Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan involved in a wide variety of cellular functions. However, its turnover in living cells remains largely unknown. In this study, CD44, a receptor for HA, and hyaluronidase-1, -2, and -3 (Hyal-1, -2 and -3) were stably expressed in HEK 293 cells and the mechanism of HA catabolism was systematically investigated using fluorescein-labeled HA. CD44 was essential for HA degradation by both endogenous and exogenously expressed hyaluronidases. Hyal-1 was not able to cleave HA in living cells in the absence of CD44. Intracellular HA degradation was predominately mediated by Hyal-1 after incorporation of HA by CD44. Although Hyal-1 was active only in intracellular space in vivo, a certain amount of the enzyme was secreted to extracellular space. This extracellular Hyal-1 was found to be incorporated by cells and such uptake of Hyal-1 was, in part, involved in the intracellular degradation of HA. Hyal-2 was involved in the extracellular degradation of HA. Hyal-2 activity was also dependent on the expression of CD44 in both living cells and enzyme assays. Immunofluorescence microscopy demonstrated that both Hyal-2 and CD44 are present on the cell surface. Without CD44 expression, Hyal-2 existed in a granular pattern, and did not show hyaluronidase activity, suggesting that localization change could contribute to Hyal-2 function. A convenient and quantitative enzyme assay was established for the measurement of Hyal-2 activity. Hyal-2 activity was detected in the membrane fraction of cells co-expressing Hyal-2 and CD44. The pH optimum for Hyal-2 was 6.0–7.0. The membrane fraction of cells expressing Hyal-2 alone did not show hyaluronidase activity. Hyal-3 did not show any hyaluronidase activity in our experimental conditions. Based on these findings, Hyal-1 and -2 contribute to intracellular and extracellular catabolism of HA, respectively, in a CD44-dependent manner, and their HA degradation occurs independently from one another.

Hyaluronic acid (HA) is a negatively charged, high molecular weight glycosaminoglycan found predominantly in the extracellular matrix. It is the simplest of the glycosaminoglycans, the only one not covalently linked to core protein, and is unbranched and composed of repeating alternating units of glucuronic acid and N-acetylgalactosamine. Despite the simplicity of its composition, HA has a great number of biological functions. It not only functions as a biological glue that participates in lubricating joints or holding together gel-like connective tissues, but also functions as a microenvironmental cue that co-regulates cell behavior during embryonic development and morphogenesis, wound healing, repair and regeneration, inflammation, and tumor progression and invasion.

There are 15 g of HA in a 70-kg individual, of which 5 g is replaced daily. In the skin, which contains 50% of the total body HA, the half-life of HA is about 1 day, and even in as seemingly inert a tissue as cartilage, HA turns over with a half-life of 1–3 weeks (10). In the bloodstream, the half-life of HA is 2–5 min (11). All such catabolism is presumably the result of hyaluronidases. Hyaluronidases and their attendant control mechanisms are apparently of critical importance in normal and abnormal biology. There are six hyaluronidase-like sequences in the mammalian genome. All are transcriptionally active with unique tissue distributions. In humans, three genes, HYAL1, HYAL2, and HYAL3 are clustered on chromosome 3p21.3, coding for Hyal-1, Hyal-2, and Hyal-3, respectively. Another three genes, HYAL4, PHYLAL1, and SPAM1, are clustered similarly on chromosome 7q31.3. They code for Hyal-4, a pseudogene transcribed but not translated in humans, and PH-20, the sperm enzyme, respectively (12, 13). Among them, Hyal-1, Hyal-2, and Hyal-3 are somatically expressed hyaluronidases. Hyal-1 known as serum hyaluronidase, is an acid-active lysosomal enzyme (14, 15) that is able to utilize HA of any size as a substrate, and which predominantly generates tetrasaccharides. Hyal-2 (16, 17) is also supposed to be an acid-active enzyme and is linked to plasma membranes by a glycosylphosphatidylinositol anchor (18). This hyaluronidase has unique substrate specificity, cleaving high molecular mass HA polymers to intermediate size fragments of ~20 kDa, or about 50 disaccharide units. Little is known about Hyal-3, whose strong hybridization expression patterns are found in mammalian testis and bone marrow (12). These two tissues retain a fetal and stem cell-like state for the life of the animal, suggesting that Hyal-3 may have an important function in vivo. However, it has not been conclusively shown to possess hyaluronidase activity in vitro to date.

Several studies have shown that the HA is endocytosed by cells (chondrocytes, macrophages, keratinocytes, etc.) for degradation and that the endocytosis is mediated via cell surface HA receptors. Endocytosis and degradation of HA was inhib-

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3 The abbreviations used are: HA, hyaluronic acid; Hyal-1, -2, and -3, hyaluronidase-1, -2, and -3; HEK 293, human embryonic kidney 293; PBS, phosphate-buffered saline; fl-HA, fluorescein-labeled HA; Vcpal, L-ascorbic acid 6-hexadecanoate; IRES, internal ribosome entry site.
Mammalian Hyaluronidases and CD44

by the anti-CD44 antibody, suggesting that CD44 has a critical role in HA turnover (19–21). A model was proposed based on observations in breast cancer cells (22, 23). The high molecular weight HA is tethered to the cell surface by the combi-

ded efforts of CD44 and Hyal-2. HA is cleaved by Hyal-2 to the 20-kDa limited sized products corresponding to about 50 disaccharide units in a specialized microenvironment. The Hyal-2-generated HA fragments are internalized, delivered to endosomes, and ultimately to lysosomes, where Hyal-1 degrades the 20-kDa fragments to small tetrasaccharides. However, the relationships and differences between these mammalian hyaluronidases have not been clearly demonstrated.

In this study, various kinds of cell lines stably expressing human Hyal-1, Hyal-2, Hyal-3, and/or CD44 were established for systematic characterization of mammalian hyaluronidases, and for better understanding the HA catabolism in somatic tissues. HA catabolism in these cell lines was analyzed in detail using HA conjugated with fluorescein. In addition, an efficient and quantitative enzyme assay method was developed for the measurement of Hyal-2 activity. Hyal-1 and -2 were revealed to contribute to intracellular and extracellular catabolism of hyaluronic acid, respectively, in a CD44-dependent manner.

EXPERIMENTAL PROCEDURES

Establishment of Cell Lines Expressing HYAL1, HYAL2, HYAL3, and CD44—HEK 293 (293) cells were maintained in a humidified incubator at 37 °C in 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cDNA of human CD44 was amplified by a PCR using a Human Lymph Node cDNA Library (Takara) as a template, and inserted into the expression vector pRK5 (BD Pharmingen). HEK 293 cells were transfected with pRK5/CD44 and pSV2neo (Clontech) by a calcium phosphate transfection method. A cell lineage expressing CD44 was selected using 500 µg/ml of hygromycin B (Invitrogen). In this way, various kinds of cell lines stably expressing human Hyal-1, Hyal-2, Hyal-3, and/or CD44 were established for systematic characterization of mammalian hyaluronidases, and for better understanding the HA catabolism in somatic tissues. HA catabolism in these cell lines was analyzed in detail using HA conjugated with fluorescein. In addition, an efficient and quantitative enzyme assay method was developed for the measurement of Hyal-2 activity. Hyal-1 and -2 were revealed to contribute to intracellular and extracellular catabolism of hyaluronic acid, respectively, in a CD44-dependent manner.

Preparation of Fluorescein-labeled HA and Biotin-conjugated HA—Fluorescein-labeled HA (fl-HA) was prepared as described by de Belder and Wik (24) with a slight modification. Briefly, 50 mg of HA (800–1200 kDa, Seikagaku Kogyo) was dissolved in 40 ml of water, and mixed with 20 ml of dimethyl sulfoxide (Me2SO). Fluorescein amine, isotype I (25 mg, Aldrich) in Me2SO (0.5 ml) containing acetaldehyde (25 µl, Fluka) and cyclohexyl isocyanide (25 µl, Fluka) was added to the mixture. The mixture was incubated at 25 °C for 5 h, and then poured into 240 ml of ethanol saturated with NaCl. The precipitated fl-HA was collected by centrifugation at 1000 x g, the supernatant (20 µg of total proteins) and the conditioned medium (5 µl) from each cell line was incubated with 40 µg of HA (800–1200 kDa, Seikagaku Kogyo) in 50 µl of 1% Triton X-100, 50 mM NaCl, 100 mM formic acid, pH 3.7, at 37 °C for 24 h. In another experiment, conditioned medium was incubated with HA in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, at 37 °C for 24 h. An aliquot of the reaction solution was electrophoresed on 0.5% agarose gel and stained with Stains-all (Polysciences, Inc.).

Characterization of fl-HA Degradation by Intact Cells Using Size Exclusion Chromatography—Cells (5 x 10⁶) were plated in 10-cm type I collagen-coated dishes and cultured for 24 h. The cells were then cultured in 10 ml of medium containing fl-HA. After incubation, the conditioned medium was collected, and the cells were detached and harvested by washing 2 times with PBS. Then, the cells were washed again with PBS and incubated in 1 ml of 0.05% trypsin at 37 °C for 5 min followed by a 5-min centrifugation at 1000 x g. This supernatant was defined as the cell surface pool. The cell pellets were washed again 3 times with PBS and then incubated in 50 mM Tris-HCl, pH 7.4, containing 400 µg/ml proteinase K (Sigma) at 37 °C for 24 h to solubilize the cells and release the total intracellular material.
The solubilized fraction was termed the intracellular pool. The fl-HA in 1 ml of conditioned medium, total cell surface pool, and the total intracellular pool was characterized by size exclusion chromatography using a Sepharose CL-2B (1.6 × 25 cm) column equilibrated with PBS. Fractions of 2.5 ml were collected at 0.5 ml/min and the fluorescence (excitation, 485 nm; emission, 535 nm) was determined by an ARVO Fluoroscan (PerkinElmer Life Sciences). The column void volume was determined as the elution volume for blue dextran 2000 (Amer sham Biosciences).

Endocytosis of Hyal-1 by Cells—Cells (5 × 10⁵) were plated on a 24-well type I collagen pre-coated plate and cultured for 24 h. The cells were then cultured for various periods of time in 1 ml of medium containing 50% conditioned medium from a 24-h confluent culture of Hyal-1/293 or 293 cells. After incubation, the cells were washed 3 times with PBS, and incubated in 1 ml of 0.05% trypsin at 37 °C for 5 min, followed by a 5-min centrifugation at 1300 × g. The cell pellets were washed again 2 times with PBS and disrupted by sonication in PBS by sonication. After centrifugation at 1300 × g, Hyal-1 activity in the supernatant (6.4 μg of total protein) was measured as described above. In another experiment, cells (5 × 10⁵) were plated in 10-cm type I collagen-coated dishes and cultured for 24 h. The cells were then cultured in 10 ml of medium containing fl-HA (10 μg/ml) and 20% conditioned medium from a 24-h confluent culture of Hyal-1/293 or 293 cells. Then the medium, the cell surface pool, and the intracellular pool were prepared and analyzed by the Sepharose CL-2B column as described above.

Immunofluorescence—Immunofluorescence for Hyal-2 was basically performed according to the protocol for glycosylphosphatidylinositol-anchored proteins (25). In brief, cells (5 × 10⁵) plated on a 96-well poly-D-lysine pre-coated plate were washed with PBS and blocked in a blocking solution (PBS supplemented with 5% normal goat serum) for 15 min at room temperature. After removing the blocking solution, the cells were incubated in primary antibodies, mouse anti-human CD44 antibody, and rabbit anti-human Hyal-2, in blocking buffer for 30 min at room temperature. After subsequent washings, the cells were stained with Cy5-labeled goat anti-rabbit IgG antibody (Zymed Laboratories Inc.), Alexa Fluor 350-labeled goat anti-mouse IgG antibody (Molecular Probes), and fl-HA in blocking buffer for 30 min at room temperature. Then the cells were washed and fixed in 10% formaldehyde in PBS for 10 min and immunofluorescence microscopy was done using an IN Cell Analyzer 1000 (GE Healthcare).

Measurement of Hyal-2 Activity—Cells were washed and harvested with PBS, and disrupted by sonicating in PBS. The suspensions of the disrupted cells were centrifuged at 400 × g for 5 min. Then, the supernatant was subjected to centrifugation at 20,000 × g for 10 min. The supernatant was applied to a Sepharose CL-2B column and eluted with PBS. The enzyme assay was further modified for more quantitative analysis in a microplate format. Biotin-conjugated HA (30 μl, 2 μg/ml in PBS) was immobilized onto 384-well, black, and high binding microplate (Greiner Bio-One) by incubation at 4 °C overnight. Then the plate was washed 3 times with 100 μl of 50 mM NaCl, 100 mM phosphate buffer, pH 6.5. The membrane fractions (40 μl, 0–100 μg/ml of total protein in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5) were added to each well and incubated at 37 °C for various periods of time. Then, each well was washed 3 times with 100 μl of 50 mM NaCl, 100 mM phosphate buffer, pH 6.5. The biotin-conjugated HA remaining on the plate was detected using DELFIA assay solutions (PerkinElmer Life Sciences). In brief, the plates were incubated with europium-labeled streptavidin for 1 h at room temperature and washed 3 times with the washing solution. Enhancement solution was added, mixed for 5 min, and the time-resolved fluorescence was determined by an ARVO Fluoroscan (PerkinElmer Life Sciences). All these solutions were prepared and used according to the manufacturer’s directions.

RESULTS

Establishment of 293 Cells Stably Expressing Hyal-1, Hyal-2, Hyal-3, and/or CD44—Various cells endogenously express hyaluronidases, but their expression level is relatively low, and several types of hyaluronidases are expressed simultaneously (12), resulting in difficulty in clear characterization of each hyaluronidase. In addition, the expression level of CD44 should be also taken into account for their characterization. These facts led us to establish various cell lines stably expressing CD44 and/or Hyal-1, -2, and -3 for functional analysis of each hyaluronidase in living cells. First, CD44-overexpressing cell lineages, named CD44/293, was established from 293 cells by transfection with human CD44 cDNA. Next, 293 and CD44/293 cells were transfected with the constructs for expressing human HYAL1, HYAL2, and HYAL3. The internal ribosome entry site (IRES) system was utilized for this purpose, as the system enabled us to establish multiple cell lines efficiently without cell cloning and to minimize the difference in background among various stably transfected cell lines for comparative studies. Consequently, hyaluronidase-expressing cell lines (Hyal-1/293, Hyal-2/293, and Hyal-3/293) and CD44-hyaluronidase co-expressing cell lines (Hyal-1/CD44/293, Hyal-2/CD44/293, and Hyal-3/CD44/293) were established. The total cell lysates were extracted from each cell line and Western blot analyses were performed using anti-human CD44, Hyal-2 and Hyal-3 antibodies to assess whether each protein was expressed appropriately (Fig. 1). Lysates from CD44-transfected 293 cells revealed a band with the same level of intensity at ~96 kDa (Fig. 1A) that corresponds to the molecular mass of the standard form of human CD44. Similarly, Hyal-2 (Fig. 1B) and Hyal-3 (Fig. 1C) were detected at the corresponding molecular masses of 55 and 46 kDa, respectively. Because we could not raise specific anti-Hyal-1 antibodies, Hyal-1 expression was assessed by the hyaluronidase activity at pH 3.7, the pH optimum of Hyal-1 (15), instead of Western blotting. Conditioned media were collected from the confluent cultures of each cell line and incubated with
high molecular weight HA. HA incubated with the conditioned media from Hyal-1/293 and Hyal-1/CD44/293 cells was degraded to a low molecular weight (Fig. 1D). Similar results were obtained when HA was incubated with the lysates from the cell lines (Fig. 1E). On the other hand, the acid-active hyaluronidase activity was not detected in conditioned media or lysates from the other cells. It should be noted that neither the conditioned media nor the lysates from any of the types of cells showed hyaluronidase activity at pH 7.4 (Fig. 1F). These results clearly indicate that only Hyal-1/293 and Hyal-1/CD44/293 cells were stably overexpressing Hyal-1 and that the expressed Hyal-1 was not only accumulated intracellularly, but was also secreted to the outside of the cells.

Degradation of Fl-HA Incubated with Cells Expressing Hyal-1, Hyal-2, Hyal-3, and/or CD44—Fl-HA, high molecular mass HA conjugated with fluoresceinamine, was prepared and used for sensitive and quantitative analysis of HA catabolism by intact cells. The eight cell lines with different expression profiles in CD44 and hyaluronidases were cultured in the presence of Fl-HA (10 μg/ml) for 48 h. After incubation, the conditioned medium (hereafter medium) was collected, and then the cells were treated with trypsin to remove pericellular-associated Fl-HA (cell surface pool). The remaining cell pellets were further subjected to exhaustive protease digestion to solubilize the cells and liberate intracellular Fl-HA (intracellular pool). The sizes of Fl-HA in these three fractions were analyzed by a size exclusion chromatography. Fig. 2 shows fluorescence derived from Fl-HA in each fraction of the Sepharose CL-2B chromatography.

Without CD44 expression, none of Hyal-1, Hyal-2, and Hyal-3 affected catabolism of Fl-HA. Most of the Fl-HA in the medium remained intact and was eluted in the void volume of the Sepharose CL-2B column (Fig. 2A–D). Only trace amounts of Fl-HA were detected in the cell surface (Fig. 2I–L) and intracellular pools (Fig. 2Q–T). Whereas, even the HA turnover derived from the endogenous hyaluronidase was accelerated...
when CD44 was expressed. The high molecular weight fl-HA in the medium of the CD44/293 cells was decreased and the partially degraded products were increased (Fig. 2E). Taken together with the fact that the conditioned medium from CD44/293 cells did not show hyaluronidase activity at neutral pH (Fig. 1E), this result indicates that the fl-HA that bound to the cells with the aid of CD44 was degraded by endogenous hyaluronidase within or on the surface of the cells, and then released into the medium. fl-HA was also incorporated into CD44/293 cells and partially degraded fl-HA was accumulated in intracellular pools (Fig. 2U).

In Hyal-1/CD44/293 cells, only fl-HA incorporated into cells by CD44 was degraded by Hyal-1 to a low molecular weight (Fig. 2, F, N, and V). In the intracellular pool, fl-HA was eluted in the total volume of the Sepharose CL-2B column, whereas the elution profiles of fl-HA from the medium and the cell surface pool were similar to those of CD44/293 cells. In contrast, almost all the fl-HA in the medium of Hyal-2/CD44/293 cells was degraded, with the majority eluted in fractions 15–18 (Fig. 2G). This result indicates that Hyal-2 was responsible for the turnover of fl-HA accumulated in the extracellular space. The presence of CD44 is critical for the Hyal-2 activity. Accumulation of degraded fl-HA was not observed in the medium from the Hyal-2/293 cells (Fig. 2C). Although fl-HA degraded by Hyal-1 had a lower molecular weight than the products in the medium by Hyal-2, it was found only in the intracellular pool. The amount of fl-HA incorporated into the cells and degraded by Hyal-1 was ~4% of fl-HA added to the dishes, whereas more than 90% of fl-HA added to the dish was degraded by Hyal-2, according to the calculation based on the fluorescence counts. Furthermore, the intracellular degradation of fl-HA was not up-regulated in the presence of Hyal-2. These observations suggest that HA degradation mediated by Hyal-2 occurs on the surface of the cells and the products were released into the medium, and that the intracellular catabolism by Hyal-1 is independent of the HA degradation on the cell surface mediated by Hyal-2.

No significant fl-HA turnover mediated Hyal-3 was detected under our experimental conditions, even though the enzyme was co-expressed with CD44 (Fig. 2, H, P, and X). Hyal-3 does not appear to possess a hyaluronidase activity. Otherwise, at least, additional components other than CD44 might be necessary for Hyal-3 to display its activity.

**Incorporation of Extracellular Hyal-1 into Cells**—Although Hyal-1 is believed to be an acid-active and a lysosomal hyaluronidase, it is well known as a plasma hyaluronidase (15). Our observation as described above also showed that a large amount of expressed Hyal-1 was found to be secreted into the medium although Hyal-1 is functional only in the intracellular space in the presence of CD44 (Fig. 1). It is possible that extracellularly secreted Hyal-1 is incorporated secondarily by cells like other lysosomal enzymes (26). To investigate this possibility, 293, CD44/293, and Hyal-2/CD44/293 cells were incubated with Hyal-1 by adding the conditioned medium of Hyal-1/293 cells. After incubation, cells were treated with trypsin to remove pericellular-associated Hyal-1, and then the presence of Hyal-1 in the intracellular pools was determined by the acid-active hyaluronidase assay. Hyal-1 was incorporated into 293 cells and its activity was detected in the intracellular pools in a time-dependent manner (Fig. 3A). The incorporation of Hyal-1 was independent of CD44 or Hyal-2 expression as no differences in the incorporation were observed among 293, CD44/293, and Hyal-2/CD44 cells (Fig. 3B).

Next, we tried to verify whether the incorporated Hyal-1 is intracellularly functional like other lysosomal enzymes. Cells of 293, CD44/293, and Hyal-2/CD44/293 were incubated with fl-HA (10 µg/ml) and the conditioned media from Hyal-1/293 or 293 cells for 48 h, and then catabolism of fl-HA in the media and intracellular pools was analyzed by Sepharose CL-2B chromatography (Fig. 3C). The added Hyal-1 did not affect extracellular degradation of fl-HA in the medium, and the activity mediated by Hyal-2 was also not influenced by Hyal-1 addition (Fig. 3C, a–c). In the intracellular pools, on the other hand, fl-HA incorporated by CD44-expressing cells was degraded to a lower molecular weight when Hyal-1 was added externally (Fig. 3C, d–f). These results show that Hyal-1 could be taken up by cells and act as an intracellular hyaluronidase.

The accumulation of high molecular non-degraded fl-HA (fractions 6–12) in the intracellular pool of CD44/293 cells was always observed when cells were incubated with conditioned media (Fig. 3C, e). The addition of the conditioned medium from 293 cells presumably enhanced the interaction between fl-HA and CD44 and the incorporation of fl-HA by CD44, which led to the apparent accumulation of non-degraded fl-HA in the intracellular pool of CD44/293 cells. On the other hand, such apparent accumulation of fl-HA was absent in Hyal-2/CD44/293 cells, as the fl-HA bound to CD44 on the cell surface was rapidly degraded by Hyal-2 and was released from the cell surface. Taken together with the fact that we could not observe any difference in the amounts of degraded fl-HA (fractions 19–25) in the intracellular pools between CD44/293 and Hyal2/CD44/293 cells (Fig. 3C, e and f), these observations also suggest that Hyal-2 mainly contributes to the extracellular degradation rather than to the intracellular degradation of HA and that intracellular degradation mediated by Hyal-1 is independent of the presence or activity of Hyal-2.

**Characterization of the Extracellular Catabolism of HA by Hyal-2/CD44/293 Cells**—For further characterization of the HA catabolism by Hyal-2, Hyal-2/CD44/293 and CD44/293 cells were cultured in the presence of fl-HA (3 µg/ml) for various periods of time, and Sepharose CL-2B chromatographic profiles of fl-HA from media, cell surface pools, and intracellular pools were compared (Fig. 4). The differences in the molecular weight of fl-HA between Hyal-2/CD44/293 and CD44/293 appeared first in the cell surface pool. After 2 h of incubation, fl-HA of the cell surface pool from the Hyal-2/CD44/293 cells was already degraded. On the other hand, the majority of the fl-HA from the CD44/293 cells retained a high molecular weight eluted in the void volume of the column (Fig. 4F). This degradation of fl-HA in the cell surface pools from Hyal-2/CD44/293 cells proceeded in a time-dependent manner, whereas the elution profile of fl-HA from CD44/293 cells did not change (Fig. 4, F–J). Following the HA degradation by Hyal-2 on the cell surface, degraded fl-HA began to be detectable in the medium after 10 h of incubation, and it was definitive after 24 h of incubation (Fig. 4, A–E). Hyal-2 activity did not
contribute to HA degradation in intracellular spaces as the size and amount of fl-HA in the intracellular pool from the Hyal-2/CD44/293 cells were not different from those of CD44/293 cells during the incubation (Fig. 4, K–O). Monensin, an inhibitor for intracellular transportation, did not affect the extracellular degradation of HA by Hyal-2 (data not shown). From these observations, it is concluded that degradation of HA by Hyal-2 takes place on the cell surface, and the degraded HA is directly released into the extracellular space. As a result, the high molecular weight of fl-HA in the medium kept decreasing and for 72 h and subjected to size exclusion chromatography (Fig. 6A). The percentage of the decrease of fl-HA that retained a high molecular weight (eluted in fraction numbers 6–10 in Fig. 6A) was calculated and plotted in Fig. 6B. Co-expression of CD44 is essential for Hyal-2 activity even under the cell-free conditions as significant hyaluronidase activity was detected only in the membrane fractions of the Hyal-2/CD44/293 cells. Neither Hyal-2/293 nor CD44/293 membrane fractions showed hyaluronidase activity. No enhancement of the enzymatic activity was observed even if the membrane fractions

FIGURE 3. Intracellular incorporation of extracellular Hyal-1. A and B, Hyal-1 activity incorporated in the cells. A, 293 cells were incubated in a medium supplemented with conditioned medium from Hyal-1/293 at 37 °C for 1–24 h. B, 293, CD44/293, and Hyal-2/CD44/293 cells were incubated with the conditioned medium from Hyal-1/293 (+) or 293 (−) cells at 37 °C for 24 h. Then intracellular pools were prepared, and total proteins were incubated with 8 μg of HA in 20 μl of 1% Triton X-100, 150 mM NaCl, 100 mM formic acid, pH 3.7, at 37 °C for 24 h. An aliquot of the reaction solution was electrophoresed on 0.5% agarose gel and stained with Stains-all. DNA markers were used for the estimation of HA size. C, fl-HA turnover by the incorporated Hyal-1. Cells of 293 (a and d), CD44/293 (b and e), and Hyal-2/CD44/293 (c and f) were incubated with fl-HA (10 μg/ml) in conditioned medium from Hyal-1/293 (a and b) or 293 (c and d) cells at 37 °C for 48 h. Then media (a–c) and intracellular pools (d–f) were applied to a Sepharose CL-2B column and eluted with PBS. The amounts of fl-HA in each fraction (2.5 ml) were determined by the fluorescence, as described under "Experimental Procedures." The arrowheads indicate the positions of the void volume (V) and the total volume (Vt) of the column.
from Hyal-2/293 cells were mixed with those of CD44/293 cells. The pH and ionic strength dependence of Hyal-2 activity are shown in Fig. 6, C and D, respectively. The pH optimum for Hyal-2 was pH 6.0–7.0. The optimum NaCl concentration was 25–100 mM, although the activity did not show a severe dependence on the salt concentration.

Although Hyal-2 activity could be defined by comparing the membrane fractions of Hyal-2/CD44/293 and other cells, the enzyme reaction was still slow and it took 72 h to detect the activity. The weak activity was presumably due to the low expression level of Hyal-2 by the IRES system. To more firmly establish the Hyal-2 activity, the cDNA of human HYAL2 was inserted into the expression vector pRK5, and introduced into CD44/293 cells. The cell line, prkHyal-2/CD44/293, expressed Hyal-2 ~20 times higher than Hyal-2/CD44/293 by Western blot analysis (Fig. 7A). Fig. 7B represents the Sepharose CL-2B chromatographic profiles of fl-HA (3 μg) incubated with the membrane fractions (1 mg of total proteins) of prkHyal-2/CD44/293 and Hyal-2/CD44/293 cells in 1 ml of 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 12 h. The higher expression level of Hyal-2 linked to higher HA degradation activity (Fig. 7C). Time-dependent fl-HA (1 μg/ml) degradation by the membrane fractions (100 μg/ml of total proteins) of prkHyal-2/CD44/293 cells is shown in Fig. 7D. The dose dependence of Hyal-2 was shown in Fig. 7E, in which fl-HA degradation was proceeded with the proportion of prkHyal-2/CD44/293 membrane fractions. Vcpal, a putative hyaluronidase inhibitor (27) inhibited Hyal-2 with an IC50 value of 72.8 μM (Fig. 7F).

Using prkHyal-2/CD44/293 cell membrane fractions, we finally developed a sensitive, high-throughput assay method in which Hyal-2 activity is measured in a microplate format (Fig. 7, G and H). In this assay, HA conjugated with biotin was used as a substrate. The microplate coated with biotin-conjugated HA was incubated with prkHyal-2/CD44/293 or CD44/293 cell membrane fractions in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C. After the reaction, biotin-conjugated HA remaining on the microplate was detected by streptavidin-conjugated europium. Hyal-2 activity showed good time and dose dependences in the microplate format.

**DISCUSSION**

CD44 and several hyaluronidases have been shown to be involved in HA turnover of somatic tissues and various cells including chondrocytes (19), keratinocytes (21), and several tumor cells (22, 28). However, the precise mechanism of HA turnover in living cells has largely remained unknown. First, we tried to characterize various hyaluronidases and CD44 by a transient expression system. However, especially in the case of Hyal-2, we could not detect any hyaluronidase activity in the cell-based assay. In the transient expression system, it was presumed that the overexpressed CD44 and Hyal-2 were not able to hold the correct conformations and/or the post-translational modifications, which would be necessary to maintain their normal localization and interaction in living cells. This preliminary finding led us to establish stable cell lines expressing CD44 and/or hyaluronidases in various combinations. The IRES system permitted us to establish these cell lines efficiently and

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**FIGURE 4. Time-dependent turnover of fl-HA by CD44/293 and Hyal-2/CD44/293 cells.** CD44/293 (○) and Hyal-2/CD44/293 (●) were incubated with fl-HA (3 μg/ml) at 37 °C for 2–24 h. Then media (A–E), cell surface pools (F–J), and intracellular pools (K–O) were applied to a Sepharose CL-2B column and eluted with PBS. The amount of fl-HA in each fraction (2.5 ml) was determined by fluorescence count as described under “Experimental Procedures.” The arrowheads indicate the positions of the void volume (V0) and the total volume (Vt) of the column.

**FIGURE 5. Distribution of Hyal-2, CD44, and fl-HA in cells.** Hyal-2/293 (A–C), CD44/293 (D–F), and Hyal-2/CD44/293 (G–I) cells were incubated with anti-Hyal-2 and anti-CD44 antibodies, and each molecule was detected by Cy5-labeled (A, D, and G) or Alexa Fluor 350-labeled (B, E, and H) secondary antibodies, respectively. Cells were incubated with fl-HA (10 μg/ml) as well as secondary antibodies (C, F, and I). After incubation, cells were fixed in 10% formaldehyde in PBS for 10 min and immunofluorescence microscopy was done using an IN Cell Analyzer 1000 (GE Healthcare). Magnification bar indicates 10 μm.
Degradation of HA by Hyal-2 takes place mainly on cell surfaces, and also depends on CD44 function(s). In Hyal-2/CD44/293 cells, but not in Hyal-2/293 cells, more than 90% of extracellular fl-HA was degraded by Hyal-2. The difference in the fl-HA molecular mass between CD44/293 and Hyal-2/CD44/293 cells appeared first in the cell surface pools, and catabolites accumulated extracellularly. Hyal-2 was localized on the cell surface, and the presence of CD44 was influenced on the localization of Hyal-2.

These observations are consistent with the previous study that reported the interaction between CD44 and Hyal-2 by co-immunoprecipitation (22). HA forms a macrocomplex in an extracellular matrix by associating with itself, cell surface proteins, or other glycosaminoglycans (31–33). In such a macrocomplex, HA degradation on cell surfaces is critical for HA catabolism, as it is quite unlikely that HA is taken up by cells as macromolecules, or other lysosomal enzymes, or other glycosaminoglycans (31–33). In such a macrocomplex, HA degradation on cell surfaces is critical for HA catabolism, as it is quite unlikely that HA is taken up by cells as macromolecules, or other lysosomal enzymes, or other glycosaminoglycans (31–33). In such a macrocomplex, HA degradation on cell surfaces is critical for HA catabolism, as it is quite unlikely that HA is taken up by cells as macromolecules, or other lysosomal enzymes, or other glycosaminoglycans (31–33).

Degradation of HA by Hyal-1 was only observed in the intracellular pool. Hyal-1 activity was limited by CD44 function because only HA taken into cells by CD44 could be the substrate for Hyal-1. These observations confirmed and emphasized previous findings in chondrocytes and other cells (15, 19) at the molecular level.

Although Hyal-1 was secreted to extracellular space like other lysosomal enzymes as reported previously (15), the evidence from patients with I-cell disease indicates that Hyal-1 is unusual and different from most lysosomal enzymes. The specific defect in I-cell disease is an enzyme essential for the synthesis of the mannose 6-phosphate recognition marker that targets enzymes to lysosomes. Plasma from patients with I-cell disease has normal levels of Hyal-1, but elevated levels of other lysosomal enzymes (29), suggesting that either Hyal-1 is not taken up secondarily by cells or that Hyal-1 is targeted to lysosomes by mechanisms other than the usual phosphomannosyl recognition pathway. In this study, the extracellular Hyal-1 was found to be incorporated into cells, and to act as an intracellular hyaluronidase. These results suggest the existence of unknown pathway(s) for Hyal-1 uptake and that enzyme replacement therapy might be effective for a disorder caused by the Hyal-1 deficiency mucopolysaccharidosis IX. It is also expected that if the specific pathway for Hyal-1 uptake is identified, it could be a potential biological target for diseases like bladder cancer, where Hyal-1 is closely related to tumor growth and progression (30).

Degradation of HA by Hyal-2 takes place mainly on cell surfaces, and also depends on CD44 function(s). In Hyal-2/CD44/293 cells, but not in Hyal-2/293 cells, more than 90% of extracellular fl-HA was degraded by Hyal-2. The difference in the fl-HA molecular mass between CD44/293 and Hyal-2/CD44/293 cells appeared first in the cell surface pools, and catabolites accumulated extracellularly. Hyal-2 was localized on the cell surface, and the presence of CD44 was influenced on the localization of Hyal-2. These observations are consistent with the previous study that reported the interaction between CD44 and Hyal-2 by co-immunoprecipitation (22). HA forms a macrocomplex in an extracellular matrix by associating with itself, cell surface proteins, or other glycosaminoglycans (31–33). In such a macrocomplex, HA degradation on cell surfaces is critical for HA catabolism, as it is quite unlikely that HA is taken up by cells as a macrocomplex and is degraded intracellularly. Accordingly, Hyal-2 is regarded to have a prominent role in extracellular HA catabolism. Based on the findings in cellular catabolism of HA, we have established quantitative enzyme assays for Hyal-2, and characterized its enzymatic properties. Hyal-2 was dependent on CD44 even under cell-free conditions. Hyaluronidase activity was detected in Hyal-2/CD44/293 cells but not in Hyal-2/293 cell membranes. A mixture of cell membranes of Hyal-2/293 cells and CD44/293 cells did not show hyaluronidase activity. The solubilization of the membranes of Hyal-2/CD44/293 cells by using the established cell lines, we have demonstrated that Hyal-1 and -2 contribute to intracellular and extracellular catabolism of hyaluronic acid, respectively.
FIGURE 7. Hyal-2 enzymatic activity in prkHyal-2/CD44/293 cell membrane fractions. A, Western blot analysis of Hyal-2 expression. The whole extracts of prkHyal-2/CD44/293 and Hyal-2/CD44/293 cells (40 μg) were analyzed by Western blotting using the anti-Hyal-2 antibody. B and C, comparison of Hyal-2 activity in the cell membrane fractions of prkHyal-2/CD44/293 and Hyal-2/CD44/293. B, the cell membrane fractions (1 mg/ml of total proteins) of prkHyal-2/CD44/293 (●), Hyal-2/CD44/293 (▲), and 293 (○), as control, were incubated with fl-HA (3 μg/ml) in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 12 h. Then, the fl-HA in the supernatant was applied to a Sepharose CL-2B column and eluted with PBS. The amount of fl-HA in each fraction (2.5 ml) was determined by fluorescence count as described under "Experimental Procedures." The arrowheads indicate the positions of the void volume (V₀) and the total volume (V_t) of the column. C, decrease of high molecular weight fl-HA in fraction numbers 6–10 was calculated and represented as the percentage of the intact fl-HA eluted in the same fractions. D, time dependence of the enzyme reaction. The membrane fractions of prkHyal-2/CD44/293 (●) and Hyal-2/CD44/293 (▲) cells (100 μg/ml of total proteins) were incubated with fl-HA (1 μg/ml) in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 0–8 h. E, dose dependence of fl-HA degradation by prkHyal-2/CD44/293 cell membrane fractions. The membrane fractions of prkHyal-2/CD44/293 cells were mixed with those of CD44/293 cells in a variety of proportions (0–100%), and total proteins (100 μg/ml) were incubated with fl-HA (1 μg/ml) in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 2 h. The control fl-HA was incubated with the membrane fractions from 293 cells. Then the fl-HA in the supernatant was applied to a Sepharose CL-2B column and eluted with PBS. The amount of fl-HA in each fraction (2.5 ml) was determined by fluorescence count as described under "Experimental Procedures." F, inhibition of Hyal-2 by Vcpal. The membrane fractions of prkHyal-2/CD44/293 cells (100 μg/ml of total proteins) were incubated with Vcpal (20–320 μM) and fl-HA (1 μg/ml) in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 2 h. After Sepharose CL-2B chromatography, the percentage of the inhibition was calculated from the fluorescence in fraction numbers 6–10. G and H, determination of Hyal-2 enzymatic activity in a microplate format. Microplates were coated by biotin-conjugated HA (Bio-HA), and used as substrate plates. The membrane fractions (25 μg/ml) of prkHyal-2/CD44/293 or CD44/293 cells were added to the plate and incubated in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 0–4 h. The membrane fractions (0–100 μg/ml) of prkHyal-2/CD44/293 or CD44/293 cells were added to the plate and incubated in 50 mM NaCl, 100 mM phosphate buffer, pH 6.5, at 37 °C for 2 h (H). The amount of released biotin-conjugated HA was estimated from the amount of the biotin-conjugated HA remaining on the microplate. The bar shows standard deviation. Each figure is illustrated using the result of one of a few experiments with similar results.
Mammalian Hyaluronidases and CD44

Triton X-100 completely abolished the enzymatic activity of Hyal-2 in cell-free conditions (data not shown). These results strongly suggest that a proper complex with CD44 on the cell surface is essential for Hyal-2 to exhibit hyaluronidase activity, and that the interaction between Hyal-2 and CD44 is unstable. The optimum pH was a weak acidic condition of pH 6.0–7.0. At pH conditions of lower than pH 5.5 and higher than 7.5, the enzymatic activity was weakened. These results are consistent with the observations in breast tumor cells that the CD44-Hyal-2 complex is enriched in specialized microdomains termed caveolae, one of the lipid rafts, where the extracellular pH was regularly dropped to pH 6.5 when HA binds to CD44 and that pretreatment of the cells with anti-CD44 antibody blocks the hyaluronidase activity at pH 6.5 (22).

It has been proposed that Hyal-1 and Hyal-2 are major mammalian hyaluronidases in somatic tissues, and that they act in concert to degrade high molecular weight HA to a tetrasaccharide (10, 13, 23). HA fragments (20-kDa intermediate size) are initially generated by the glycosylphosphatidylinositol-anchored Hyal-2 near the cell surface, transported intracellularly and delivered ultimately to lysosomes, where Hyal-1 degrades the 20-kDa fragments to smaller ones. However, our observations indicate that Hyal-1 and Hyal-2 could act independently on HA degradation for the following reasons: (i) Hyal-1 can cleave large HA. (ii) Most of the degradation products by Hyal-2 were released to the medium, and not incorporated into the cells as substrates for Hyal-1. (iii) HEK 293 cells possess the endogenous intracellular hyaluronidase presumably derived from Hyal-1, and the activity was enhanced by the expression of CD44. However, additional expression of Hyal-2 did not influence the endogenous intracellular hyaluronidase activity. (iv) Exogenously added Hyal-1 into medium was incorporated into cells and acted as an intracellular hyaluronidase. However, the intracellular activity of the incorporated Hyal-1 in Hyal-2/CD44/293 cells was not distinguished from that in CD44/293 cells although similar amounts of Hyal-1 were incorporated into both cells. If Hyal-1 could work cooperatively with Hyal-2, the intracellular degradation of HA by the incorporated Hyal-1 in Hyal-2/CD44/293 cells would be higher than that in CD44/293 cells. From these observations, we could propose that Hyal-1 and Hyal-2 are involved in different pathways for HA degradation rather than work cooperatively in the same pathway.

The enzymatic activity of Hyal-3 still remains to be determined. We could not detect its activity to any degree of significance. Hyal-3 as well as Hyal-2 is up-regulated by inflammatory cytokines, such as interleukin-1 and tumor necrosis factor, whereas Hyal-1 is not (34). It might be possible that Hyal-3 has to be coupled with an unknown molecule(s) to show its function, like the coupling seen for Hyal-2 and CD44. Alternatively, there might be unknown substrate(s) for Hyal-3 other than HA.

Hyal-2 is a potential future biological target where modulation of HA catabolism may be of therapeutic value in the treatment of disease. It has been shown that Hyal-2 could function as an oncogene. Overexpression of Hyal-2 accelerates the tumor formation of murine astrocytoma cells (35). Although Hyal-2 is not expressed in normal adult brains (16, 36), its expression was detected from several primary human brain tumor specimens (35). Hyal-2 is a cell surface receptor for some retroviruses, the envelope protein of which mediates oncogenic transformation (18, 37). In highly invasive breast cancer cell lines, preferentially expressing hyaluronan synthase 2 and Hyal-2, both synthesis and degradation of HA are up-regulated (27). Hyal-2 has also been implicated in the pathogenesis of inflammatory joint diseases including rheumatoid arthritis and osteoarthritis. The concentration and average molecular weight of HA in the synovial fluid are reduced in both types of arthritis (38, 39) and the expression of Hyal-2 in the synovial fluid of both types of arthritis was significantly up-regulated (40). The level of mRNA of Hyal-2 in chondrocytes was also up-regulated by IL-1 and TNF stimulation (34), thereby implicating cartilage-derived hyaluronidase activity as a factor contributing to cytokine-induced extracellular matrix degradation during joint disease. The 20-kDa HA fragments, products of Hyal-2 cleavage, are highly angiogenic (41). This finding does not conflict with the fact that progression of cancer and osteoarthritis is associated with angiogenesis. The 20-kDa fragments also stimulate synthesis of inflammatory cytokines (5), induce transcription of matrix metalloproteases (42), and stimulate endothelial recognition of injury (43). In this context, it is significant that the Hyal-2 activity under cell-free conditions was well characterized in this study, and the sensitive and high-throughput Hyal-2 enzyme assay method would be valuable to provide therapeutics for these diseases. It was clearly demonstrated in this study that Hyal-2-mediated HA catabolism is progressed not only by Hyal-2 but also by CD44 up-regulation. In addition, it was suggested that Hyal-2 distribution change and pH condition change on the cell surfaces also could be factors for Hyal-2-mediated HA degradation. These findings might enable us to reach further potential therapeutic targets.

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