to detect the anti-CD44cyto pAb, or with HRP-conjugated anti-mouse IgG to detect the anti-β-tubulin mAb. The secondary Abs were detected using ECL Western blotting detection reagents.

Immunofluorescence Microscopy —Immunofluorescence microscopy was performed as reported previously (29).

Migration Assay —Cell migration was analyzed using 12-well Costar Transwell chambers (Corning Inc., Corning, NY) containing polycarbonate filters with a 12-μm pore size (29). Both sides of the filter were coated with 500 μg/ml 1,000-kDa HA. MIA PaCa-2 cells (2 x 10⁶ cells/ml) were added to the upper compartment and were incubated at 37 °C for 3 h so that the cells became attached to the filter. The cells were then incubated with or without 10 μg/ml BRIC235 or mouse IgG 20 min prior to and during the migration assay. Finally, various HA preparations were added to the upper compartment of the wells at a final concentration of 50 μg/ml. The chambers were subsequently incubated at 37 °C for 15 h. After the cells on the upper side of the filters were gently wiped off, the filters were fixed in methanol, stained with hematoxylin and eosin, and mounted on glass slides. Cells that had migrated to the lower side of the filters were counted under a light microscope. The number of cells in 10 defined high-power fields (x200) was counted, and the average was determined. Each assay was performed five times.

RESULTS

MIA PaCa-2 Cells Express Hyaluronidases and Secrete Them into the Culture Supernatant —We have previously reported that HA oligosaccharides of a certain size range up-regulate CD44 cleavage in CD44-expressing pancreatic carcinoma cell line, MIA PaCa-2 (29). Because the MIA PaCa-2 cells show CD44 cleavage in the absence of any exogenous stimulation at a readily detectable level, we investigated the possibility that these tumor cells may themselves generate CD44 cleavage-inducible HA oligosaccharides by expressing HA-degrading enzymes. As shown in Fig. 1A, the MIA PaCa-2 culture supernatant was found to contain potent HA-degrading activities, which digested HA in a highly pH-dependent manner with the optimal pH of 4.0. These activities were abrogated by heat inactivation of the culture supernatant (Fig. 1B) and by a hyaluronidase inhibitor, apigenin (Fig. 1C), in accordance with the
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hypothesis that MIA PaCa-2 cells produce hyaluronidases. The presence of HA-degrading activities in the culture supernatant was also confirmed by a substrate-gel electrophoresis analysis (data not shown). Furthermore, as shown in Fig. 2, MIA PaCa-2 cells expressed two of the known hyaluronidases, Hyal-1 (41) and Hyal-2 (42), at both the mRNA and protein levels and secreted both of these proteins into the culture supernatant (Fig. 2). Consistent with previous data, Hyal-1 and Hyal-2 expression were detected in a human prostate cancer cell line, LNCaP (17) and in a human breast cancer cell line, MDA-MB231 (37), respectively, at both mRNA and protein levels (data not shown).

MIA PaCa-2 Cells Generate HA Oligosaccharides—Tumor cells often demonstrate an up-regulated production of HA (9, 10), and MIA PaCa-2 cells were no exception. As shown in Fig. 3A, we could detect high levels of HA in the MIA PaCa-2 culture supernatant. We then investigated the size profiles of the HA contained in the MIA PaCa-2 culture supernatant by gel filtration chromatography, in which the HA sizes were estimated by calibrating the column with three different sizes of HA preparations: 1000-kDa HA, HA 36-mers (6.9-kDa HA), and HA 10-mers. As shown in Fig. 3B, MIA PaCa-2 cells generated HA oligosaccharides ranging from ~10- to 40-mers (Fig. 3B), which were very similar in size to those that have been shown to enhance CD44 cleavage in CD44-expressing tumor cells (29). These results are thus consistent with the hypothesis that MIA PaCa-2 cells degrade HA by hyaluronidases they produce and that the HA oligosaccharides that are generated have the ability to enhance CD44 cleavage and tumor cell motility (29).

Two other pancreatic tumor cell lines we examined (Capan-1 and BxPC-3) also expressed substantial levels of LMW-HAs (data not shown).

MIA PaCa-2 Cell-derived HA Oligosaccharides Up-regulate CD44 Cleavage and Promote Cell Motility of the MIA PaCa-2 Cells Themselves—We next examined whether MIA PaCa-2 cells actually generate molecules that can enhance CD44 cleavage in MIA PaCa-2 cells. As shown in Fig. 4A, the addition of MIA PaCa-2 culture supernatant induced the up-regulation of CD44 cleavage in MIA PaCa-2 cells, as evidenced by an increase in the membrane-bound 25-kDa cleavage product in Western blotting analysis; this cleavage was strongly inhibited by the Fab fragment of the anti-CD44 neutralizing monoclonal antibody BRIC235 (29, 43), indicating that the up-regulated CD44 cleavage was because of the interaction between CD44 and its ligand. Furthermore, as shown in Fig. 4B, the addition of the MIA PaCa-2 culture supernatant induced numerous filopodia and actin filament remodeling in MIA PaCa-2 cells to a level comparable with that induced by the addition of HA 36-mers (29). These changes, which reflect increased cell motility, were almost completely abrogated by the Fab fragment of BRIC235, indicating that they were also induced by the interaction of cell-surface CD44 and its ligand. In addition, as shown in Fig. 4C, the MIA PaCa-2 culture supernatant also enhanced the migration of MIA PaCa-2 cells in a Transwell migration assay; this migration was almost completely obliterated by the anti-CD44 monoclonal antibody BRIC235 but not by mouse IgG (Fig. 4C), indicating that the enhanced tumor cell motility was also dependent on the CD44-HA interaction. The results that the morphological changes (Fig. 4B) and the increased cell motility (Fig. 4C) were observed on HA-coated materials but not on fibronectin-coated materials (data not shown), again suggest the CD44 dependence of these changes.

Together, these results support the possibility that the HA oligosaccharides produced by the MIA PaCa-2 cells themselves can up-regulate CD44 cleavage and cell motility in the MIA PaCa-2 cells.

**FIGURE 2.** MIA PaCa-2 cells express hyaluronidases and secrete them into the culture supernatant. The expression levels of Hyal-1 (upper panel) and Hyal-2 (lower panel) were examined by reverse transcriptase (RT)-PCR (lanes 1 and 4) and Western blotting (lanes 2–4). Note that both Hyal-1 and Hyal-2 were expressed at the mRNA level (lane 1) and at the protein level (cell lysate, lane 2; and M-sup/conc, lane 4) in MIA PaCa-2 cells.

**FIGURE 3.** MIA PaCa-2 culture supernatant (M-sup) contains HA oligosaccharides. A, the concentration of HA in M-sup was determined by an ELISA as described under “Materials and Methods.” B, the size profile of HA contained in the MIA PaCa-2 culture supernatant was determined by gel-filtration chromatography on a Sepharose CL-6B column, which was standardized using 1000-kDa HA, HA 36-mers (6.9-kDa HA), and HA 10-mers. HA fragments ranging from ~10- to 40-mers were detected in the culture supernatant.
FIGURE 4. MIA PaCa-2 culture supernatant (M-sup) up-regulates CD44 cleavage and promotes tumor cell migration in MIA PaCa-2 cells. A, MIA PaCa-2 cells were preincubated with (lane 3) or without (lanes 1, 2, and 4) BRIC235 Fab fragments for 30 min, followed by further incubation with MG132 for another 30 min. The culture supernatant was then changed to 1 ml of fresh RPMI medium (lanes 1 and 4) or M-sup (lanes 2 and 3). After adding BRIC235 Fab fragments and MG132, the cells were incubated with (lane 4) or without (lanes 1–3) 50 μg/ml HA 36-mers for 1 h. The cells were lysed with SDS sample buffer, and samples containing equal amounts of cell lysate were analyzed by immunoblotting with anti-CD44cyto pAb (upper panel) or anti-β-tubulin mAb (lower panel). B, MIA PaCa-2 cells were seeded onto HA-coated cover glasses placed in a 6-well plate and incubated overnight. The cells were preincubated with (G–I) or without (A–F and J–L) BRIC235 Fab fragments, followed by further incubation without (A–C) or with 2.5 ml of M-sup (D–I) or 50 μg/ml HA 36-mers (J–L). After stimulation, the cells were double stained with anti-CD44cyto pAb (A, D, G, and J) and rhodamine-conjugated phalloidin (C, F, I, and L). The samples were analyzed with a confocal microscope. Merged images are also shown (B, E, H, and K). The results shown are representative of at least three independent experiments. C, the effect of M-sup on the MIA PaCa-2 cell migration was assessed by a Boyden chamber-type migration assay. Cells were placed on the upper side of the HA-coated filters and incubated in the absence of antibodies (columns 1, 4, and 7), or in the presence of BRIC235 (columns 2, 5, and 8) or mouse IgG (columns 3, 6, and 9) for 20 min. The cells were then combined with culture medium alone (columns 1–3), M-sup (columns 4–6), or HA 36-mers (columns 7–9) and cultured for 12 h in the presence of the antibodies. Columns and bars represent the mean ± S.D. obtained from five independent experiments. Statistical differences were determined with Student’s t-test; asterisk, p < 0.05.

FIGURE 5. MIA PaCa-2 cell-derived HA oligosaccharides (M-HA) up-regulate CD44 cleavage by interacting with CD44 in MIA PaCa-2 cells. A, MIA PaCa-2 cells were treated with 10 μM MG132 for 30 min, followed by further incubation with PBS alone (lane 1) or with M-HA at various concentrations (lane 2, 10 μg/ml; lane 3, 25 μg/ml; lane 4, 50 μg/ml; lane 5, 100 μg/ml). Cells were preincubated with (lane 3) or without (lanes 1 and 2) 10 μg/ml BRIC235 Fab fragments for 30 min. The cells were then incubated with MG132 for another 30 min, and then with (lanes 2 and 3) or without (lane 1) 50 μg/ml M-HA. C, the HA concentration of fresh M-HA (column 1) or M-HA treated with intact hyaluronidase SD (column 2), or boiled hyaluronidase SD (column 3) was determined by an ELISA as described under “Experimental Procedures.” D, MIA PaCa-2 cells were treated with MG132, and were further incubated with PBS alone (lane 1), M-HA (lane 2), M-HA/HAase (lane 3), or M-HA/HAase-boiled (lane 4). MIA PaCa-2 cells in A, B, and D were finally lysed with SDS sample buffer, and samples containing equal amounts of cell lysate were analyzed by immunoblotting with anti-CD44cyto pAb (upper panel) or anti-β-tubulin mAb (lower panel).
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We next examined whether the HA oligosaccharides isolated from the MIA PaCa-2 culture supernatant could enhance CD44 cleavage. As shown in Fig. 5A, the MIA PaCa-2-derived HA oligosaccharides indeed induced the up-regulation of CD44 cleavage in MIA PaCa-2 cells in a concentration-dependent manner. The enhancement was almost completely inhibited by the Fab fragment of BRIC235 (29, 43) (Fig. 5B), indicating that the cleavage was induced by the interaction between CD44 and the HA oligosaccharides. When completely digested by the hyaluronidase derived from S. dysgalactiae (Fig. 5, C and D), the MIA PaCa-2-derived HA oligosaccharides lost their ability to enhance CD44 cleavage, whereas the same treatment with heat-inactivated hyaluronidase did not (Fig. 5, C and D). In addition, as shown in Fig. 6, the MIA PaCa-2-derived HA oligosaccharides induced virtually the same changes in the cell morphology and motility of MIA PaCa-2 cells as those observed by the addition of MIA PaCa-2-derived culture supernatant (Fig. 4): the tumor-cell-derived HA oligosaccharides enhanced filopodia and actin filament formation (Fig. 6A) and tumor cell migration in vitro (Fig. 6B). Very similar changes were observed with HA 36-mers (Fig. 6, A and B), and as expected, all these changes in cell morphology and motility were abrogated by Fab BRIC235 (Fig. 6, A and B), confirming that they were dependent on the interaction between CD44 and HA. The tumor-derived HA did not appear to affect cell proliferation in vitro (data not shown). Collectively, these results strongly indicate that the MIA PaCa-2 cells themselves constitutively generate HA oligosaccharides that can enhance CD44 cleavage and tumor cell motility via the action of HA-degrading enzymes that the tumor cells also produce.

**DISCUSSION**

In this report, we propose a novel autocrine/paracrine activation mechanism for tumor cells. We demonstrated that human pancreatic carcinoma MIA PaCa-2 cells produce active hyaluronidases and HA oligosaccharides of ~10–40-mers, and that the tumor-derived HA oligosaccharides enhance CD44 cleavage and tumor cell motility via interaction with CD44 molecules. Thus, tumor cells appear to generate HA oligosaccharides that bind to their own tumor cell CD44 by expressing hyaluronidases constitutively. This in turn enhances their own CD44 cleavage and cell motility that subsequently promotes tumor progression and invasion.

Six human hyaluronidase genes have been reported, and five of them are translated into hyaluronidase proteins (16). Among them, Hyal-1 and Hyal-2 act as the major hyaluronidases for HA degradation in somatic tissues (15). Both Hyal-1 and Hyal-2 are reported to be involved in tumor progression. For example, Hyal-1 is detected in bladder cancer (17) and prostate cancer tissues, whereas the Hyal-2 expression level is increased in breast tumor metastases (20), and the overexpression of Hyal-2 accelerates intracerebral tumor formation in astrocytoma cells (44). In the present study, we found that both Hyal-1 and Hyal-2 were secreted by MIA PaCa-2 tumor cells (Fig. 2), and inhibition of the hyaluronidase activities resulted in complete abrogation of the HA-degradation activities of the culture supernatant (Fig. 1). These findings indicated that hyaluronidase-mediated degradation plays a major role in HA oligosaccharide generation in MIA PaCa-2 cells. Other mechanisms, such as those induced by oxygen-derived free radicals (45) or the de novo synthesis of low molecular mass HA (46), may play only a minor role in MIA PaCa-2 cells. Given that the HA oligosaccharides detected in the culture supernatant ranged from 10- to 40-mers, either Hyal-1 or Hyal-1-like hyaluronidase (18) may be the main enzyme involved in HA degradation in these tumor cells, although the exact identity of the hyaluronidases awaits determination.

With regard to the regulation of hyaluronidase expression, we addressed the possibility that the HA oligosaccharides produced by the tumor cells might stimulate hyaluronidase expression by acting on the cell-surface HA receptors. Although we found that the exogenous addition of HA oligosaccharides induced small increases in hyaluronidase expression levels in the tumor cells (data not shown), the endogenous expression level was so high that it was difficult to judge whether this was a significant increase or not. It remains therefore unclear whether the tumor-derived HA oligosaccharides actually contribute to the regulation of the hyaluronidase expression level in MIA PaCa-2 cells.

Hyaluronidases are acid-active HA-degrading enzymes (pH optima 3.7–4.5). It was recently reported that in breast tumor cells, the HA-CD44 interaction activates Na+/H+ exchange activity, which creates an acidic extracellular matrix environment, leading to Hyal-2 and cathepsin B activation (37). It is tempting to imagine that such a mechanism also occurs in MIA PaCa-2 cells, promoting an acidic microenvironment where hyaluronidases can act properly, enabling the cells to generate HA oligosaccharides readily.

Low molecular weight HA oligosaccharides induce various biological activities (21). For instance, HA 4–6-mers enhance the expression of metalloproteases and urokinase plasminogen activator in murine tumor...
cells (47), HA 6–20-mers induce angiogenesis in the chick choioallantoic membrane (25), and HA 36-mers (6.9-kDa HA) up-regulate Fas expression in human rheumatoid synovial cells (48) and activate LFA-1 in human colon cancer cells (28). An HA preparation containing mainly 10–12-mers inhibits the anchorage-independent in vitro growth of human lung carcinoma cells (49) and the in vivo growth of murine melanoma (50), mammary carcinoma (49), and human lung carcinoma (49). We therefore examined effects of the tumor-derived HA oligosaccharides (~10–40-mers) on Mia PaCa-2 cells. The results demonstrate that the tumor-derived HA oligosaccharides strongly enhanced CD44 cleavage (Fig. 5), induced prominent filopodia formation (Fig. 6A), and promoted cell motility (Fig. 6B) in Mia PaCa-2 cells, and all these activities were abrogated by a neutralizing anti-CD44 mAb. Growth inhibition was not obvious. These results suggest the presence of an autocrine/paracrine activation mechanism in which tumor cells generate HA oligosaccharides themselves by constitutively expressing hyaluronidases, and the HA degradation products bind to tumor cell CD44 to enhance the CD44 cleavage and motility of the tumor cells. This would subsequently promote tumor progression and invasion, probably without affecting tumor cell growth. Interruption of the CD44-HA interaction resulted in complete abrogation of the increased CD44 cleavage, cell motility, and invasion. These results are consistent with the concept that the CD44-HA interaction plays an essential role in the regulation of tumor cell motility and invasion (2) and may be extended to suggest that the tumor-derived HA oligosaccharide-CD44 interaction provides an autocrine/paracrine-like mechanism in the regulation of cell motility and invasion. Thus, tumor cells may sustain their own invasive activities through the actions of hyaluronidases, CD44, and HA oligosaccharides.

The serum level of soluble CD44 that results from CD44 cleavage has been reported to correlate with tumor burden in patients with gastric and colon carcinomas (51). Enhanced CD44 cleavage was reported in invasive tumors such as glioma, breast carcinoma, non-small cell lung carcinoma, colon carcinoma, and ovarian carcinoma (34). Stimuli that up-regulate CD44 cleavage, such as phorbol esters (52) and HA oligosaccharides (29), promote tumor cell migration. Accordingly, the proteolytic cleavage of CD44 has been implicated in tumor progression. The induction of CD44 cleavage by tumor cell products such as HA oligosaccharides would thus provide the tumor cells with another significant advantage in the process of invasion and metastasis.

In conclusion, a mechanism consisting of constitutively high hyaluronidase expression, constitutive generation of HA oligosaccharides, sustained CD44 stimulation, and the sustained induction of tumor motility and invasion may represent a novel autocrine/paracrine activation mechanism in tumor cells, which would lead to the promotion and maintenance of their own malignant properties. In addition, because HA oligosaccharides show divergent pro-tumoral functions by binding to CD44 and non-CD44 HA receptors of not only tumor cells but also the surrounding stromal cells (2), inhibition of their generation and function may provide novel approaches for the treatment of cancer.
Hyaluronan Oligomers as Autocrine Tumor Progression Factors

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