Cancer Science
The official journal of the Japanese Cancer Association

Cleavage of hepatocyte growth factor activator inhibitor-1 by membrane-type MMP-1 activates matriptase

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(Received October 24, 2011/Revised November 21, 2011/Accepted November 23, 2011/Accepted manuscript online November 26, 2011/Article first published online December 28, 2011)

Co-expression of membrane-type 1 (MT1)-MMP with hepatocyte growth factor activator inhibitor-1 (HAI-1) in HEK293T cells resulted in cleavage of HAI-1 to produce three fragments. Recombinant MT1-MMP was shown to cleave HAI-1 protein *in vitro*. Hepatocyte growth factor activator inhibitor-1 was initially identified as the cognate inhibitor of matriptase, a transmembrane serine protease that processes urokinase-type plasminogen activator (uPA). Co-expression of HAI-1 with matriptase suppressed matriptase protease activity, and co-expression of MT1-MMP with them resulted in recovery of matriptase activity by stimulating shedding of HAI-1 fragments. Matriptase protein was detected in squamous carcinoma-derived HSC-4 cells, however, matriptase protease activity was undetectable. Transfection of siRNA for HAI-1 enhanced serine protease activity, which was suppressed by cotransfection of matriptase siRNA. Collagen-gel culture or treatment with concanavalin A (ConA) of HSC-4 cells enhanced MT1-MMP activity, which induced shedding of HAI-1 fragments and conversely stimulated uPA activation by these cells. Serine protease activity, including uPA activation of cells treated with ConA, was abrogated by downregulation of either matriptase or MT1-MMP through the transfection of each siRNA. These results suggest that MT1-MMP induced by collagen-gel culture or ConA treatment causes cleavage and shedding of HAI-1 protein, which allows activation of matriptase in HSC-4 cells. HSC-4 cells showed a characteristic invasive growth by forming vacuole-like structures in collagen gel, which was suppressed by transfection of siRNA for either MT1-MMP or matriptase, suggesting that activation of matriptase through the cleavage of HAI-1 is one of the MT1-MMP multifunctions essential for invasive growth of HSC-4 cells. (*Cancer Sci* 2012; 103: 448–454)

Hepatocyte growth factor activator inhibitor-1 is a membrane-associated Kunitz-type serine protease inhibitor.[1–5] It was initially identified as the cognate inhibitor of HGFA,[6] and purified from human milk as a complex with matriptase, a multidomain, transmembrane serine protease of the S1 trypsin-like family.[7–11] Matriptase was detected in a variety of human tumors of epithelial origin or phenotype and has been implicated in the initiation and progression of human carcinomas.[12–14] Matriptase mediates the degradation of ECM components and activates growth and angiogenic factors, which not only facilitates cellular invasiveness but may also activate oncogenic pathways. These functions are partially attributed to its role in the activation of HGF and uPA.[15–17] Both HGF and uPA have been implicated in cancer invasion and metastasis for their roles in cellular motility, ECM degradation, and tumor vascularization.[1,18] The HAI-1 fragments are often identified in culture supernatant of cells in complex with proteases, suggesting that proteolytic processing of HAI-1 may play roles in regulation of inhibitory activity.[19,20] However, the molecular mechanism of HAI-1 shedding and its pathophysiological significance still remain unclear.

Matrix metalloproteinases make up a family of Zn^{2+}-dependent enzymes that are known to cleave ECM proteins in normal and pathological conditions.[21–23] Twenty-six MMP genes have been identified in humans, and they can be subgrouped into soluble-type and MT-MMPs. Membrane-type-1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered, and was identified as the first physiologic activator of latent MMP-2 (pro-MMP-2).[24] In addition to pro-MMP-2, a variety of substrates of MT1-MMP were identified, including ECM proteins, cell adhesion molecules, cytokines, and others.[25–28] Processing of these proteins by MT1-MMP alters their activities and thereby regulates a variety of cellular functions, such as motility, invasion, growth, differentiation and apoptosis. As membrane proteases, MT1-MMP and matriptase share similar functions for malignant progression of tumors, however, their functional and physiological interactions have not been examined.

Previously, we identified type II transmembrane MSP as an MT1-MMP-binding molecule by an expression cloning strategy.[29] In the course of study, we examined the possible interaction of HAI-1 as an inhibitor of MSP within the MT1-MMP/MSP complex, and observed reduced HAI-1 expression in the presence of MT1-MMP. This led us to examine the physiological significance of HAI-1 shedding by MT1-MMP. While we were studying the cleavage of HAI-1 by MT1-MMP, Niiya *et al.*[30] identified HAI-1 as one of many MT1-MMP-associated proteins by proteomics screening.

In the present study, we showed for the first time that MT1-MMP activates matriptase through the cleavage of its cognate inhibitor HAI-1, which contributes, in collaboration with MT1-MMP, to the invasive growth of tumor cells.

Materials and Methods

Materials. The DMEM was from Sigma (St. Louis, MO, USA), and Opti-MEM serum reduced medium was from Invitrogen (Carlsbad, CA, USA). Primers were synthesized by Greiner Japan (Tokyo, Japan). Monoclonal antibodies against HA and GST were purchased from Wako Pure Chemical Industries (Osaka, Japan). Monoclonal antibodies against FLAG epitope and tubulin were purchased from Sigma. The mAb against MT1-MMP (222-3ER) was a gift from Daiichi Fine Chemical (Takaoka, Japan). Polyclonal antibodies against HAI-1 ectodomain and matriptase catalytic domain were purchased from R&D Systems (Mineapolis, MN, USA).

Plasmids. Expression plasmids for MT1-MMP, inactive mutant-type MT1-MMP, and MT1-MMP tagged with FLAG or HA epitope were constructed as described previously.[31] The cDNAs encoding HAI-1, HAI-mut-I with amino acid

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doi: 10.1111/j.1349-7006.2011.02162.x

substitution of Lue[452] to Gly and HAI-mut-II with deletion of Gly[306]–Val[330] were amplified using sets of PCR primers listed in Table 1. The expression plasmid for HAI-1 tagged with FLAG epitope was constructed by inserting HAI-1 cDNA fragment amplified using a set of primers into pEAK-FLAG plasmid.[31] The expression plasmids for matriptase, matriptase-FLAG, and uPA-FLAG were also constructed using PCR primers listed in Table 1.

Recombinant proteins. Recombinant MT1-MMP catalytic domain (Tyr[112]–Val[335]) was expressed in *Escherichia coli* BL21 strain in the form of a fusion protein with a SUMO using pE-SUMO vector (LifeSensors, Malvern, PA, USA). The SUMO-MT1-MMP fusion protein was purified using a Ni-chelate column. Expression plasmids for HAI-1 fusion proteins with GST, which contain Ile[348]–Glu[465] and Leu[241]–Cys[354] of HAI-1 (HAI-GST-I and HAI-GST-II, respectively) were constructed using THE PCR primers listed in Table 1. HAI-1-GST fusion proteins were purified using glutathione-conjugated beads (Amersham Biosciences, Uppsala, Sweden).

Cell culture. HEK293T cells and oral squamous cell carcinoma HSC-4 cells were cultured as described previously.[32] Type I collagen Cellmatrix Type I(A) was purchased from Nitta Gelatin (Osaka, Japan). ConA (30 μg/mL; Sigma) was added to the culture of HSC-4 cells, and cells were incubated for 5 h before use. Cell growth was examined using a Cell Counting Kit-8 (Dojin Laboratory, Kumamoto, Japan). The kit reagent of 25 μL was added to the collagen gel culture in a 24-well microplate, and absorbance at 450 nm was measured 2 h later.

Table 1. Polymerase chain reaction primers used in this study

Protein: (nucleotide no. of cDNA fragment; GenBank accession no.) Vector Primer sequence, restriction enzyme site (restriction enzyme)
HAI-1 (nt, 71–794; NM_181642) pEAK8
GATCAAGCTTCGCCACCATGAGAGCCCTGC (*Hin*dIII)
GATCTAGATCAGAGGGCCAGGCCATTCTCTTC (*Xba*I)
Matriptase (nt, 184–2761; NM_021978) pEAK8
GATCAAGCTT GAGGAAGGCGATGGCCCCTGC (*Hin*dIII)
GATCTAGACTATACCCCAGTGTTCTCTTTG (*Xba*I)
Matriptase-FLAG (nt, 184–2758; NM_021978) pEAK-FLAG
GATCAAGCTT GAGGAAGGCGATGGCCCCTGC (*Hin*dIII)
TCTCTAGATACCCCAGTGTTCTCTTTGA (*Xba*I)
uPA-FLAG (nt, 70–1369; M15476) pEAK-FLAG
GATCAAGCTTCGCCACCATGAGAGCCCTGC (*Hin*dIII)
GATCTAGAGAGGGCCAGGCCATTCTCTTC (*Xba*I)
SUMO-MT1 (nt, 568–1239; BC064803) pE-SUMO
TTGGTCTCAAGGTTACGCCATCCAGGGACTCAAATG (*Bsa*I)
GATCTAGAGACAAACATCTCCCCTCGGAGC (*Xba*I)
HAI-GST-I (nt, 1246–1599; NM_181642) pGST-CTC
TCGAATTCCATCGACAGTTTCCTGGAGTG (*Eco*RI)
CTCGGTACCCTCCACAGAGCCTGTGCTGG (*Kpn*I)
HAI-GST-II (nt, 925–1266; NM_181642) pGST-CTC
TCGAATTCCCTGTCCACCAAGCAGACAGA (*Eco*RI)
TCGGTACCACACTCCAGGAAACTGTCGAT (*Kpn*I)
Mutation primers (mutation)
HAI-1-mut I (substitute Lue[452] with Gly)
TGTTTGGCGGGAGGCGGGAAATC
CCCGCCTCCCGCCAAACACATCC
HAI-1-mut II (delete Gly[306]–Val[330])
CCTGTCGGGGTGTGCAAGGTTGCTCTGGCACCTGTCAGCC
GGCTGACAGGTGCCAGAGCAACCTTGCACACCCCGACAGG

HAI-1, hepatocyte growth factor activator inhibitor-1; SUMO, small ubiquitin-like modifier; uPA, urokinase-type plasminogen activator.

Cell surface biotinylation. Control plasmid or matriptase-FLAG expression plasmid (400 ng) was cotransfected with HAI-1-FLAG (800 ng) and/or MT1-MMP-FLAG (800 ng) plasmid into HEK293T cells cultured in a 35-mm-diameter dish coated with poly-L-lysine. Cell surface labeling with biotin and immunoprecipitation were carried out as described previously.[33]

Protease activity. Fluorescence-quenching substrate for serine protease (Boc-Gln-Ala-Arg-AMC) (Peptide Institute, Osaka, Japan) diluted with Opti-MEM (1 μM) was incubated with cells for 1 h, and the fluorescence was monitored. Pro-uPA-rich supernatant prepared from HEK293T cells transfected with an expression plasmid for uPA-FLAG was diluted twofold with Opti-MEM, and incubated with cells for 8 h. After TCA precipitation, uPA processing was examined by Western blotting with anti-FLAG antibody.

RNA interference. RNA interference technology was used to generate specific knockdown of MT1-MMP, HAI-1, and matriptase mRNA transcription. Small interfering RNA was prepared by Nippon EGT (Toyama, Japan). The siRNA target sequences were as follows:

MT1-MMP(I), CAGGCAAAGCTGATGCAGA; MT1-MMP(II), GCGAUGAAGUCUUCACUUA; HAI-1(I), CTGCAAGAGTTTCGTTTAT; HAI-1(II), GGGAAGAAGAGTGCATTCT; matriptase(I), CCGGCTTCTTAGCTGAATA; and matriptase(II) CGTCGTCACTTGTACCAAA. Transfection was carried out using Lipofectamin RNAiMAX (Invitrogen) by the reverse transfection method.

Zymography. Pro-MMP-2 supernatant was prepared from MMP-2-transfected HEK293T cells as previously described.[29] Pro-MMP-2 supernatant was incubated with cells for 1 h, then subjected to gelatin zymography using Alexa Fluor 680-labeled gelatin as described previously.[31] Detection of matriptase by gelatin zymography was carried out by modifying the protocol for MMP.[34]

Results

Cleavage and shedding of HAI-1 by MT1-MMP. HEK293T cells do not express either MT1-MMP or HAI-1 endogenously. An expression plasmid for HAI-1 was cotransfected into HEK293T cells with either control plasmid or MT1-MMP plasmid, and HAI-1 protein was detected by Western blotting using anti-HAI-1 antibody (Fig. 1A). Cell-associated HAI-1 protein was detected as a 66 kDa band in cells transfected with HAI-1 plasmid alone. Co-expression of MT1-MMP induced shedding of 58, 42, and 16 kDa fragments of HAI-1, and the cell-associated HAI-1 protein level was considerably reduced. Treatment of cells with the MMP inhibitor BB94 abrogated MT1-MMP-induced HAI-1 shedding. Expression of a catalytically inactive MT1-MMP mutant failed to induce HAI-1 shedding. Observation of a shed 58 kDa fragment suggested that one of the cleavage sites is close to the transmembrane domain. To identify the MT1-MMP cleavage site in this region, a recombinant HAI-GST fusion protein containing the juxtamembrane domain (HAI-GST-I) was prepared, and incubated with recombinant MT1-MMP (Fig. 1B,C). HAI-GST-I protein was cleaved to produce a 25 kDa fragment. The NH2-terminal amino acid sequence of the fragment revealed cleavage at the Gly[451]–Leu[452] peptide bond of HAI-1 by MT1-MMP. To identify another cleavage site, HAI-GST-II protein, which contained KD-1 and the LDLR-like domains, was digested with recombinant MT1-MMP, and the cleavage product of 28 kDa was isolated. The amino acid sequence analysis identified two possible cleavage sites (Pro[307]–Leu[308] and Pro[329]–Val[330]) in the flanking region between the KD-1 and LDLR-like domains. In order to confirm HAI-1 cleavage by MT1-MMP, HAI-1 mutant protein with either amino acid substitution at Leu[452] (HAI-mut-I) or

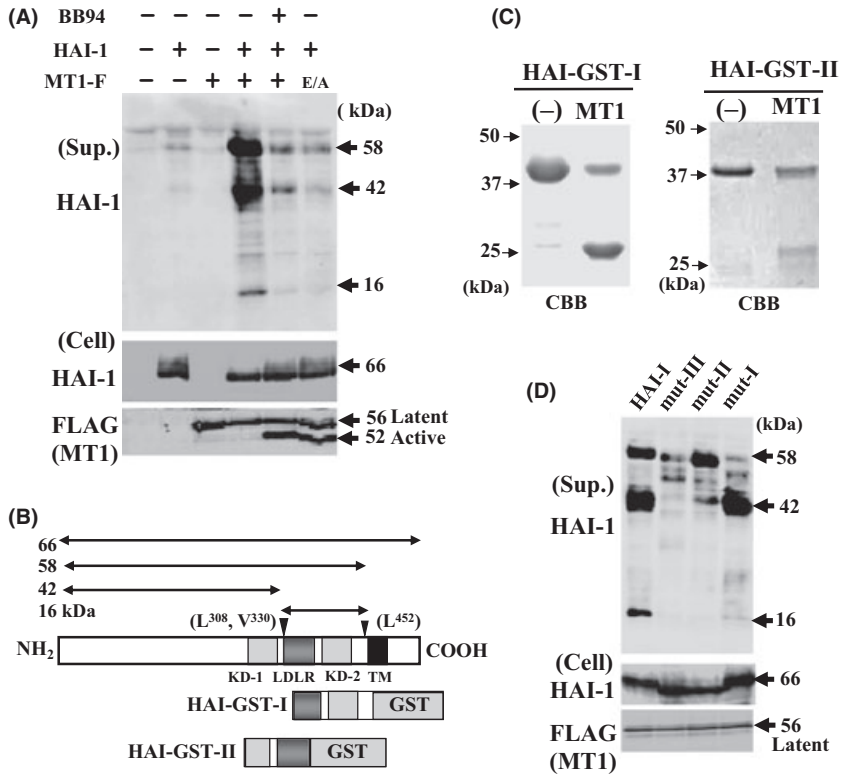

Fig. 1. Membrane-type-1 (MT1)-MMP cleaves hepatocyte growth factor activator inhibitor-1 (HAI-1). (A) Control plasmid or expression plasmid for MT1-MMP-FLAG (MT1-F; 1 μg) or its inactive mutant form (E/A) was cotransfected with HAI-1 or control plasmid (1 μg) into HEK293T cells cultured in a 35-mm dish. Twenty-four hours after transfection, culture medium was replaced with 1 mL Opti-MEM, and cells were incubated for a further 12 h. After TCA precipitation of the supernatant, HAI-1 processing was examined by Western blotting with anti-HAI-1 antibody (upper panel). Cell lystes were subjected to Western blotting with anti-HAI-1 or anti-FLAG antibody as indicated. BB94 (1 μM) was included in the indicated culture. Note that the 66 kDa HAI-1 protein was cleaved to 58, 42, and 16 kDa fragments, and shed into culture medium, when co-expressed with MT1-MMP. (B) Schematic representation of HAI-1 and HAI-1-GST fusion proteins. Arrowheads indicate possible MT1-MMP cleavage sites. HAI-I-GST-I contains amino acid residues 348–465 of the HAI-1 protein, and HAI-GST-II contains amino acid residues 241–376. KD, Kunitz domain; LDLR, low-density lipoprotein receptor-like domain; TM, transmembrane domain. (C) HAI-GST-I or HAI-GST-II was incubated with or without (–) recombinant MT1-MMP protein for 3 h, separated on SDS-PAGE, and visualized by Coomassie Brilliant Blue R-250 (CBB) staining as indicated. (D) Expression plasmid for HAI-1 or its mutant protein (HAI-mut-I, HAI-mut-II or HAI-mut-III) was cotransfected with MT1-MMP-FLAG plasmid, and HAI-1 fragments in the supernatants and full-length HAI-1 or MT1-MMP-FLAG protein in cell lysates were examined as described above.

deletion of the Gly^{306}–Val^{330} region (HAI-mut-II) was co-expressed with MT1-MMP, and shed HAI-1 fragments were examined. In contrast to wild-type HAI-1 protein, which was cleaved to generate 16, 42, and 58 kDa fragments, HAI-mut-I and HAI-mut-II were cleaved to shed only a 42 kDa fragment and a 58 kDa fragment, respectively. HAI-mut-III, which contains both amino acid substitution at Leu^{452} and deletion of Gly^{306}–Val^{330} was no longer cleaved and shed by MT1-MMP (Fig. 1D). These results indicate that MT1-MMP cleaves HAI-1 at the juxtamembrane site and flanking region between the KD-1 and LDLR domains.

MT1-MMP abrogates inhibition of matriptase by HAI-1. Cell surface protein biotinylation assay was carried out to examine the cell-surface localization of HAI-1, MT1-MMP, and matriptase (Fig. 2A). The cell surface HAI-1 level was reduced by co-expression of MT1-MMP. Matriptase protein level at the cell surface and in the cell extract was quite low in cells transfected with matriptase plasmid alone. Matriptase accumulated in cells cotransfected with matriptase and HAI-1 plasmids, with the processed form of matriptase dominant at the cell surface and the pro-form dominant in the cell extract. Co-expression of MT1-MMP with HAI-1 and matriptase reduced the cell surface level of not only HAI-1 but also matriptase. The intracellular pro-matriptase level was less affected by MT1-MMP expression than the level of cell surface processed form. The protein concentration of the active form of matriptase was so low that it was not detected by Western blotting. Next, zymography was carried out to examine the matriptase active form level. Active form matriptase accumulated in cells co-expressing matriptase and HAI-1, which was reduced by co-expression of MT1-MMP. Concomitant with HAI-1 reduction by co-expression of MT1-MMP, active form matriptase was shed into the culture supernatant. Matriptase was not detected in cells transfected with matriptase plasmid alone by either zymography or Western blotting. These results indicate that matriptase undergoes rapid autoprocessing, activation, and degradation, and these are partially suppressed by HAI-1, resulting in an accumulation of not only active form matriptase but also pro and processed forms. This is abrogated by cleavage of HAI-1 by MT1-MMP, suggesting that MT1-MMP would restore matriptase protease activity inhibited by HAI-1.

In order to confirm this, cell-associated matriptase protease activity was examined using an artificial peptide substrate for serine proteases and the physiological substrate pro-uPA (Fig. 2C). Matriptase activates pro-uPA through processing at the consensus cleavage site Lys^{158}–Ile^{159}. Both substrates were processed by the cells transfected with matriptase plasmid alone, which produced only a trace level of active form of matriptase

doi: 10.1111/j.1349-7006.2011.02162.x

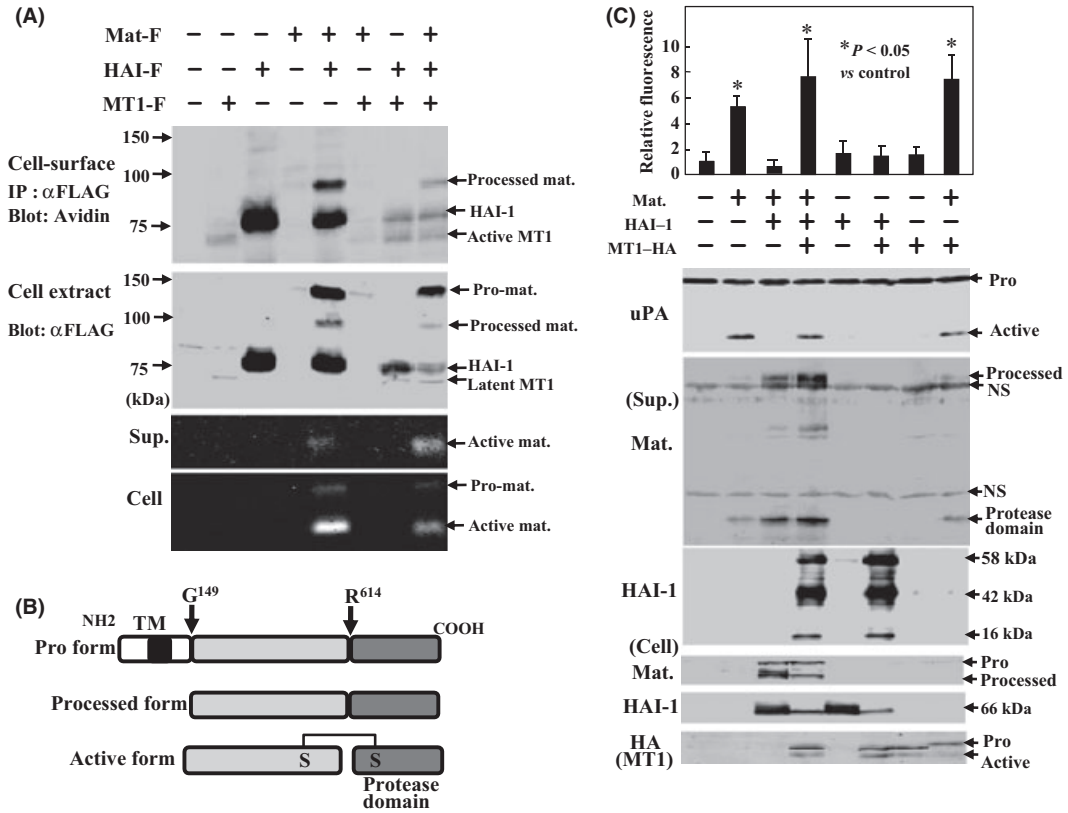

Fig. 2. Membrane-type-1 (MT1)-MMP abrogates inhibition of matriptase by hepatocyte growth factor activator inhibitor-1 (HAI-1). (A) Control plasmid and/or expression plasmid for HAI-1, matriptase, and MT1-MMP tagged with FLAG epitope (HAI-F, Mat-F, and MT1-F, respectively) were cotransfected into HEK293T cells as indicated. After biotin-labeling, cells were lysed in TritonX-100-containing buffer, immunoprecipitated with anti-FLAG antibody beads, separated by 12% SDS-PAGE, then blotted with IRDye 800-conjugated streptavidin. Aliquots of TritonX-100 extracts were analyzed by Western blotting using anti-FLAG antibody. Production of matriptase in culture supernatants (Sup.) and cell lysates (Cell) was examined by zymography. IP, immunoprecipitation. (B) Schematic representation of the structures of pro, processed, and active forms of matriptase. TM, transmembrane domain. (C) Expression plasmid for matriptase or control plasmid was cotransfected with control plasmid or expression plasmids for HAI-1 and/or MT1-MMP tagged with HA (MT1-HA) into HEK293T cells. Twenty-four hours after transfection, fluorescence-quenching substrate or pro-urokinase-type plasminogen activator (uPA) was incubated with the transfected cells for 1 or 12 h, respectively, and their processing was monitored. The fluorescence intensity obtained from the cells transnfected with control plasmid alone was arbitrarily set to 1, and the processing levels of other transfections adjusted accordingly. Matriptase and HAI-1 in the conditioned medium were precipitated with TCA, and analyzed by Western blotting using anti-matriptase and anti-HAI-1 antibodies, respectively. Cell lysates were also examined for expression of matriptase, HAI-1, and MT1-MMP by Western blotting. NS, non-specific band.

protein into the conditioned medium. Co-expression of HAI-1 with matriptase suppressed protease activity, and matriptase pro-form and processed form accumulated in these cells as observed above. Active and processed matriptase protein forms also accumulated in the conditioned medium of these cells. Co-expression of MT1-MMP with matriptase and HAI-1 stimulated shedding of HAI-1, reduced the level of cell-associated HAI-1 protein, and restored the matriptase activity that was downregulated by HAI-1 expression. Production of matriptase processed form and active form by cells co-expressing matriptase and HAI-1 was augmented by co-expression of MT1-MMP. The cell-associated matriptase activity of each sample was proportional to that in the conditioned medium (data not shown). These results indicate that cleavage of HAI-1 by MT1-MMP abrogates inhibition of matriptase by HAI-1.

HSC-4 cells. Squamous carcinoma-derived HSC-4 cells express HAI-1 and matriptase, as detected by Western blotting (Fig. 3A). Serine protease activity of HSC-4 cells was enhanced by knockdown of HAI-1 expression through transfection of siRNA targeting HAI-1. Protease activity induced by HAI-1 knockdown was aborted by transfection of matriptase siRNA. These results indicate that HSC-4 cells express matriptase, the protease activity of which is masked by HAI-1. Endogenous MT1-MMP activity of HSC-4 cells as indicated by pro-MMP-2 activation was faint (Fig. 3B). Type I collagen and ConA are both well-known regulators of MT1-MMP, and were applied to HSC-4 cells. Collagen gel culture and ConA treatment of HSC-4 cells induced pro-MMP-2 activation, which was accompanied by enhanced pro-uPA activation and secretion of HAI-1 fragments. These results suggest that MT1-MMP induced by ConA treatment or collagen gel culture of HSC-4 cells caused cleavage and shedding of the HAI-1 ectodomain, which consequently activated matriptase.

In order to examine the involvement of MT1-MMP in matriptase activation, HSC-4 cells were transfected with siRNAs targeting either MT1-MMP or the matriptase gene, and tested for processing of peptide substrate and pro-uPA (Fig. 3C). Suppression of matriptase expression by transfection of matriptase siRNA significantly reduced processing of the peptide substrate and pro-uPA by ConA treated cells, indicating that matriptase is the major serine protease induced by ConA treatment of HSC-4 cells. Knockdown of the MT1-MMP expression in ConA treated cells reduced HAI-1 shedding and increased cell-associated HAI-1 level. Consistent with HAI-1 accumulation by knockdown of MT1-MMP expression, processing of peptide substrate and pro-uPA was downregulated. These results suggest that

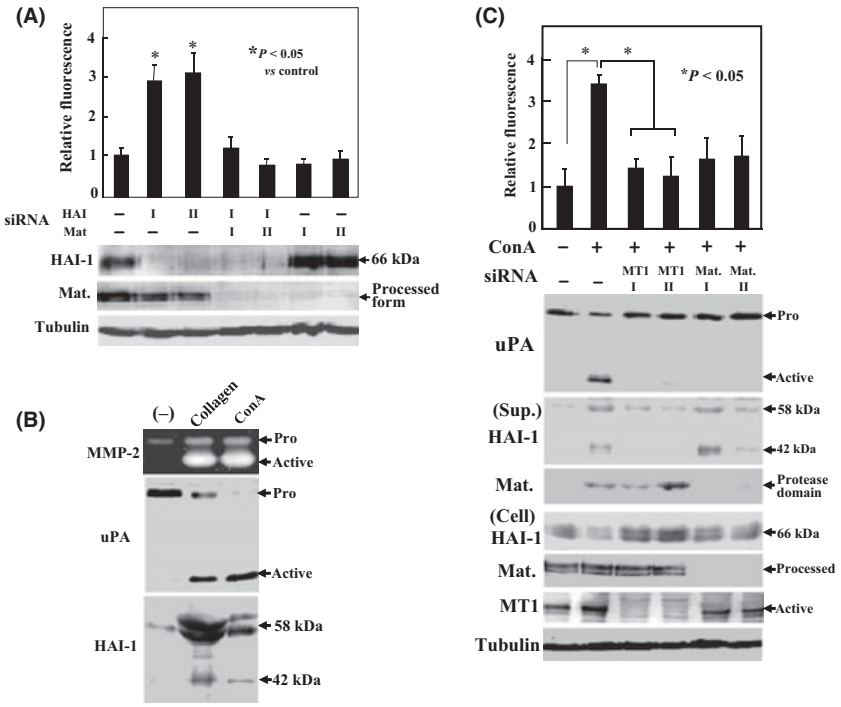

Fig. 3. Downregulation of hepatocyte growth factor activator inhibitor-1 (HAI-1) restores matriptase activity in HSC-4 cells. (A) HSC-4 cells were transfected with control RNA or siRNA targeting the HAI-1 (HAI-I, -II) and/or matriptase (Mat-I, -II) gene. Twenty-four hours after transfection, cells were incubated with fluorescence-quenching substrate for 1 h. Cell lysates were examined for HAI-1, matriptase, and tubulin expression by Western blotting as indicated. (B) HSC-4 cells were mock treated, cultured in collagen gel, or treated with concanavalin A (ConA), then incubated with pro-MMP-2 for 1 h. Cell-associated MMP-2 was examined by gelatin zymography. Cells were incubated with pro-uPA for 12 h to examine its processing. HAI-1 shed into conditioned medium was examined by Western blotting after TCA precipitation. (C) HSC-4 cells transfected with the indicated siRNA were either treated or untreated with ConA, then incubated with either fluorescence-quenching substrate or pro-uPA. Shedding of HAI-1 and matriptase protease domain and expression of HAI-1, matriptase, membrane-type-1 (MT1)-MMP, and tubulin were examined as indicated. Sup., supernatant.

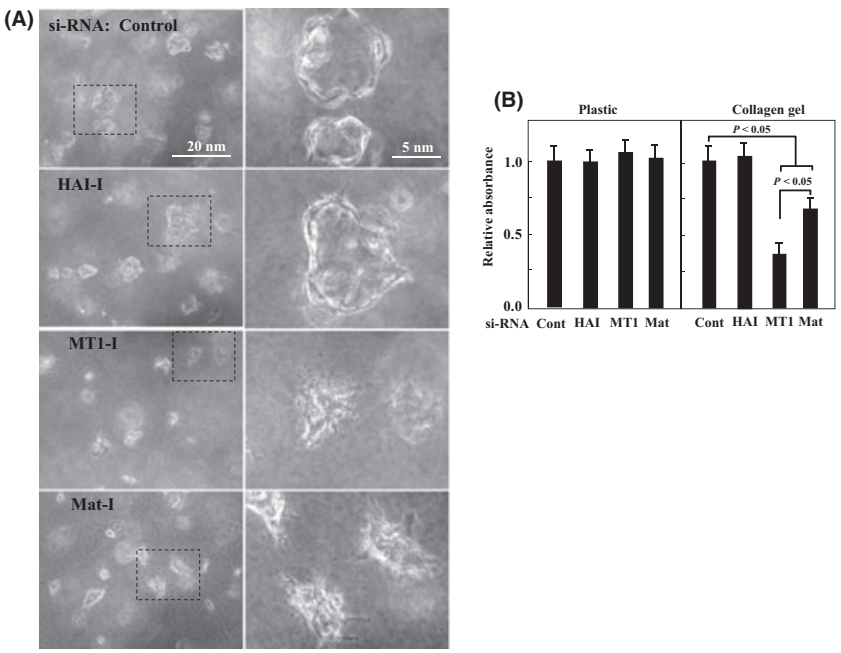

Fig. 4. Activation of matriptase by membrane-type-1 (MT1)-MMP induces invasive growth. (A) HSC-4 cells transfected with the indicated siRNA were cultured in collagen gel for 4 days (left panel). (B) HSC-4 cells transfected as above were either cultured on plastic or in collagen gel for 4 days, then cell numbers were examined using cell-counting reagent. The absorbance obtained from cells transfected with control RNA was arbitrarily set to 1. Cont, control; HAI, hepatocyte growth factor activator inhibitor-1; Mat, matriptase.

MT1-MMP induced by ConA treatment activated matriptase through the cleavage of HAI-1, which in turn caused pro-uPA activation.

HSC-4 cells transfected with control, HAI-I, MT1-MMP, or matriptase siRNA were cultured in collagen gel (Fig. 4A). HSC-4 cells treated with control or HAI-1 siRNA caused intensive digestion of collagen gel, and showed an invasive growth by forming vacuole-like structures. Knockdown of MT1-MMP or matriptase expression did not affect cell growth on plastic, but suppressed it in collagen gel (Fig. 4B). Knockdown of MT1-MMP expression suppressed more severely than that of matriptase. These results suggest that matriptase activated by MT1-MMP may collaborate with MT1-MMP for collagen degradation and subsequent invasive growth in collagen gel.

Discussion

Both MT1-MMP and matriptase are overexpressed in a variety of tumors and thought to be closely associated with their malignant progression, however, their functional interaction and combined pathophysiological relevance has never been examined.[13,14,24,25,27]

In this study, we showed for the first time that MT1-MMP activates matriptase through the cleavage of HAI-1. Proteases are normally produced in an inactive zymogen form, and their activation is one of the important regulatory steps, another being specific inhibitors that regulate the active proteases. Unlike soluble-type MMPs, MT-MMPs are activated by an intracellular processing enzyme (e.g. furin) and transferred to the cell

doi: 10.1111/j.1349-7006.2011.02162.x

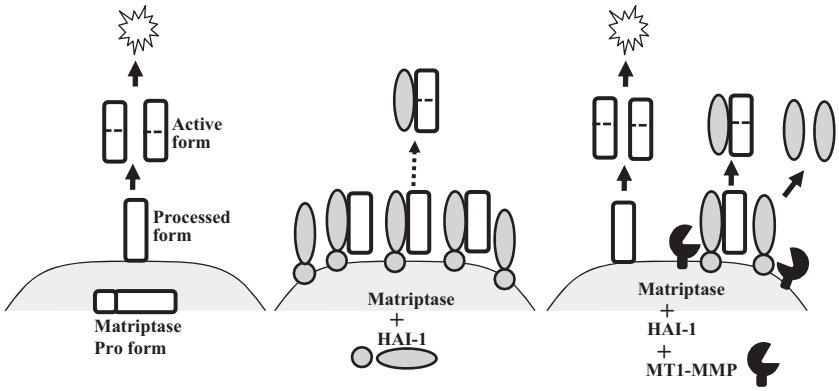

Fig. 5. Schematic illustration of interaction between matriptase, hepatocyte growth factor activator inhibitor-1 (HAI-1), and membrane-type-1 (MT1)-MMP. Matriptase causes rapid autoprocessing and degradation in cells expressing matriptase alone (left panel). Processed forms of matriptase accumulate on the cell surface in complex with HAI-1 (middle panel). MT1-MMP sheds HAI-1 and reduces cell surface concentration of HAI-1 in cells expressing matriptase, HAI-1, and MT1-MMP, which in turn produces active matriptase. A residual matriptase/HAI-1 complex is shed by MT1-MMP (right panel).

membrane in an active form.[35] Among numerous serine proteases, matriptase is also unique in that its activation cleavage site is autocatalytic, which suggests that matriptase can act upstream of a serine protease activation cascade, just as MT1-MMP activates pro-MMP-2 and pro-MMP-13.[24,36]

The activation of the matriptase zymogen is extraordinarily complex, and results in cleavage after Gly^{149} and Arg^{614} to produce a catalytically active protease domain (reviewed by List *et al.*).[13] HAI-1 is the cognate inhibitor of matriptase, however, paradoxically it has been thought to be required for activation of matriptase[36] and involved in its expression and trafficking.[37] Again, this has a parallel in the role of TIMP-2 in the MT1-MMP-mediated activation of MMP-2.[38] However, transfection experiments by us and others showed that HAI-1 is not always necessary for production of catalytically active matriptase,[39,40] and several lines of evidence from HAI-1-deficient animal models clearly show the importance of HAI-1 as an inhibitor, rather than an activator, of matriptase activity.[41] Indeed, HAI-1 regulates matriptase autoprocessing, activation, and/or degradation (Fig. 2A), but the matriptase produced by HAI-1-expressing cells was catalytically inactive (Fig. 2C). Co-expression of HAI-1 suppressed autoprocessing and degradation of matriptase, resulting in an accumulation of matriptase pro-form in cytoplasm, and processed and active forms on the cell surface. Matriptase in these cells was catalytically inactive and might be complexed with HAI-1. In contrast, matriptase activity was quite high in cells expressing matriptase alone, although only a trace level of active form protein was detected in these cells. This may be due to a rapid turnover rate of active matriptase in the absence of HAI-1. Co-expression of MT1-MMP with HAI-1 and matriptase effectively cleaved and shed HAI-1, and reduced the cell-surface HAI-1 concentration (Fig. 2). Thus, matriptase produced by these cells was active. MT1-MMP also shed HAI-1 which was in a complex with matriptase, and the shed matriptase-HAI-1 complex was stable and accounted for the accumulated matriptase protein seen in the conditioned medium, as shown in Figure 5.

Reduced expression of HAI-1 by siRNA induced spontaneous activation of matriptase in mammary epithelial cells[37] and squamous-carcinoma derived HSC-4 cells (Fig. 3), and matriptase-mediated epithelial to mesenchymal transition in pancreatic cancer cells.[42] It has been emphasized that the balance of matriptase and HAI-1 levels is a critical factor for malignant phenotype of tumors.[1,18,37] Kataoka *et al.*[20,43] reported that shedding of an HGFA-HAI-1 complex was induced by phorbol ester, and was inhibited by metalloprotease inhibitor in HLC-1 lung carcinoma cells, suggesting a possible regulatory mechanism of HAI-1 function through shedding by metalloprotease.

In this study, MT1-MMP was shown to disrupt the balance by cleaving HAI-1 and increasing matriptase activity. Collagen gel culture of HSC-4 cells stimulated MT1-MMP activity, and recovered matriptase inactivated by HAI-1. HSC-4 cells caused intensive collagen gel digestion to allow invasive growth by forming vacuole-like structures. Knockdown of either MT1-MMP or matriptase significantly suppressed their invasive growth. HSC-4 cells might use the combined action of MT1-MMP and matriptase for intensive degradation of collagen gel. Knockdown of MT1-MMP not only suppressed its expression but also abrogated matriptase activation, resulting in more severe growth suppression in collagen gel than matriptase knockdown. Both MT1-MMP and matriptase show broad substrate specificities, and the combination of MT1-MMP and matriptase may have synergistic effects on malignant progression of tumors *in vivo*. As pro-MMP-2 activation by MT1-MMP stimulates tumor invasive growth, matriptase acts upstream of a serine protease activation cascade, and the downstream uPA system plays a potent role in the malignant phenotype of tumors. Serine protease cascade also activates a variety of MMPs, which further accelerates ECM degradation. In conclusion, MT1-MMP triggers not only MMP activation but also a serine protease cascade through the cleavage of HAI-1, and may cause a protease storm.

Acknowledgments

We thank Dr. E.W. Thompson (St. Vincent's Institute of Medical Research, Melbourne, Australia) for critical reading of the manuscript. This work was supported in part by a Grant in Aid for Scientific Research (21390091 to H. Sato and T. Takino) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan.

Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

ConA	concanavalin A
HAI-1	hepatocyte growth factor activator inhibitor-1
HGF	hepatocyte growth factor
HGFA	HGF activator
KD	Kunitz domain
LDLR	low-density lipoprotein receptor
MSP	mosaic serine protease
MT-MMP	membrane-type MMP
SUMO	small ubiquitin-like modifier
TCA	trichloroacetic acid
TIMP	tissue inhibitor of metalloproteinase
uPA	urokinase-type plasminogen activator

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doi: 10.1111/j.1349-7006.2011.02162.x