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Research Article

HAb18G/CD147-mediated calcium mobilization and hepatoma metastasis require both C-terminal and N-terminal domains

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Abstract. HAb18G/CD147 is a heavily glycosylated protein containing two immunoglobulin superfamily domains. Our previous studies have indicated that overexpression of HAb18G/CD147 enhances metastatic potentials in human hepatoma cells by disrupting the regulation of store-operated Ca^{2+} entry by nitric oxide (NO)/cGMP. In the present study, we investigated the structure-function of HAb18G/CD147 by transfecting truncated HAb18G/CD147 fragments into human 7721 hepatoma cells. The inhibitory effect of HAb18G/CD147 on 8-bromo-cGMP-regulated thapsigargin-induced Ca^{2+} entry was reversed by the expression of either C or N terminus truncated HAb18G/CD147 in T7721δC and T7721δN cells, respectively. The potential effect of HAb18G/CD147 on metastatic potentials, both adhesion and invasion capacities, of hepatoma cells was abolished in T7721δC cells, but not affected in T7721δN cells. Release and activation of matrix metalloproteinases (MMPs), MMP-2 and MMP-9, were found to be enhanced by the expression of HAb18G/CD147, and this effect was abolished by both truncations. Thapsigargin significantly enhanced release and activation of MMPs (MMP-2 and MMP-9) in non-transfected 7721 cells, and this effect was negatively regulated by SNAP. However, no effects of thapsigargin or SNAP were observed in T7721 cells, and expression of HAb18G/CD147 enhanced secretion and activation of MMPs at a stable and high level. Taken together, these results suggest that both ectodomain and intracellular domains of HAb18G/CD147 are required to mediate the effect of HAb18G/CD147 on the secretion and activation of MMPs and metastasis-related processes in human hepatoma cells by disrupting the regulation of NO/cGMP-sensitive intracellular Ca^{2+} mobilization although each domain may play different roles.

Key words. HAb18G; CD147; store-operated Ca^{2+} entry; hepatoma cell; metastasis.

A number of studies have demonstrated that CD147 (HAb18G, basigin, EMMPRIN, neurothelin) plays roles in cell-cell and cell-extracellular matrix interactions during tumor progression, spermatogenesis, implantation of the uterus, maturation of the oocyte, and development processes [1–7]. CD147 is a highly glycosylated transmembrane protein belonging to the immunoglobulin superfamily with two Ig domains (fig. 1). As an adhesion

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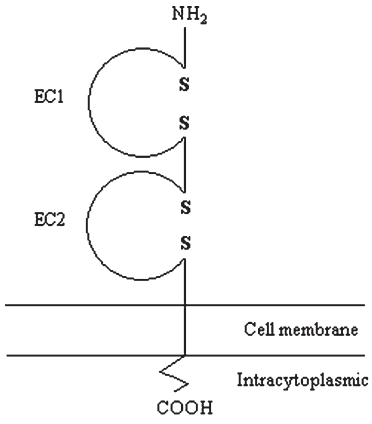

Figure 1. Schematic diagram of HAb18G/CD147 protein. EC, extracellular Ig domain.

molecule, CD147 has been reported to interact with a variety of cell types including endothelial cells and fibroblasts, as well as the extracellular matrix [8].

HAb18G, a new hepatoma-associated antigen, has an identical nucleotide acid and amino acid sequence as CD147 [9]. HAb18G/CD147 has an extracellular region with two Ig domains and an N-terminal with several enzyme catalytic sites. A homo-oligomer can be formed on the plasma membrane which may play crucial roles in enabling the functions of the molecule in a cis-dependent manner and may be sufficient for CD147 homo-oligomer formation [10]. CD147 molecules associate with each other probably via hydrophobic interactions between their N-terminal Ig-like domains. The intracellular domain of CD147 is well conserved between species (70% and 60% in mouse vs human and chicken CD147, respectively) [11]. The transmembrane domain of CD147 contains a glutamic acid residue, a charged residue which could be involved in the protein association on the plasma membrane. Three leucines are repeated every seventh amino acid residue in the CD147 transmembrane domain similar to the leucine zipper motif [12, 13]. This motif may be involved in the association between membrane proteins. As the transmembrane domain is highly conserved between species (96% between mouse and human or chicken CD147 [11], it may serve critical biological functions.

Our previous studies have demonstrated that HAb18G promotes the invasion activity of human hepatoma cells by stimulating fibroblast cells to produce elevated levels of several matrix metalloproteinases (MMPs) [4]. We have also demonstrated that overexpression of HAb18G/CD147 enhances the metastatic potential of human hepatoma cells by disrupting the regulation of Ca^{2+} entry by nitric oxide (NO)/cGMP [14]. However, how the expression of HAb18G/CD147 eventually leads to attenuation of the response of store-operated Ca^{2+} entry to NO/cGMP and enhanced metastatic potentials remains to be elucidated. The present study, therefore, investigated the relationship between the structure and function of HAb18G/CD147 by examining the effects of extracellular and intracellular domains of HAb18G/CD147 on calcium mobilization and metastatic processes after transfecting truncated fragments of HAb18G/CD147 into human hepatoma cells.

Materials and methods

Materials

Fura2/AM was obtained from Molecular Probes, (Eugene, Ore.). Thapsigargin and 8-bromo-cGMP were obtained from Calbiochem (La Jolla, Calif.). Culture medium RPMI 1640, fetal bovine serum (FBS), geneticin (G418 sulfate) and lipofectin transfection reagents were purchased from Gibco BRL, Life Technologies (Gaithersburg, Mass.). Trizol for total RNA isolation was from Gibco BRL, Life Technologies and ExpressHyb hybridization solution for Northern blot was from Clontech Laboratories (Palo Alto, Calif.). All restriction endonucleases were purchased from Promega (Madison, Wis.) and ^{32}P-dCTP was from Amersham Pharmacia Biotech (Amersham, UK). FITC-conjugated rat anti-mouse IgG and Matrigel were obtained from Becton Dickinson Labware (Bedford, Mass.). Other reagents were from Sigma (St. Louis, Mo.).

Cells

Human 7721 hepatoma cells (obtained from the Institute of Cell Biology, Academic Sinica) and T7721 cells stably expressing HAb18G [14] were cultured with RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in a humidified atmosphere of 5% CO_2.

Molecular construction and transfections

The mammalian expression vector pCI-neu (Promega) was used for these studies. The extracellular and cytoplasmic truncated fragments of HAb18G were generated by a PCR method with the following primers, and subcloned into expression vector pCI-neu. The forward primer, 5′-GGGGAATTCATGTCAGAACACATCAAC GAG-3′, and the reverse primer, 5′-GGGGTCGACA GCGTCCTCGGGCCACCTG-3′, were used to amplify the partial extracellular and transmembrane fragments which were subsequently cloned to pCI-neu to generate *δ*C-HAb18G-pCI-neu. To amplify the cytoplasmic and transmembrane fragments, the forward primer, 5′-GGG GAATTCATAGGAATCATGGCGGCTGC-3′, and reverse primer, 5′-GGGGTCGACTTACTTCAGGGGTG CAGAGCCGG -3′, were used and cloned into pCI-neu to generate *δ*N-HAb18G-pCI-neu.

The constructs were transfected into human 7721 hepatoma cells according to the procedure we used previously [14] and stable transfectants were selected with G418 (400 μg/ml) in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Northern blot and immunocytochemical staining analysis of the expression of truncated HAb18G/CD147

Total RNA was isolated from cells using Trizol reagent and 20 μg was resolved on 1% agarose/formaldehyde gels and transferred to nitrocellulose membranes. Hybridization was done using the HAb18G cDNA fragment as the probe in ExpressHyb hybridization solution according to the manufacturer's instructions. Membranes were exposed to Cyclone film for 2–4 h and scanned using the Cyclone Storage Phosphor System (Packard Instrument Company, Meriden, Conn.).

For immunocytochemical staining, human hepatoma monoclonal antibody HAb18 was used. Cell preparation and staining procedures were the same as previously described [14]. After cells had been mounted in glycerol, they were examined by fluorescence microscopy.

Measurement of intracellular free calcium concentration

Intracellular free calcium ($[Ca^{2+}]_i$) was measured using Fura2 acetoxymethyl ester (Fura2/AM). Cells were loaded with the dye by incubation with 5 μM Fura2/AM for 45 min in the dark at 37 °C in normal PBS (NPBS) containing 2 mM $CaCl_2$, pH 7.4. Cells were then washed and resuspended in NPBS. To start the experiment, cells were pretreated with 4 μM thapsigargin for 20 min or were without pretreatment with thapsigargin. Then, the cells were washed with and maintained briefly in PBS containing no Ca^{2+} and 2 mM EGTA. Unless stated otherwise, the cells were pretreated with or without 2 mM 8-bromo-cGMP for 5 min. The fluorescent signal was monitored and recorded by an LS-50B luminescent spectrometer (Perkin Elmer, Wiesbaden, Germany). The excitation light at 340 and 380 nm was provided by a 150-W Xenon arc lamp (Perkin Elmer) and a filter wheel (Perkin Elmer) containing 340- and 380-nm interference filters (Perkin Elmer). The emitted fluorescence at 510 nm was collected by a photomultiplier tube and recorded.

Adhesion assay

The wells of a 96-well culture plate were coated with Matrigel at a concentration of 5 μg/ml and incubated at 4 °C overnight. The coated wells were blocked with PBS containing 2% BSA for 30 min and then washed with PBS. Cells suspended in serum-free medium containing 0.1% BSA were added to the wells (2×10^4/well) and incubated at 37 °C, 5% CO_2 for 30 ~ 60 min. After removing medium and non-attached cells, 0.2% crystal violet was added for 10 min. The plate was gently washed with tap water and dried in air for 24 h; 0.1 ml 5% SDS/50% ethanol was added for 20 min and then the plate was read at 540 nm.

Invasion assay

The chemotactic cell invasion assay was performed using 24-well Transwell units (Costar Corning Inc., N. Y., USA) with an 8-μm pore size polycarbonate filter according to the method described by Mensing et al. [15]. Each lower compartment of the Transwell contained 600 μl of 0.5% FBS as chemoattractant or 0.5% BSA as negative control in RPMI 1640. The upper side of a polycarbonate filter was coated with Matrigel (5 μg/ml in cold medium) to form a continuous thin layer. Prior to addition of the cell suspension, the dried layer of Matrigel matrix was rehydrated with medium without FBS for 2 h at room temperature. Cells (1×10^5) in 0.1 ml RPMI 1640 containing 0.1% BSA were added into the upper compartment of the Transwell unit and incubated for 36 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cells remaining in the upper compartment were completely removed with gentle swabbing. The number of cells that had invaded through the filter into the lower compartment was determined using a colorimetric crystal violet assay.

Zymography experiments

To check the expression and activation of MMPs, the 7721, T7721, T7721δC and T7721δN cells were cultured in serum-free medium and incubated at 37 °C for 5–20 h. The conditioned media were collected by centrifugation, concentrated, and dialyzed. The dialyzed samples containing an equal amount of total proteins were mixed with sample buffer, incubated in a water bath (~55 °C) for 3 ~ 5 min, and loaded onto a zymographic SDS gel containing gelatin (1 mg/ml) as previously described [16]. The gels were washed in 2.5% Triton X-100 for 15 min twice and incubated in incubation buffer [50 mM Tris-HCl (pH 8.8) containing 5 mM $CaCl_2$, and 0.02% (w/v) NaN_3] for 16 h. The gels were stained with Coomassie blue and destained. The zones of gelatinolytic activity were shown by negative staining.

Results

Stable expression of truncated δC-HAb18G and δN-HAb18G in human hepatoma cells

Previously, we have stably transfected the full length of HAb18G into human 7721 hepatoma cells, to produce T7721 [14]. In the present study, we constructed two mammalian expression vectors containing C and N terminus truncated fragments, δC-HAb18G-pCI-neu and δN-HAb18G-pCI-neu, respectively, and transfected them

into 7721 cells. After G418 selection, T7721δC and T7721δN cell lines, which express δC-HAb18G and δN-HAb18G respectively, were obtained. The expression of truncated HAb18Gs was demonstrated by Northern blot (fig. 2A) and immunocytochemistry (fig. 2B). Since HAb18G/CD147 is an antigen expressed on the membrane, and the antibody raised against HAb18G only recognized its extracellular domain, as shown in figure1, while mRNA expression was positive for all the cell lines, the immunofluorescent staining was negative in the N-terminus-lacking T7721δN cells.

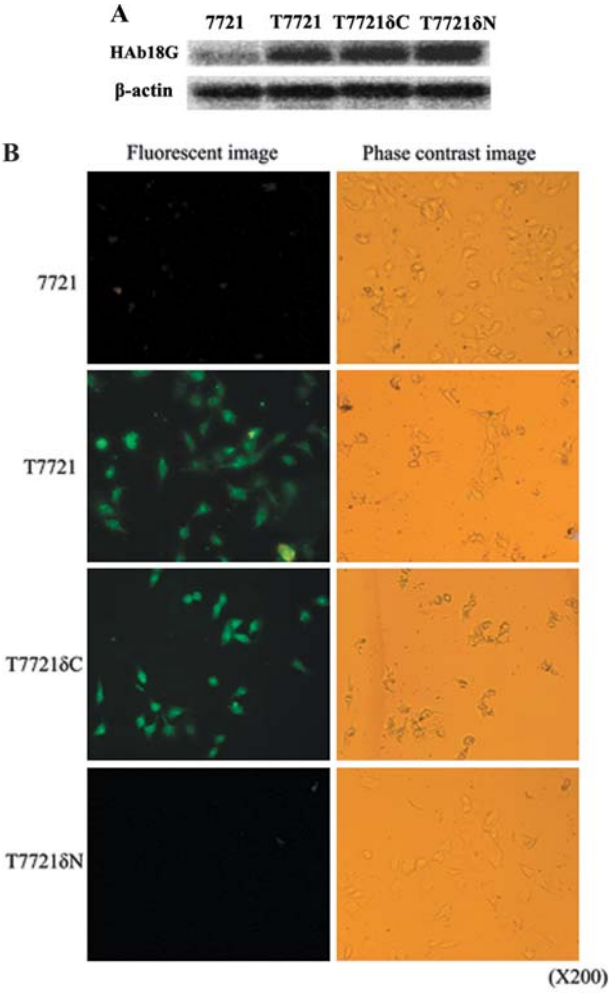

Figure 2. Stable transfection of HAb18G into human hepatoma cells. (*A*) Northern blot analysis of HAb18G and its fragments in transfected cells. Total RNA was isolated and fractionated on a 1% agarose gel containing 0.22 M formaldehyde, transferred by capillary blotting, and cross-linked to a nitrocellulose membrane. The blot was then probed with ^{32}P-labeled HAb18G cDNA. The results demonstrate expression of HAb18G and its mRNA fragments in transfected cell lines. The densities were normalized by human β-actin. (*B*) Immunocytochemical demonstration of expression of HAb18G and its fragments. 7721 and transfected cells (T7721, T7721δC and T7721δN) were fixed and stained with monoclonal antibody to HAb18, followed by FITC-conjugated rat anti-mouse IgG antibody. T7721 and T7721δC cells were positive by staining.

Effects of truncation of HAb18G on cGMP-sensitive store-operated Ca^{2+} entry

Our previous studies have demonstrated that expression of HAb18G attenuated the sensitivity of store-operated Ca^{2+} entry to NO/cGMP [14]. In the present study, we examined the effects of truncation of HAb18G on this Ca^{2+} entry. Figure 3 shows that 8-bromo-cGMP (2 mM) reduced the thapsigargin-induced $[Ca^{2+}]_i$ rise in 7721 cells and expression of HAb18G in T7721 cells abolished the inhibitory effect of cGMP, similar to what we observed previously [14]. However, the effect of HAb18G was opposed by truncations of HAb18G in T7721δC and T7721δN cells with 30.8 ± 4.1% and 35.5 ± 6.0% inhibition, respectively (as compared to T7721 cells, $p < 0.001$). However, there was no significant difference between the cells expressing the two truncations ($p > 0.05$).

Effects of truncated HAb18G expression on metastatic potential

Matrigel-coated plates and chambers are models used widely to investigate cancer metastatic processes in vitro and we have previously used these methods to demonstrate the role of HAb18G in metastasis-related processes in human hepatoma cells [14]. The same procedures were used in the present study. A significant increase in the number of attached cells, expressed as percent of total seeded cells, was observed in both T7721 and T7721δN cells, 63.6 ± 7.2% and 75.6 ± 5.8%, respectively, compared to the control 7721 cells, 45.6 ± 3.3% ($p < 0.05$; fig. 4). However, the number of attached T7721δC cells was significantly reduced, 31.2 ± 5.7%, compared to T7721 and T7721δN, ($p < 0.05$; fig. 4).

As shown in figure 5, the ability of HAb18G/CD147-expressing T7721 cells to invade through Transwell chambers, expressed as percent of total seeded cells, was significantly greater than that observed in control 7721

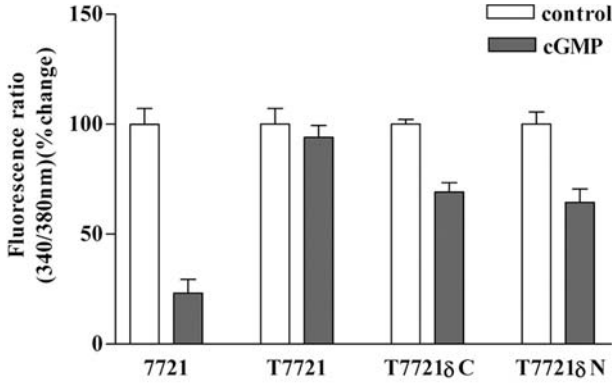

Figure 3. Effects of truncation of HAb18G on 8-bromo-cGMP-regulated thapsigargin-induced Ca^{2+} influx in hepatoma cells. Cells were incubated in NPBS with 4 μM thapsigargin for 20 min. 2 mM 8-bromo-cGMP was introduced 5 min prior to the measurement. Values are the percent change from baseline and are expressed as the means ± SE ($n = 4 \sim 6$).

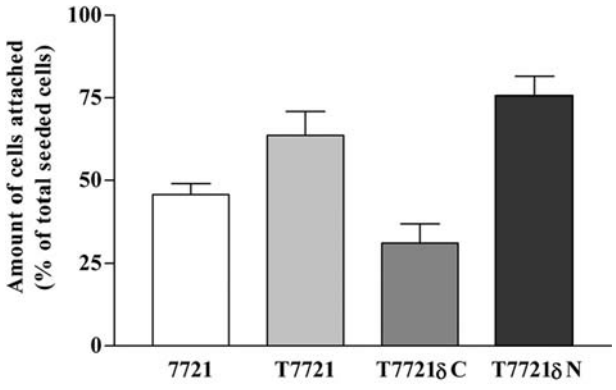

Figure 4. Adhesion potential of human 7721 hepatoma cells and transfected T7721, T7721δC and T7721δN cells to Matrigel. Cells were suspended in serum-free medium and seeded into the Matrigel (5 µg/ml)-coated wells. After incubation for 30 ~ 60 min at 37 °C, the percentage of adhered cells was determined using the colorimetric crystal violet assay. Values are the means ± SE (n = 6 ~ 8).

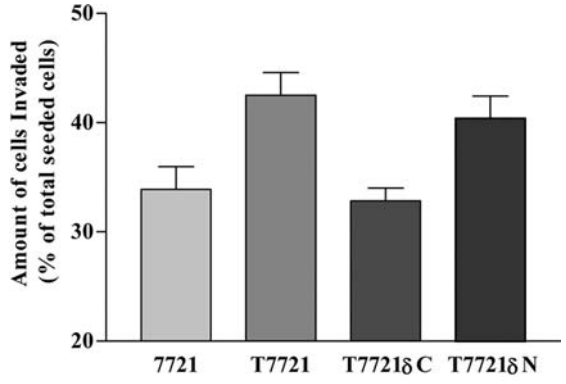

Figure 5. Invasive potential of human 7721 hepatoma cells and transfected T7721, T7721δC and T7721δN cells. The invasive potential was evaluated in Transwell chambers as described in Materials and methods. Briefly, cells were suspended in serum-free medium supplemented with 4 µM thapsigargin and seeded into the upper side of the Matrigel (5 µg/ml)-coated chambers. After incubation for 36 h at 37 °C, the percentage of invaded cells was determined using a colorimetric crystal violet assay. Values are the means ± SE (n = 3 ~ 4).

cells, 42.5 ± 2.1% compared with 33.8 ± 2.1% after 36 h, respectively ($p < 0.01$). The invasion-enhancing effect of HAb18G/CD147 was reduced in C-terminus-lacking T7721δC (32.8 ± 1.2%) but not in N-terminus-lacking T7721δN (40.5 ± 2.0%) cells.

Effect of HAb18G on MMP release

Since MMPs are well known for promoting cancer metastasis by degradation of the extracellular matrix, the effect of HAb18G/CD147 on metastatic potential could be mediated by MMPs. We tested this hypothesis in the present study using zymography. As shown in figure 6, MMP-2 and MMP-9 activities were significantly enhanced in T7721 cells compared to those observed in control 7721

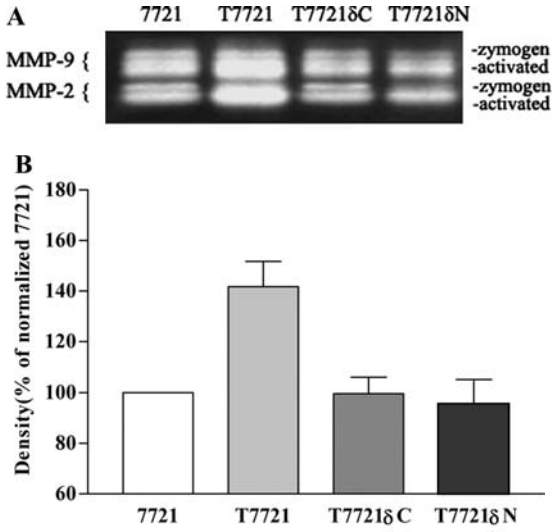

Figure 6. Gelatin zymography of culture medium conditioned by 7721 cells and transfected T7721, T7721δC and T7721δN cells. Cells were cultured in serum-free medium for 5–20 h, and the conditioned media were collected and analyzed for MMP activity by gelatin zymography. (*A*) Image of gelatin zymography. Top bands correspond to MMP-9 gelatinase, lower bands to MMP-2 gelatinase. (*B*) Densitometric analysis of total MMP activity. The value of MMP activity of 7721 cells is shown as a control (100%). Mean ± SE of at least six independent samples per group. $p < 0.01$, Student's t test.

cells ($p < 0.01$), but the truncations of HAb18G resulted in reduced activities/levels of MMPs in T7721δC and T7721δN cells. There was no significant difference between these two cell types ($p > 0.05$). Because the ratio of pro- to activated MMPs was the same in all cases (both 7721 control cells and transfected cells; fig. 6), this finding suggests that truncation of HAb18G may play a role in reducing the expression of MMPs.

Effect of extracellular Ca^{2+} addition on MMP release

MMP expression and activation are regulated complicatedly by a number of processes including zymogen activation, enzyme-inhibitor binding, endocytosis, and shedding. However, the biological factors involved in these processes are poorly understood. Calcium effects on MMP activation have been evaluated in some tumor cells. In the present study, human 7721 cells were plated in medium with serial doses of Ca^{2+}, and incubated for a certain time. Zymography results showed that an increasing Ca^{2+} concentration resulted in dose-dependent MMP-2 and MMP-9 release and activation. These results indicate that extracellular Ca^{2+} addition can mediate regulation of MMP release and activation (especially MMP-2) (fig. 7).

Involvement of thapsigargin-induced NO/cGMP-sensitive store-operated calcium entry in MMP release and activation

Although calcium addition resulted in rapid changes in MMP activation, the intracellular calcium regulation

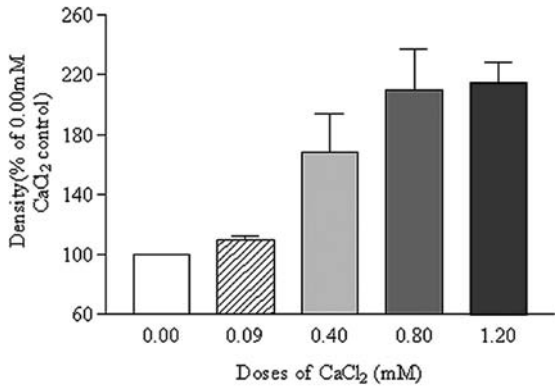

Figure 7. Dose-dependent induction of extracellular Ca^{2+} of MMP release and activation in 7721 cells. 7721 cells were cultured in serum-free medium with different concentrations of Ca^{2+}. MMP activity of 7721 cells with 0 mM Ca^{2+} induction is shown as a control (100%). Mean ± SE of at least six independent samples per group. $p < 0.01$, Student's t test.

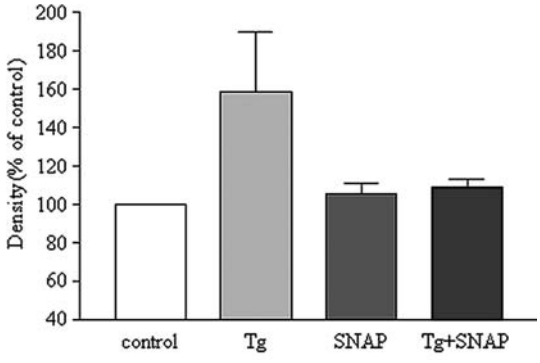

Figure 8. Effects of thapsigargin (Tg) and SNAP on MMP release and activation in 7721 cells. Tg, cells treated with 4 μM thapsigargin; SNAP, cells treated with 200 μM SNAP; Tg+SNAP, cells treated with 4 μM thapsigargin and 200 μM SNAP. MMP activity of untreated 7721 cells is shown as a control (100%). Mean ± SE of at least six independent samples per group. $p < 0.05$, Student's t test.

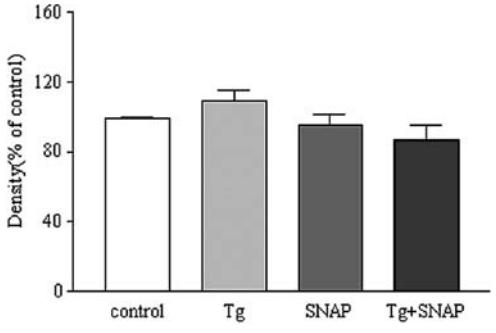

Figure 9. Effects of thapsigargin and SNAP on MMP release and activation in T7721 cells. Tg, cells treated with 4 μM thapsigargin; SNAP, cells treated with 200 μM SNAP; Tg+SNAP, cells treated with 4 μM thapsigargin and 200 μM SNAP. MMP activity of untreated 7721 cells is shown as a control (100%). Mean ± SE of at least six independent samples per group. $p > 0.05$, Student's t test.

should be determined. In the present study, 4 μM thapsigargin was used to deplete the intracellular Ca^{2+} store and subsequently to induce Ca^{2+} entry, and then 200 μM SNAP was introduced into the culture system. Conditioned medium was analyzed by zymography. Figure 8 showed a significant high level of release and activation of MMPs by thapsigargin treatment in 7721 cells (58.63 ± 31.04% higher than that of the untreated control cells, $p < 0.05$), while SNAP inhibited the effects of thapsigargin. These results suggest that MMP expression and activation were regulated by a cGMP/NO-sensitive intracellular calcium signal pathway. But when the treatment of thapsigargin and SNAP was carried out on T7721 cells, expression of HAb18G induced a sustained high level of MMP activity and release. There was no significant difference between the treatments and untreated control (fig. 9).

Discussion

The present study has demonstrated that both the C and N terminus of HAb18G/CD147 are important for its effect on metastasis-related processes in human hepatoma cells. Our previous study demonstrated that HAb18G/CD147 enhances the metastatic potential in human hepatoma cells by disrupting the regulation of Ca^{2+} entry by NO/cGMP [14]. The present study has further investigated the involvement of the two terminal domains of HAb18G/CD147 in these processes. While truncation of the C terminus abolished the effect of HAb18G/CD147 on cGMP-regulated Ca^{2+} entry, MMP release/activation as well as metastatic potential, deletion of the N terminus only reversed the effect of HAb18G/CD147 on Ca^{2+} entry and MMP release, but not metastatic potential, indicating differential effects of the two domains in metastasis-related processes.

HAb18G/CD147 is a highly glycocylated transmembrane protein belonging to the Ig superfamily with two Ig domains. These Ig ectodomains in different species have low homology and high variability, whereas the transmembrane and intracellular domains show high homology and are conserved in different species. In the ectodomain and intracellular domain there are several kinase phosphorylation sites, such as those for N-glycosylation and N-myristoylation [17]. Also interesting to note is that the transmembrane domain contains a glutamic acid residue and a leucine zipper motif which is implicated in the protein association with the plasma membrane and the dimerization of DNA-binding proteins [12, 13]. These structural characteristics may enable HAb18G/CD147 to interact with other membrane proteins, intracellular enzymes/factors as well as extracellular factors that are involved in the signaling pathways pertinent to the process of metastasis.

Cellular Ca^{2+} mobilization is a frequent common denominator of different signal transduction pathways and is considered to play an important role in the process of cancer metastasis [18–20]. The present study demonstrating the reversal of the effect of HAb18G/CD147 on cGMP-regulated store-operated Ca^{2+} entry by truncations of either the extracellular or intracellular domain of HAb18G suggests that both are required to mediate the effect of HAb18G/CD147 on Ca^{2+} entry. While detailed information concerning the entire apparatus for the store-operated Ca^{2+} entry is not yet available, evidence has been provided to suggest the involvement of Ca^{2+} channels on the plasma membrane as well as transporters associated with the intracellular Ca^{2+} pools. For example, specific store-operated Ca^{2+} channels (SOCCs) have been shown to be present in a number of cell types since Ca^{2+} entry can be activated in response to depletion of the intracellular Ca^{2+} store by physiological Ca^{2+}-mobilizing agents in the absence of the well-known L-type calcium channel [21–23]. Endoplasmic reticulum Ca^{2+}-APTase (SERCA) is also known to be able to sequester Ca^{2+} within the endoplasmic or sarcoplasmic reticulum in some cell types and affect the kinetics of cytosolic and organellar $[Ca^{2+}]$ changes [24–26]. The interaction of the C or N terminus of HAb18G/CD147 with the component proteins/factors of the store-operated Ca^{2+} entry apparatus, either intra- or extracellular, may be essential for the inhibitory effect of HAb18G/CD147 on Ca^{2+} entry. The reversal of this inhibitory effect by either C or N truncations could be due to alterations in direct interactions at these termini. For example, the C terminus may directly interact with an enzyme or component factor of the NO/cGMP signaling pathway thereby exerting the inhibitory effect of HAb18G/CD147 on the cGMP-regulated Ca^{2+} entry. Alternatively, as a single transmembrane protein, the conformation of HAb18G/CD147 as a whole could be altered when either the C- or N-terminal domain is truncated, such that the transmembrane leucine zipper, a motif known to enable protein-protein interaction, of HAb18G/CD147 is disabled leading to changes in structure and function of other interacting channel proteins, such as SOCCs and SERCA, in the Ca^{2+} entry pathway. Thus, C- or N-terminal domains are either directly or indirectly required for the action of HAb18G/CD147 on cGMP-regulated store-operated Ca^{2+} entry.

Our previous study investigating the effect of HAb18G/CD147 suggested a link between cGMP-regulated store-operated Ca^{2+} entry and the metastatic potential of human hepatoma cells [14], but the intermediate mechanism(s) leading to the potentiation of the metastatic potential by HAb18G/CD147 was not elucidated. The results of the present study indicate that the release and activation of MMPs may be considered to be one of the important events involved in cancer metastasis. In fact, we, as well as others, have clearly demonstrated that HAb18G/CD147 promoted fibroblast and cancer cells to release and activate MMPs, resulting in enhanced invasion and metastasis [1, 3–5]. In the present study, overexpression of HAb18G/CD147 increased the levels of proMMP-2, proMMP-9, and their activated forms; however, both C- and N-terminal truncations reversed the effect of HAb18G/CD147 on MMP activities. The results suggest that the integrity of HAb18G is necessary for MMP expression and activation, consistent with the present finding that both terminals are required for mediating the effect of HAb18G/CD147 on cGMP-regulated store-operated Ca^{2+} entry. The data obtained with an extracellular Ca^{2+} induction assay and a gelatin zymography analysis using antagonist (cGMP and SNAP) or activator (thapsigargin) showed that extracellular Ca^{2+} addition and intracellular Ca^{2+} activation induced elevated MMP release and activation and, in addition, the antagonist inhibited MMP expression. However, no effects of antagonist and activator were observed in HAb18G-expressing cells and the expression of HAb18G/CD147 enhanced the secretion of MMP with stable and high levels. The present study demonstrating that both the C and N terminus were required for the effect of HAb18G/CD147 on Ca^{2+} entry and MMP activities suggests that HAb18G may regulate MMP activity via intracellular Ca^{2+} mobilization. This notion is consistent with previous reports that posttranslational MMP dynamics is regulated by localized changes in extracellular calcium and thus affects the cellular behaviors of cancer cells such as invasion and metastasis [27–29]. The leucine zipper motif in the transmembrane domain of HAb18G/CD147 may also affect the expression of MMPs, since the motif has been implicated in dimerization of DNA-binding proteins [12, 13].

The results of the present study suggest that the potential effect of HAb18G/CD147 on metastatic potentials in hepatoma cells was mediated by its direct or indirect actions on cGMP-regulated Ca^{2+} entry, subsequent activation of MMPs and, finally, metastatic behaviors, e.g., cell attachment and invasion. This is supported by the observed effects of HAb18G/CD147 on all these events, as well as the abolishment or reversal of its effects on all these events by truncation of its C terminus. While both intra- and extracellular terminals appear to be important for both cGMP-regulated store-operated Ca^{2+} entry and MMP release, the metastatic potential was demonstrated to be dependent on the C terminus but not the N terminus. This suggests that the metastatic potential may not be tightly coupled to cGMP-regulated store-operated Ca^{2+} entry and, even more surprisingly, not coupled to MMP activities, at least when the N terminus is absent. This suggests that novel alternative mechanism may be involved in mediating the potential effect of HAb18G/CD147, the details of which are not clear at the moment. One should note, however, that the processes involved in

cancer metastasis consist of a cascade of very complex events including adhesion and invasion steps which involve multiple signaling pathways and a variety of molecular and cellular mechanisms including the extracellular matrix [30–33]. Adhesion or arrest of cancer cells to capillary endothelial cells and the subendothelial matrix is the very first step for cancer cells to invade through basement membrane, depending highly on an increased production of proteases by tumor cells. Considering the important role of extracellular matrix in the metastasis process as well as its structural complexity, the absence of the extracellular terminal of HAb18G/CD147 is bound to alter the extracellular complex, the delicate structure of which determines the metastatic behavior of cancer cells. Therefore, the uncoupling of Ca^{2+} entry/MMPs and metastatic potential observed in the absence of the extracellular terminal may not reflect the physiological mechanism underlying the process of metastasis.

In summary, the present study has provided further evidence supporting a role for HAb18G/CD147 in cancer metastasis, and additional information regarding possible mechanisms underlying its effect on metastatic potential in human hepatoma cells. The requirement of both extra- and intracellular terminals for important cellular processes related to metastasis, such as Ca^{2+} mobilization and MMP activities, has indicated possible sites of interaction which may be important determinants of metastatic behaviors. The observed differential effects of the C and N terminus on metastatic potentials suggest that they may play different roles in the process of metastasis. Further studies with site-directed mutagenesis may enable further elucidation of the detailed mechanism and sequence of events underlying the effect of HAb18G/CD147 on cancer metastasis.

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