A recombinant matriptase causes an increase in caspase-3 activity in a small-intestinal epithelial IEC-6 line cultured on fibronectin-coated plates

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Abstract Matriptase is an epithelial-derived type-II transmembrane serine protease. This protease is expressed prominently in the villus tip of small-intestinal epithelia at which senescent cells undergo shedding and/or apoptosis. The basement membrane of epithelial cells, including small-intestinal epithelial cells, contains extracellular matrix (ECM) proteins such as fibronectin and laminin. We found previously that high concentrations of a recombinant matriptase catalytic domain (r-MatCD) (e.g. 1 μ M) caused an increased detachment of and increases in the activity of apoptotic effector caspase-3 in a rat small-intestinal epithelial IEC-6 line cultured on laminin-coated plates and proposed that at sites with its high level of expression, matriptase contributes to promoting shedding and/or detachment-induced death of epithelial cells through a mechanism mediating loss of cell-ECM adhesion. In this study, we found that even without increasing cell detachment, a high concentration of r-MatCD causes an increase in caspase-3

activity in IEC-6 cells cultured on fibronectin-coated plates, suggesting that the recombinant matriptase can cause apoptosis by a mechanism unrelated to cell detachment. Also, r-MatCD-treated IEC-6 cells on fibronectin were found to display spindle-like morphological changes. We suggest that r-MatCD causes apoptosis of IEC-6 on fibronectin by a mechanism involving the disruption of cell integrity.

Keywords Caspase-3 · Fibronectin · IEC-6 · Matriptase · Metalloprotease

Abbreviations

Ac	Acetyl
ADAM	A disintegrin and metalloprotease
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
MMP	Matrix metalloprotease
SFM	Serum-free DMEM supplemented with insulin and bovine serum albumin
MCA	4-Methylcoumaryl-7-amide
r-EK	Recombinant form of enterokinase catalytic domain
SIECs	Small-intestinal epithelial cells

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Introduction

Matriptase is a member of the type-II transmembrane serine protease group, which is characterized by an

N-terminal cytosolic domain followed by a trans-membrane domain and an extracellular domain, including a C-terminal catalytic domain (Bugge et al. 2007; Lin et al. 2008). Matriptase is expressed by the epithelial elements of almost all organs examined such as the small-intestinal epithelial cells (SIECs) and keratinocytes (Oberst et al. 2003; List et al. 2007). To date, a number of potential *in vivo* substrates for matriptase, including the precursor of a membrane-bound serine protease prostaticin, have been identified (Bugge et al. 2007; Lin et al. 2008). These characteristics, in line with observations using matriptase gene (*hypo*/-) mice (List et al. 2009; Kosa et al. 2012), suggest that this protease essentially serves to maintain the integrity of epithelial and epidermal barriers.

We have found that (1) in rats, matriptase mRNA is abundant in the epithelium of the villus tip in the small intestine at which terminally differentiated (or senescent) cells frequently undergo apoptosis followed by shedding into the lumen or first lose adhesion to surrounding cells and the underlying basement membrane, composed of various extracellular matrix (ECM) proteins, and then undergo death (called detachment-induced apoptosis or anoikis) and that (2) a recombinant (or r-) matriptase cleaves ECM proteins such as fibronectin and laminin (Satomi et al. 2001; Tsuzuki et al. 2005). We also reported that a recombinant form of matriptase catalytic domain (hereinafter r-MatCD) at high concentrations (e.g. 1 μ M) causes an increased detachment and apoptosis (as assessed by an increase in cellular activity of apoptotic effector caspase-3) of a rat small-intestinal epithelial IEC-6 line cultured on laminin-coated plates (Mochida et al. 2010). In small-intestinal villi, epithelial cells (including senescent cells at villus tip) adhere to the underlying basement membrane in which laminin but not fibronectin is contained, and cell binding to laminin is believed to mediate the differentiation (Potten 1998). Therefore, the IEC-6 line cultured on laminin could be regarded as a model of differentiated and/or senescent SIECs. Altogether, we proposed that matriptase contributes to the clearance (i.e., anoikis) of senescent epithelial cells at sites with its high levels of expression, such as the villus tip in the small intestine. This idea does not necessarily contradict the beliefs regarding the protective role of matriptase in epithelia (List et al. 2009; Kosa et al. 2012), because the continuous removal of senescent

cells in the superficial zone serves to contribute essentially to the maintenance of overall epithelial and epidermal architectures.

SIECs located at the mid and upper crypt zones adhere to the underlying basement membrane containing fibronectin, cell binding to which mediates the proliferation (Potten 1998). Given moderate expression of matriptase mRNA in SIECs at the proliferating crypt zone and the ability of r-matriptase to cleave fibronectin *in vitro* (Satomi et al. 2001), we previously assessed whether r-MatCD causes an increased detachment of IEC-6 cells on fibronectin (Mochida et al. 2010). However, we found no evidence for an increased detachment and suggested that the recombinant matriptase may not gain access to or cleave fibronectin molecules attached to plates or the molecules that are already associated with their specific integrins (Mochida et al. 2010). In the present study, we addressed whether r-MatCD causes apoptosis of IEC-6 cells cultured on fibronectin-coated plates. For this, we tested to determine whether the r-matriptase increases caspase-3 activity in the cell line cultured on the ECM component. For reference, the data obtained using the cells cultured on laminin-coated plates were also presented.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) (high glucose, supplemented with L-Glu) and penicillin–streptomycin mixture were from Invitrogen (Carlsbad, CA, USA). HisLink™ resin was from Promega (Madison, WI, USA). A recombinant form of enterokinase catalytic domain (hereinafter r-EK) was from Novagen (Madison, WI, USA). Bovine serum albumin (BSA) (protease free), fibronectin (human plasma), insulin (bovine pancreas), and laminin (from Engelbreth–Holm–Swarm murine sarcoma basement membrane) were from Sigma (St. Louis, MO, USA). Acetyl (Ac)-L-Asp–L-Glu–L-Val–L-Asp-methylcoumaryl-7-amide (MCA) (Ac-DEVD-MCA) and Ac-DEVD aldehyde were from the Peptide Institute (Osaka, Japan). GM6001 was purchased from Calbiochem (San Diego, CA, USA). All other reagents were of analytical grade and were from Nacalai Tesque (Kyoto, Japan).

Cell culture

IEC-6 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 13, and were maintained in DMEM supplemented with 5 % fetal bovine serum, 4 µg/ml insulin, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37 °C in 5 % CO₂.

Preparation of r-MatCD

A proteolytically inactive form of r-MatCD (pro-r-MatCD) was expressed by and secreted from *Pichia pastoris*, KM71H strain (Invitrogen). Procedures for purification of pro-r-MatCD from yeast medium by Ni²⁺-chelating chromatography using HisLinkTM resin, treatment with r-EK for in vitro conversion of pro-r-MatCD to r-MatCD, removal of r-EK, and determination of the concentrations of r-MatCD were described previously in detail (Mochida et al. 2009; 2010). A 0.5-ml aliquot of the solution containing r-MatCD was subjected to gel filtration in serum-free DMEM containing 4 µg/ml insulin and 0.1 mg/ml BSA (hereinafter SFM) using an NAP-5 column (GE Healthcare, Tokyo, Japan), filter sterilized, and stored at 4 °C until use. The sample of r-MatCD was used for cultured cell experiments within 10 days.

Preparation of and treatments of cells with a metalloprotease inhibitor

GM6001 (Calbiochem, San Diego, CA, USA) was dissolved in dimethylsulfoxide at a concentration of 10 mM and stored at −20 °C until use. When used for cultured cell experiments, the inhibitor was included in the medium at a concentration of 10 µM.

Coating of cell culture plates with fibronectin and laminin

Fibronectin and laminin were diluted with phosphate buffered saline (PBS, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 136 mM NaCl, 2.7 mM KCl, pH 7.4): the final concentration of each protein was 10 µg/ml. Wells of polystyrene 12 well plates (Asahi Techno Glass, Tokyo, Japan) were exposed to 500 µl of diluted pure matrices and incubated for 1 h at 37 °C. After incubation, each well was washed three times with 3 ml of PBS. Each well was then incubated for 30 min at 37 °C with 500 µl of PBS containing

0.5 mg/ml BSA to block sites not bound by the matrices. After incubation, wells were washed three times with 3 ml of PBS and used for experiments.

Caspase-3 activity assay

Passaged cultures of IEC-6 were detached by incubation for 5 min at 37 °C with 0.25 % trypsin-1 mM EDTA solution, immediately washed four times with PBS and suspended in SFM. Aliquots of 5×10^5 cells were incubated for 4 h at 37 °C to enable them to adhere to the wells of plates coated with fibronectin or laminin. After removing non-adherent cells by washing the wells twice with PBS, cells were exposed to 500 µl of SFM with or without test substances (e.g. 1 µM r-MatCD) and incubated for an additional 24 h.

After microscopic observation, samples for determination of cellular caspase-3 activities were prepared as follows: Detached cells included in the conditioned medium of a well were sedimented in a 1.5-ml microcentrifuge tube by centrifugation at 5,000g for 2 s at 22 °C. The sedimented cells were washed twice with 1 ml of PBS. The cells that remained attached were washed twice with 2 ml of PBS. Both the sedimented and attached cells were exposed to 100 µl of 50 mM HEPES buffer at pH 7.4 containing 0.2 % CHAPS (HEPES buffer B) and incubated for 30 min on ice with occasional agitation. The extracts were pooled and centrifuged at 10,000g for 20 min at 4 °C to remove insoluble material. The resulting supernatants (CHAPS extracts) were collected, and the protein concentration was determined as described previously (Mochida et al. 2010). Each CHAPS extract was diluted with HEPES buffer B to a final protein concentration of 0.15 mg/ml and placed on ice.

Caspase-3 activities in the CHAPS extracts were determined as follows: Ac-DEVD-MCA (a substrate for caspase-3) and Ac-DEVD aldehyde (an inhibitor for caspase-3) had been individually dissolved in dimethylsulphoxide at a concentration of 10 mM and stored at −20 °C until use. The reactions were initiated by adding 20 µl of diluted CHAPS extract to 80 µl of the substrate mixture (25 mM PIPES buffer with pH 7.3 containing 2 mM EGTA, 2 mM MgCl₂, 5 mM dithiothreitol, and 62.5 µM Ac-DEVD-MCA), and of the mixture containing 250 µM Ac-DEVD aldehyde. After incubation for 30 min at 22 °C, fluorescence of 4-methyl- coumaryl-7-amine released from Ac-DEVD-MCA was measured using a plate

reader (PowerScan, Dainippon Sumitomo Pharma, Osaka, Japan) at an excitation wavelength of 380 nm and an emission wavelength range of 460 nm. Under these conditions, no fluorescence was produced in any sample in which Ac-DEVD aldehyde was included.

Statistical analysis

Data were analyzed by two-tailed, unpaired *t* test and two-way analysis of variance and post-hoc Bonferroni's test with the aid of a commercially available software (Prism, GraphPad, San Diego, CA). A *P* value of less than 0.05 was considered to be statistically significant.

Results and discussion

We reported previously that after 24-h incubation, the number of IEC-6 cells detached was significantly higher in wells exposed to SFM containing 1 μ M r-MatCD than in those exposed to SFM alone when using cells cultured on laminin-coated plates, whereas those were similar irrespective of the presence or absence of the r-matriptase at the same concentration when using cells on fibronectin (Mochida et al. 2010). These results were confirmed in this study (data not shown). Consistent with our previous data (Mochida et al. 2010), after 24-h of incubation the level of caspase-3 activity was significantly higher in the wells exposed to SFM containing 1 μ M r-MatCD than in those exposed to SFM alone when using cells on laminin (Fig. 1). Strikingly, a significant increase in caspase-3 activity in cells on fibronectin was also found by incubation with the r-matriptase (Fig. 1). Although additional assays (e.g. DNA fragmentation and annexin-V labeling assays) are required to confirm the occurrence of apoptosis in r-MatCD-treated cells on fibronectin, here we suggest that at a high concentration, the r-matriptase can cause cell death by a mechanism unrelated to cell detachment. Also, it is thought that r-MatCD-induced death of IEC-6 cells attached on laminin which we observed previously (Mochida et al. 2010) could not necessarily be accounted for by detachment per se. SIECs in the proliferating crypt zone are known to undergo apoptosis in response to toxic drugs (Potten 1998). It is interesting to speculate that matriptase in the crypt epithelia, if the expression is highly increased under such environmental conditions, contributes to the induction of apoptosis.

We next show the morphological appearances of IEC-6 cells on laminin and fibronectin after 24 h of incubation with SFM alone or SFM containing 1 μ M r-MatCD (Fig. 2). After incubation with SFM alone, IEC-6 cells on laminin exhibited the appearance of monolayer epithelium (LN, –MatCD). Cells that remained attached to the plates even after incubation with r-MatCD displayed a flat and elongated shape, on which cells (or cell aggregates) with rounded morphology attached (LN, +MatCD). Note that cells on laminin detached as singles or small aggregates upon treatment with r-MatCD (refer to Mochida et al. 2010). After incubation with SFM alone, the cells on fibronectin also grew as a monolayer epithelium, but the cell–cell boundaries were obscure compared to those of cells on laminin (FN, –MatCD). Upon treatment with r-MatCD, most of the cells on fibronectin underwent spindle-like morphological changes (FN, +MatCD). Together with an earlier report showing that the formation of spindle-like structures of bovine endothelial cells induced by treatment with an anti-tumor drug is accompanied by apoptosis

Fig. 1 Elevation of the caspase-3 activity by r-MatCD in IEC-6 cells cultured on laminin- and fibronectin-coated plates. IEC-6 cells attached to laminin (LN)- or fibronectin (FN)-coated plates were incubated with SFM alone (–) and SFM containing 1 μ M r-MatCD (+) for 24 h. After incubation, cellular caspase-3 activities were assessed as described in the text. Caspase-3 activity in cells on laminin incubated with SFM alone was assigned a value of 100 %. Data were obtained from a single experiment and are expressed as the mean $\pm$ SD from three independent wells per each treatment condition. Data were analyzed by two-tailed, unpaired *t* test. **P* < 0.01; compared to cells exposed to SFM alone under each experimental condition

Fig. 2 Morphological changes of IEC-6 cells on laminin and fibronectin upon incubation with r-MatCD. IEC-6 cells were attached to plates precoated with laminin (LN) or fibronectin (FN). After attachment, cells were exposed to SFM alone (–MatCD) and SFM containing 1 μ M r-MatCD (+MatCD). After 24 h of incubation, cells were analyzed by light-microscopy and photographed. Bars 50 μ m

(Meng et al. 1999), we suggest that a high concentration of the r-matriptase induces IEC-6 death on fibronectin (and possibly on laminin) by a mechanism involving the disruption of cell integrity.

Metalloproteases are known to often mediate the function of matriptase (Fang et al. 2011; Milner et al. 2010; Mochida et al. 2010). For instance, r-MatCD-induced detachment and morphological changes of Madin–Darby canine kidney epithelial cells were found to highly depend on matrix metalloprotease (MMP)-2 (Mochida et al. 2010). In this study, we addressed whether the effect of r-MatCD on the increase in caspase-3 activity in IEC-6 cells on fibronectin is mediated by metalloproteases. GM6001 is a broad spectrum metalloprotease inhibitor. When the inhibitor was present, the levels of caspase-3 did not differ significantly between cells incubated with SFM alone and those incubated with SFM containing r-MatCD at a high concentration (Fig. 3). This suggests that certain metalloproteases mediate the effect of r-MatCD. IEC-6 cells were previously found to express the mRNA for MMP-2 upon attachment to laminin-coated plates (Mochida et al. 2010). More notably, we found recently that the mRNAs for some members of a disintegrin and

metalloprotease (ADAM) family, such as ADAM-9, -15, and -17 are readily detected in IEC-6 cells cultured in DMEM supplemented with fetal bovine serum and insulin (Mochida S, Tsuzuki S, Inouye K, and Fushiki T, unpublished data). Of these, ADAM-17 (also known as a pro-tumor necrosis factor- α -converting enzyme) has emerged as a key player in the induction of male germ cell apoptosis (Moreno et al. 2011). The involvements of MMP(s) and ADAM(s) in r-MatCD-induced increases in caspase-3 activity in IEC-6 cells await further investigation.

In summary, we show that r-MatCD increases the caspase-3 activity in IEC-6 on fibronectin without increasing cell detachment. However, the precise mechanism(s) involved are unclear. To date, a defective integrity of cell–cell contacts has emerged as the onset

Fig. 3 Inhibition by GM6001 of r-Mat-induced increase in caspase-3 activity in IEC-6 cells attached on fibronectin. IEC-6 cells were attached to plates pre-coated with fibronectin. After attachment, cells were exposed to SFM alone and SFM containing 0.5 μ M r-MatCD in the presence and absence of 10 μ M GM6001. Note that any of the test solutions contain 0.5 μ l of dimethylsulfoxide. After 24 h of incubation, cellular caspase-3 activity was assessed as described in the text. Caspase-3 activity in cells incubated with SFM alone in the absence of GM6001 was assigned a value of 100 %. Data were obtained from a single experiment and are expressed as the mean $\pm$ SD from three independent wells per each treatment condition. Data were analyzed by two-way analysis of variance and post hoc Bonferroni's test. The *P* values obtained were 0.0002 for the r-MatCD effect, 0.6456 for the GM6001 effect, and 0.0023 for the r-MatCD $\times$ GM6001 interaction. **P* < 0.05; N.S. not significant

of death in SIECs of mice (Fouquet et al. 2004). We also found (i) that IEC-6 cells detached as aggregates by treatment with a serine protease granzyme A and displayed no characteristics of apoptosis (Hirayasu et al. 2008) and (ii) that after detachment by treatment with r-MatCD, single IEC-6 cells were mostly positive for annexin-V staining, whereas the ones maintaining cell-cell contact (i.e., small cell aggregates) were not (Mochida et al. 2010). Judging from the spindle-like morphology, r-MatCD-treated IEC-6 cells on fibronectin might undergo loss of cell-cell adhesions. Altogether, we hypothesize that at a high concentration, r-MatCD induces IEC-6 death primarily via a mechanism involving the disruption of cell-cell contacts. In various epithelial-derived cancer cell lines, loss of cell-cell adhesion (or loss of *E-cadherin*) is known to function as a mediator of epithelial-to-mesenchymal transition (EMT), a process during which cells lose their polarized organization and acquire mesenchymal characteristics (i.e., motile and/or invasive properties) (Tiwari et al. 2012). In addition, disruption of *E-cadherin* by MMP(s) has been found to induce EMT of a renal tubular epithelial cell line (Zheng et al. 2009). Thus, it would not be surprising if r-MatCD, together with certain metalloproteases, should cause the breakdown of molecules essential for cell-cell adhesion (e.g., *E-cadherin*) leading to EMT or EMT-like events in IEC-6 cells on fibronectin. However, it is not generally accepted that EMT is followed by or combined with apoptosis or loss of cell viability. A possible explanation for cell death following or concurrently with EMT-like events in our experimental model is that treatment of r-MatCD simultaneously results in the breakdown of fibronectin molecules onto culture supports that are not involved in the adhesion of cells, thereby not affording new substratum adhesions at the leading edge of motile cells. It will be a challenge to prove this hypothesis.

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