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Biochimica et Biophysica Acta 1591 (2002) 37–44
BIOCHIMICA ET BIOPHYSICA ACTA BBA
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CD44 stimulation by fragmented hyaluronic acid induces upregulation and tyrosine phosphorylation of c-Met receptor protein in human chondrosarcoma cells

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Received 21 August 2001; received in revised form 25 March 2002; accepted 24 May 2002

Abstract

Hepatocyte growth factor/scatter factor (HGF/SF) can induce proliferation and motility and promote invasion of tumor cells. Since HGF/SF receptor, c-Met, is expressed by tumor cells, and since stimulation of CD44, a transmembrane glycoprotein known to bind hyaluronic acid (HA) in its extracellular domain, is involved in activation of c-Met, we have studied the effects of CD44 stimulation by ligation with HA upon the expression and tyrosine phosphorylation of c-Met on human chondrosarcoma cell line HCS-2/8. The current study indicates that (a) CD44 stimulation by fragmented HA upregulates expression of c-Met proteins; (b) fragmented HA also induces tyrosine phosphorylation of c-Met protein within 30 min, an early event in this pathway as shown by the early time course of stimulation; (c) the effects of HA fragments are critically HA size-dependent. High molecular weight HA is inactive, but lower molecular weight fragments (M_r 3.5 kDa) are active with maximal effect in the μg/ml range; (d) the standard form of CD44 (CD44s) is critical for the response because the effect on c-Met, both in terms of upregulation and phosphorylation, is inhibited by preincubation with an anti-CD44 monoclonal antibody; and (e) phosphorylation of c-Met induced by CD44 stimulation is inhibited by protein tyrosine kinase inhibitor, tyrphostin. Therefore, our study represents the first report that CD44 stimulation induced by fragmented HA enhances c-Met expression and tyrosine phosphorylation in human chondrosarcoma cells. Taken together, these studies establish a signal transduction cascade or cross-talk emanating from CD44 to c-Met.

Keywords: CD44; c-Met; Hepatocyte growth factor/scatter factor (HGF/SF); Hyaluronic acid; Signal transduction

1. Introduction

The cellular functions are dependent upon a number of cell surface adhesion proteins and CD44 has been implicated as one such molecule [1,2]. CD44 is a transmembrane glycoprotein known to bind hyaluronic acid (HA) in its extracellular domain [3]. CD44 is known to exist in many different isoforms arising by alternative splicing events [4]. HA is a nonsulfated, linear glycosaminoglycan consisting of repeating units of (β,1-4)-D-glucuronic acid-(β,1-3)-*N*-acethyl-D-glucosamine [5]. Several studies have suggested that high and low molecular weight species of HA exhibit different biological effects on cells [6,7]. Low molecular weight, but not native, HA has been shown to stimulate the expression of cytokines, proinflammatory chemokines and cell adhesion molecules possibly through a mechanism involving the cellular HA receptor, CD44 [8–15].

In common with other cell surface molecules, there is evidence that ligand binding may be enhanced by the multimerization of CD44 [16]. It is now apparent that CD44 interacts with the HER2 (p185, c-ErbB2) cell surface

Abbreviations: BSA, bovine serum albumin; c-Met, HGF/SF receptor; DMEM, Dulbecco's minimum essential medium; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HGF/SF, hepatocyte growth factor/scatter factor; HA, hyaluronic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidine difluoride; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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PII: S0167-4889(02)00246-X

tyrosine kinase protein. HA binding stimulates HER2 tyrosine kinase activity, leading to an increase in tumor cell growth [17]. The cytoplasmic domain of CD44 has been shown to bind directly to a number of intracellular proteins, including ankyrin [18,19], ERM family [20] and protein 4.1 [21]. These proteins connect elements of the plasma membrane with the actin filament network of the cell and thus a direct link between CD44 and intracellular scaffold structures can be envisioned, facilitating cell motility and migration [22].

It has also been reported that, although precise mechanisms are not known, CD44 stimulation is involved in activation of c-Met, which is a specific receptor for hepatocyte growth factor/scatter factor (HGF/SF) [23]. HGF/SF can induce proliferation and motility in epithelial cells and promotes invasion of tumor cells [24–26]. It also stimulates upregulation of urokinase-type plasminogen activator (uPA) in tumor cells [27,28], which is considered to be a biomarker of tumor invasion. Furthermore, it has been established that stimulation of endothelial monolayers with HGF/SF resulted in an upregulation of CD44 expression together with enhancement of tumor cell–endothelial cell adherence and this factor may act to facilitate metastatic spread [29,30]. Thus, HGF/SF plays a key role in the initial adhesion mechanism between tumor cells and endothelial cells via upregulation of CD44. A recent study by Kawakami-Kimura et al. [31] has shown that HGF/SF upregulates the expression of tumor cell integrin molecules, resulting in enhancement of their adhesive properties towards extracellular matrix (ECM) components. HGF/SF upregulates the production of not only uPA but also matrix metalloproteinases (MMP) [32]. These observations further implicate HGF/SF in the enhancement of tumor cell interactions necessary for successful metastasis. These data allow us to hypothesize that there is a cross-talk between CD44 and c-Met system.

In the present study, we have studied the effects of CD44 stimulation by ligation with HA upon the expression and tyrosine phosphorylation of c-Met in human chondrosarcoma cell line HCS-2/8.

2. Materials and methods

2.1. Chemicals and reagents

Purified native HA and HA fragments (kindly provided by Dr. M. Ikeda, Seikagaku Kogyo Co. Ltd., Tokyo, Japan) were derived from rooster comb and had an average molecular weight of 2200, 1000, 640, 350 and 3.5 kDa. These HA preparations are free of protein (<2%) and free of chondroitin sulfate (<3%). Anti-CD44 monoclonal antibody (mAb), chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate and heparan sulfate were also from Seikagaku Kogyo. Fetal calf serum (FCS) was obtained from Life Technologies (Rockville, MD). Anti-CD44 mAb specifically inhibits HA binding to CD44. The ECL enhanced chemiluminescence detection kit was purchased from Amersham Japan (Tokyo). PY20 antiphsphotyrosine mAb was from Zymed (South San Francisco, CA, USA). Anti-c-Met rabbit polyclonal antibody (pAb) was from Santa Cruz Biotechnology, CA, USA (an affinity-purified polyclonal rabbit IgG raised against the synthetic peptide corresponding to the 28 C-terminal amino acids of the human c-Met protein). Protein A/G-agarose beads and tyrphostin A25 were from Calbiochem (Cambridge, MA).

2.2. Cells and culture conditions

Human chondrosarcoma cell line HCS-2/8 was grown and cultured as previously described [33]. Briefly, the HCS-2/8 cells were harvested and aliquoted into tissue culture plates (2×10^6 cells/well) in Dulbecco's minimum essential medium with Eagle's Salts (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated FCS. On the next day, the cells were washed three times with phosphate-buffered saline (PBS) to remove serum, and the medium was replaced with DMEM supplemented with antibiotics. Serum-free medium plus the test drugs were added and incubation was continued for different time lapses. After culture, medium was aspirated and cells were harvested and washed extensively.

In a parallel experiment, serum-starved HCS-2/8 cells were preincubated without or with different concentrations (0–50 μM) of tyrphostin for 4 h in DMEM and subsequently stimulated with fragmented HA.

2.3. Western blot and immunoprecipitation to detect c-Met protein

Serum-starved cells were treated for different periods of time with high molecular weight and fragmented HA (0–1000 μg/ml). Cells were washed three times with PBS, and the cell membrane proteins were solubilized at 4 °C for 16 h with 1 ml of lysis buffer [0.5% Nonidet P-40 and 0.02% sodium azide in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The solubilized cell preparation was processed by centrifugation at 8000 × *g* for 5 min, and the supernatant (50 μg of protein) was boiled for 5 min in nonreducing sample buffer and resolved in a 10% gel. Protein determinations were performed using the BCA protein assay [34]. Western blot was performed with polyvinylidine difluoride (PVDF) membrane using a transblot system (Marisol, Tokyo). The protein bands were then revealed using the ECL detection system (Amersham Japan) according to the manufacturer's protocol. Anti-c-Met pAb or anti-CD44s mAb was used at a final concentration of 0.5 μg/ml. Protein molecular weight standards were run simultaneously on the gels.

Total protein (200 μg) from each specimen was used for immunoprecipitation. Protein A/G-agarose beads (20 μl) and 2 μg of nonimmune IgG were added to each protein sample.

The reaction mixture was incubated for 2 h at 4 °C in a shaker and centrifuged at 8000 × *g* for 5 min. The supernatant was combined with 1 μg of anti-c-Met pAb or nonimmune rabbit IgG. Protein A/G-agarose beads (20 μl) were added, and the mixture was incubated in a shaker for an additional 2 h at 4 °C. The mixture was again centrifuged at 8000 × *g* for 5 min. The pellet was washed four times with 100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM sodium vanadate with 2 μg/ml aprotinin, 2 μg/ml leupeptin and 0.1% Triton X-100. The pellet was resuspended in 2 × sample buffer, boiled for 10 min, centrifuged at 8000 × *g*, and 20 μl of sample was loaded on an SDS–10% polyacrylamide gel under nonreducing conditions. Immunoblot was performed as described above. The membranes were blocked with 2% bovine serum albumin (BSA), and tyrosine-phosphorylated proteins were immunodetected by incubating the blots with anti-phosphotyrosine PY-20 mAb at a final concentration of 0.5 μg/ml. Protein molecular weight standards were run simultaneously on the gels.

3. Results

3.1. Involvement of CD44 in the upregulation of c-Met protein by fragmented HA

When a human chondrosarcoma cell line HCS-2/8 was probed for expression of CD44 with anti-CD44 antibody, cells were positive for the standard form of CD44 (CD44s) expression as indicated by a band detected at 85 kDa by Western blot (Fig. 1). In a parallel experiment, we also assessed the expression of CD44 protein on HCS-2/8 cells using flow cytometry. The CD44 was highly and consistently expressed on cells (data not shown). To characterize whether the 85-kDa CD44s is functionally active, we first investigated the ability of fragmented HA to induce expression of c-Met protein in HCS-2/8 cells. We confirmed in the present study that HA, especially when fragmented, is a possible ligand involved in CD44-mediated expression of c-Met protein (see below).

Fig. 2 shows that treatment of HCS-2/8 cells with fragmented HA, at a peak molecular mass of 3.5 kDa, upregulates c-Met protein, as evidenced by immunoblotting. A pAb against the C-terminal region of human c-Met receptor protein, whose specificity for detecting the mature and the precursor forms of the c-Met receptor is well characterized, was used. We detected two forms of immunoreactive c-Met, corresponding to a 170-kDa precursor form and a β chain of M_r 145 kDa. The 170-kDa precursor c-Met represents the newly synthesized and unprocessed form. The mature protein (p190), under reducing conditions, gives rise to an α chain of M_r 50 kDa and a β chain of M_r 145 kDa. Both the 70- and 50-kDa bands seem to represent nonspecific forms, since anti-c-Met antibody used in this study does not recognize the α chain of M_r 50 kDa, and since the intensity of both the 70- and 50-kDa bands does not

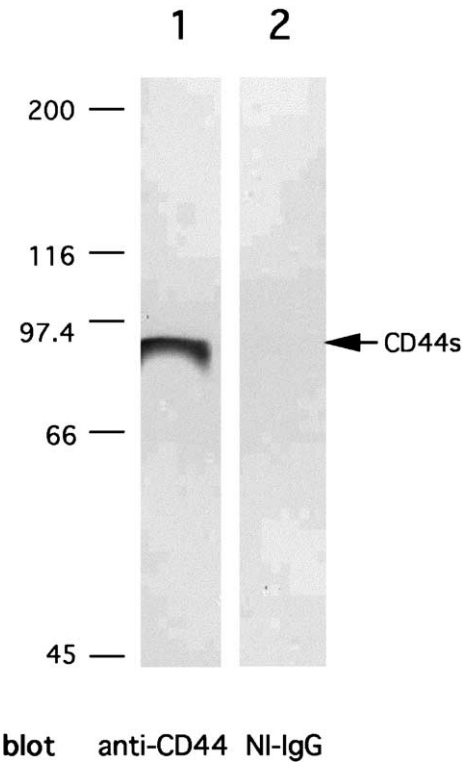

Fig. 1. Expression of CD44 protein in HCS-2/8 cells. Solubilized cell preparations (50 μg/lane) from HCS-2/8 cells were probed for CD44 immunoreactivity using a monoclonal anti-CD44 antibody (lane 1) or nonimmune mouse IgG (lane 2). Only one band of 85 kDa was detected and corresponded to standard form of CD44 (CD44s). Results are representative of two separate experiments.

change during the entire period. Fig. 2A shows a time course of upregulation of c-Met, the effect being evident from 6 h and peaking at 24 h. The c-Met upregulation was still detectable after 48 h. Furthermore, the effect of fragmented HA on the c-Met protein upregulation was dose-dependent over the dose range of 3.1–50 μg/ml as shown in Fig. 2B.

We investigated directly the role of CD44s in mediating HA stimulation of c-Met protein. When HCS-2/8 cells were preincubated with anti-CD44 mAb, which has been shown to block the binding of HA, the M_r 3.5 kDa fragmented HA-induced upregulation of c-Met protein was significantly reduced (Fig. 3). However, fragmented HA-induced c-Met protein upregulation was unaffected by preincubation with nonimmune mouse IgG. These data clearly establish that CD44s plays an important role in mediating the effects of fragmented HA on upregulation of c-Met protein.

3.2. The effect of HA size and concentration in the upregulation of c-Met protein

We asked whether the upregulation of c-Met protein is dependent on HA size. The HCS-2/8 cells were first exposed for 12 h to HA with different molecular masses [M_r 2200 kDa (1 mg/ml), M_r 1000 kDa (500 μg/ml), M_r 640 kDa (320 μg/ml), M_r 350 kDa (175 μg/ml) or M_r 3.5 kDa

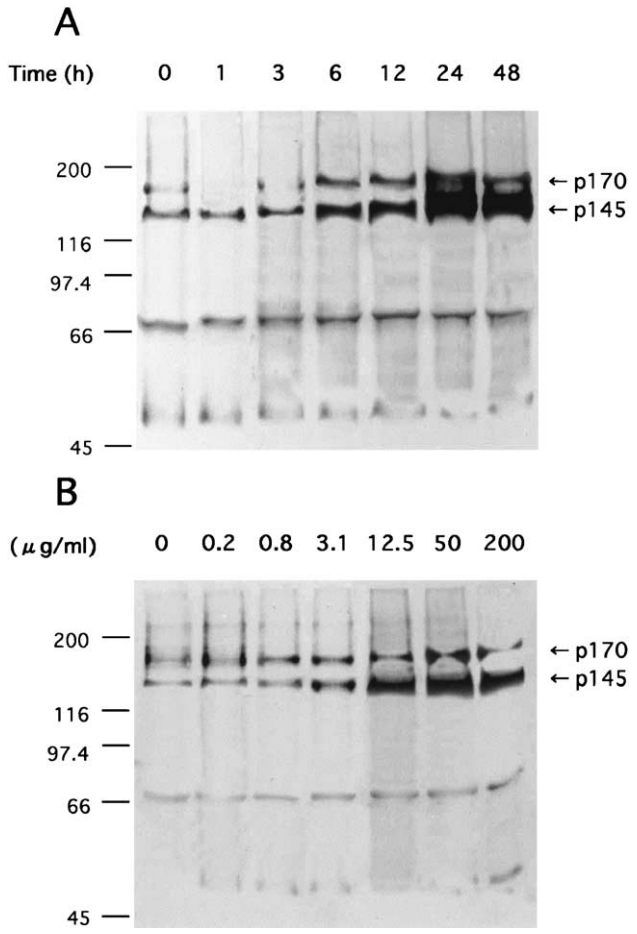

Fig. 2. Fragmented HA induces upregulation of c-Met receptor protein in HCS-2/8 cells in a time- and dose-dependent manner. (A) Serum-starved HCS-2/8 cells were treated with the M_r 3.5 kDa fragmented HA (1.8 μg/ml) from 0 to 48 h as indicated. (B) Cells were treated with fragmented HA from 0.2 to 200 μg/ml for 12 h. The cells were lyzed by the addition of lysis buffer. Total protein (50 μg) was subjected to 10% SDS-PAGE and analyzed with Western blot using commercially available antihuman c-Met pAb. The 170- and 145-kDa protein bands corresponding to intact c-Met receptor and β-subunit of c-Met protein, respectively, were recognized in solubilized preparations by the c-Met pAb. The control nonimmune rabbit IgG did not recognize these bands. Nonspecific bands (70 and 50 kDa) are presented to show the equal loading of each sample. Results are representative of two separate experiments.

(1.8 μg/ml)]. Fig. 4A demonstrates that expression of c-Met protein was significantly induced only by the addition of the M_r 3.5 kDa fragment of HA [1.8 μg/ml (= ~ 0.5 μM)]. In contrast, other HA compounds (M_r 350, 640, 1000 and 2200 kDa) (all ~ 0.5 μM) failed to induce the upregulation of c-Met protein. Further, we treated cells with the different HA sizes at different concentrations, and c-Met proteins were visualized in Western blots using anti-c-Met antibody. Fig. 4B demonstrates that the stimulatory activity of the 3.5-kDa HA fragments was dose-dependent, with maximal effect in the μg/ml range. The effect of the 3.5-kDa HA fragments is observed at a concentration as low as 1.0 μg/ml. The higher-molecular-mass forms of HA (from 350 to 2200 kDa) were unable to induce the c-Met expression also when used at any concentration used (up to 1000 μg/ml).

Related glycosaminoglycans, including chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate and heparan sulfate, all failed to induce the expression of c-Met protein in HCS-2/8 cells (data not shown). These data suggest that HA, especially when fragmented, is a possible ligand involved in CD44-mediated expression of c-Met protein. Therefore, this effect was markedly dependent on the molecular weight and concentration of the HA.

3.3. Effect of CD44 stimulation by fragmented HA on tyrosine phosphorylation of c-Met protein

We next examined the effect of CD44 stimulation by HA compounds on tyrosine phosphorylation of c-Met protein, a critical event in the activation of this receptor. HCS-2/8 cells treated with HA with different molecular masses were examined for phosphorylation of c-Met protein. The cell lysate from HCS-2/8 treated with high-molecular-mass forms of HA or fragmented HA was subjected to immunoprecipitation with anti-c-Met pAb, and then immunoblotted

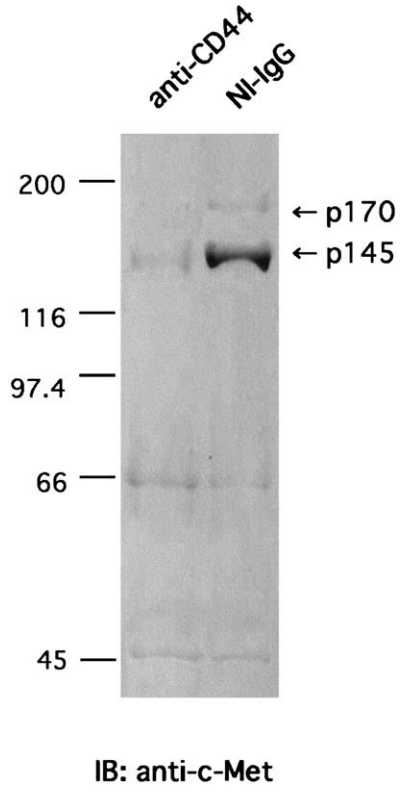

Fig. 3. Fragmented HA induces c-Met upregulation via CD44. Serum-starved HCS-2/8 cells were preincubated with anti-CD44 mAb (50 μg/ml; lane 1) or nonimmune mouse IgG (NI-IgG; 50 μg/ml; lane 2) for 1 h before stimulation with the M_r 3.5 kDa fragmented HA (1.8 μg/ml) for 12 h. The cell lysates (50 μg/lane) were prepared from HCS-2/8 cells and assessed for expression of c-Met protein. Data are representative of two separate experiments.

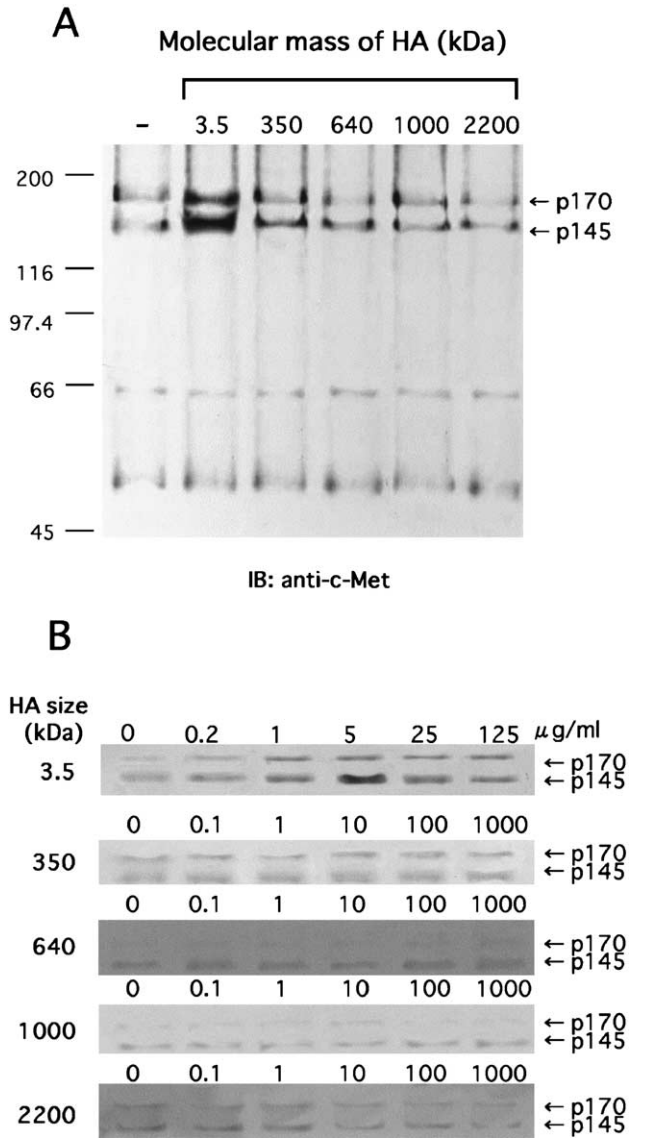

Fig. 4. HA fragment induced upregulation of c-Met protein is size- and concentration-dependent. (A) Serum-starved HCS-2/8 were treated with medium alone (–) or HA with different molecular mass [M_r 3.5 kDa (1.8 μg/ml), M_r 350 kDa (175 μg/ml), M_r 640 kDa (320 μg/ml), M_r 1000 kDa (500 μg/ml) or M_r 2200 kDa (1 mg/ml)] for 12 h. (B) Cells were treated with the different HA sizes at different concentrations for 12 h. Cell extracts (50 μg/lane) were prepared and assessed for c-Met expression by Western blot. Results shown are representative of two separate experiments.

using anti-phosphotyrosine mAb (Fig. 5). Exposure of the cells to the 3.5-kDa fragmented HA caused a time-dependent increase in phosphorylation of c-Met protein (Fig. 5A). Tyrosine phosphorylation of c-Met was most pronounced at 30 min after CD44 stimulation. Furthermore, the effect of fragmented HA on c-Met phosphorylation was dependent on concentrations of fragmented HA (Fig. 5B). Maximal induction of c-Met phosphorylation was observed with 3.1–12.5 μg/ml fragmented HA in two separate experiments. The relationship between CD44 stimulation and c-Met phosphorylation was further determined by application of a specific inhibitor of tyrosine phosphorylation, tyrphostin. As expected, tyrphostin dose-dependently inhibited the fragmented HA-induced c-Met phosphorylation; ~ 70%

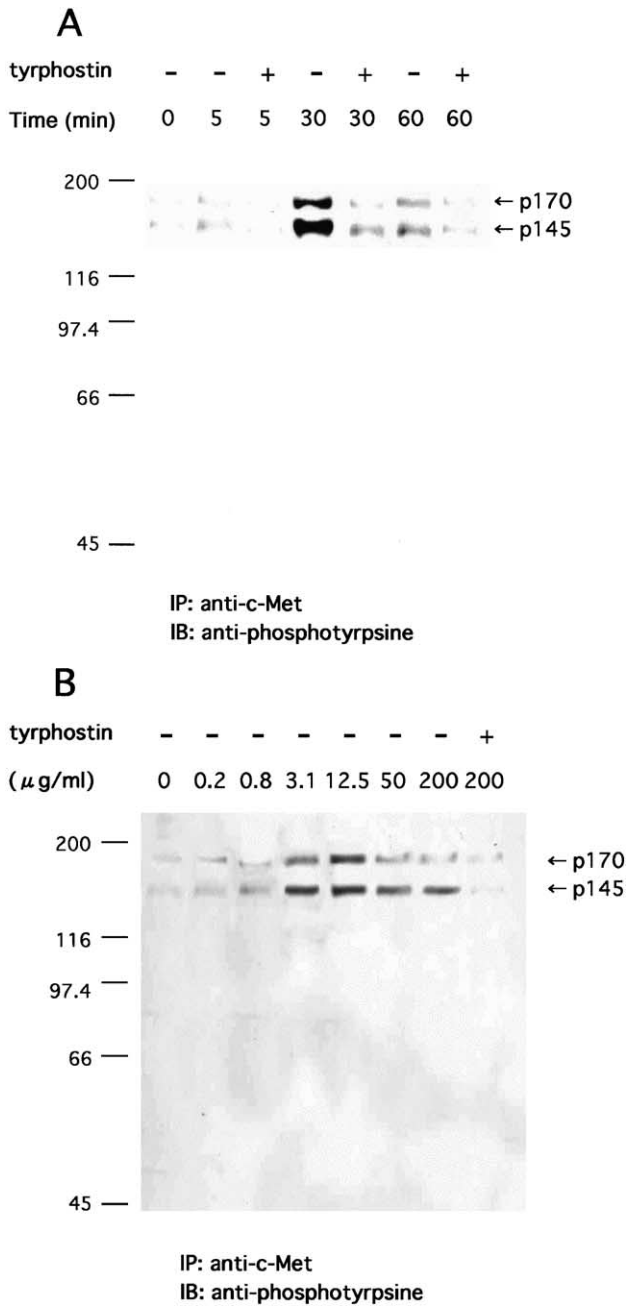

Fig. 5. Effect of CD44 stimulation by fragmented HA on tyrosine phosphorylation of c-Met protein. (A) Serum-starved HCS-2/8 cells were left untreated or treated with the 3.5-kDa fragmented HA for different periods of time in the absence or presence of tyrophostin (25 μM). (B) HCS-2/8 cells were left untreated or treated with different concentrations (0.2–200 μg/ml) of the 3.5-kDa fragmented HA for 30 min in the absence or presence of tyrphostin (25 μM). Cell lysates (50 μg/lane) were immunoprecipitated with anti-c-Met pAb followed by immunoblotted using anti-phosphotyrosine antibody. Specific phospho c-Met bands of 170 and 145 kDa were detected. No other protein bands except those shown were observed. Data are representative of two separate experiments.

at 25 μM and ~90% at 50 μM (data not shown). In a parallel experiment, the effect on c-Met, in terms of tyrosine phosphorylation, is also inhibited by preincubation with anti-CD44 mAb (data not shown). Again, the high-molecular-mass forms of HA failed to stimulate the phosphorylation of the c-Met proteins (data not shown).

4. Discussion

The main findings of the present study were the following: (a) the standard form of CD44 (CD44s) is expressed on HCS-2/8 cells; (b) CD44 stimulation by fragmented HA upregulates expression of c-Met protein. Upregulation of c-Met receptor protein after CD44 stimulation takes several hours; (c) HA fragments induce tyrosine phosphorylation of c-Met protein, an early event in this pathway as shown by the early time course of stimulation; (d) the effect of HA fragments is critically HA-size-dependent. High-molecular-mass HA (from 350 to 2200 kDa) is inactive, but lower molecular mass fragmented HA (M_r 3.5 kDa) is active; (e) the stimulatory activity of the 3.5-kDa HA fragments was dose-dependent, with maximal effect in the μg/ml range, whereas the higher-molecular-mass forms of HA (>350 kDa) were unable to induce the c-Met expression at any concentration used (up to 1000 μg/ml); (f) the effect on c-Met, both in terms of upregulation and phosphorylation, is inhibited by preincubation with the anti-CD44 mAb, which has been shown to inhibit HA binding to the CD44; and (g) upregulation and phosphorylation of c-Met induced by CD44 stimulation is inhibited by protein tyrosine kinase inhibitor, tyrphostin. Therefore, a role for CD44 in fragmented HA-induced upregulation and phosphorylation of c-Met receptor is clearly indicated in human chondrosarcoma cell line HCS-2/8.

HA has been shown to influence several aspects of cell behavior (proliferation, differentiation, migration and adhesion), but its effect greatly depends on at least three parameters: the size and concentration of the HA and the type of cells. It has been reported that the effects of HA are sometimes multivalent: higher-molecular-weights of HA are inhibitory at high concentration but stimulatory at low concentrations, e.g. its effect on cell proliferation [35]. Therefore, a wide range of concentrations were tested at each molecular weight to determine whether this is really an effect of "'fragments" or of size vs. concentration. Here, we report that the 3.5-kDa HA fragments specifically induced the expression of c-Met in a dose–response manner with maximal effect in the μg/ml range, while the higher-molecular-mass forms of HA (from 350 to 2200 kDa) were ineffective.

In the Western blot/immunoprecipitation procedures, the anti-c-Met pAb specifically recognizes the protein bands corresponding to intact 170 kDa and β subunit 145 kDa proteins. Since the pAb was raised against the synthetic peptide corresponding to the C-terminal amino acids of the human c-Met receptor protein, the reactivity with both the intact and b subunit of the c-Met receptor was expected. However, we used nonreducing conditions that should not dissociate the disulfide-linked 145-kDa β subunit from the 50-kDa α subunit. Our results are in agreement with the data reported by Herness and Naz [36], in which they reported that the c-Met receptor protein is present both as an intact as well as an isolated β subunit.

Our study represents the first report that CD44 stimulation induced by fragmented HA enhances c-Met upregulation and tyrosine phosphorylation in human chondrosarcoma cells. The effect of fragmented HA on the tyrosine phosphorylation was rapid and was seen as early as 30 min after stimulation. It is likely that a conformational change of CD44 is induced that allows interaction with fragmented HA to occur within minutes [37] for induction of tyrosine phosphorylation of c-Met receptor itself. In contrast, upregulation of c-Met receptor protein after fragmented HA binding takes several hours and may be accompanied by de novo synthesis of c-Met receptor.

It has been recently recognized that CD44 functions as a signaling receptor in a variety of cell types. Cell stimulation by anti-CD44 antibodies or natural CD44 ligands activate several signaling pathways that culminate in cell proliferation, cytokine secretion, chemokine gene expression and cytolytic effector functions [38]. There are some mechanisms whereby CD44 assembles the signaling machinery required for activation of c-Met receptor. One of the earliest signaling events following stimulation via CD44 is tyrosine phosphorylation of intracellular protein substrates. A large body of evidence has accumulated that stimulation via CD44 using antibody or HA fragments increases tyrosine phosphorylation of intracellular protein substrates [39–43]. Since CD44 lacks an intrinsic protein tyrosine kinase activity, increased tyrosine phosphorylation could only arise from direct or indirect interaction with c-Met. It is possible that there is a functional cross-talk between CD44 and c-Met. CD44 physically and functionally interacts with the Src-family protein tyrosine kinases, which are involved in c-Met signaling [44,45]. Also, ezrin may be involved in intracellular cross-talk between CD44 and c-Met. Ezrin binds directly to the cytoplasmic domain of CD44 and to the actin cytoskeleton [19,20]. Furthermore, ezrin is a substrate for c-Met shown to be involved in HGF/SF-induced cell migration [46]. Thus, CD44 may promote c-Met signaling by an intracellular mechanism.

It has been clearly shown that partial degradation products of HA had an angiogenic potential that was lacking in macromolecular HA [47] and that the cell-stimulatory effects of HA–CD44 interaction are indeed mediated by smaller HA fragments that stimulated macrophages to secrete the proinflammatory cytokines [8–15]. Our results are in agreement with that observed for induction of proinflammatory gene expression by HA fragments [8–15]. The basis for the difference between HA fragments and native HA is unclear. One possible explanation for the failure of

high-molecular-mass HA to activate c-Met receptor is that the high-molecular-mass forms may bind to cells in such a way as to prevent receptor cross-linking or dimerization. These evidences allow us to hypothesize that stimulation by CD44 ligation with fragmented HA induces outside-in signaling, which may augment phosphorylation and upregulation of c-Met by intracellular signal transduction or inside-out signaling. This plays a pivotal role in the HA-mediated adhesion of cells and subsequent invasion and metastasis.

The significance of this discovery is heightened by the fact that c-Met protein tyrosine phosphorylation is capable of inducing upregulation of uPA [32]. This has been identified as a potent proteinase digesting ECM components. It has also been reported that uPA participates in the membrane-associated activation process of MMP. Upregulated uPA and MMP has been observed in a number of aggressive tumor cells [48–52]. Preliminary data from our laboratory (M. Suzuki and H. Kobayashi; unpublished data, 2001) showed that CD44 stimulation promotes HCS-2/8 cell invasion and that phosphorylation and overexpression of surface c-Met in fragmented HA-treated cells result in a dramatic enhancement of uPA expression. Thus, the upregulation in HCS-2/8 cell invasion seen in response to CD44 stimulation involved, at least in part, the uPA–MMP fibrinolytic system. These data allow us to hypothesize that CD44 stimulation plays a key role in the invasion of HCS-2/8 cells via upregulation of uPA through c-Met-dependent activation mechanism.

In summary, the present study reveals a novel function of CD44 stimulation by fragmented HA in HCS-2/8 cells, including c-Met upregulation and activation, which further supports the role of c-Met as an invasion-promoting factor. These findings expand our understanding of the role of CD44 in tumor cell adhesion and activation that can in turn stimulate tumor cell invasion. Among tumor cells expressing autocrine loop (CD44 stimulation → c-Met activation → uPA/MMP overexpression), there may be a significant correlation between the magnitude of CD44 stimulation and invasiveness. Identification of CD44-dependent c-Met induction and c-Met-dependent uPA/MMP induction as well as reagents capable of inhibiting these processes may prove useful in retarding tumor metastasis. We are now examining in detail the signal transduction pathway activated by HA fragments leading to c-Met upregulation and phosphorylation using HCS-2/8 cells as a model system.

Acknowledgements

The authors thank Drs. M. Fujie, K. Shibata, T. Noguchi, A. Suzuki, Guang W. Sun and T. Kobayashi (Hamamatsu University School of Medicine) for their continuous and generous support of our work. We are also thankful to Drs. S. Miyauchi and M. Ikeda (Seikagaku Kogyo, Tokyo), Drs. H. Morishita, Y. Kato and K. Kato (BioResearch Institute, Mochida Pharmaceutical Co., Tokyo), and Drs. Y. Tanaka and T. Kondo (Chugai Pharmaceutical Co. Ltd., Tokyo) for helping with the biochemical analysis.

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