Soluble EMMPRIN (extra-cellular matrix metalloproteinase inducer) stimulates the migration of HEP-2 human laryngeal carcinoma cells, accompanied by increased MMP-2 production in fibroblasts

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Summary. The basement membrane functions as a barrier against the invasion of cancer cells. It is therefore important to investigate the mechanism of basement membrane degradation by matrix metalloproteinases (MMPs). Previously, cancer cells were long considered to be the major source of MMPs; however, current evidence indicates that most MMPs in cancer tissue are produced by stromal rather than cancer cells. A glycoprotein highly expressed on the cancer-cell membrane, EMMPRIN (extra-cellular matrix metalloproteinase inducer), exhibits the potential role of the MMP inducer in stromal cells. Depending on the cell type, EMMPRIN can stimulate the production of MMP-1, MMP-2, and MMP-3.

We here report that soluble full-length EMMPRIN is liberated from HEP-2 human laryngeal epidermoid carcinoma cells, probably *via* microvesicle shedding. Soluble EMMPRIN stimulates human fibroblasts to produce MMP-2, after which the augmented migration of HEP-2 cells occurs, as observed in an invasion chamber assay with separately cultured fibroblasts. An anti-EMMPRIN function-blocking antibody reduced

MMP-2 activity in the conditioned medium and inhibited the migration of HEP-2; obviously, EMMPRIN activity contributes to cancer-cell migration. We postulate that soluble EMMPRIN probably triggers the promotion of cancer invasion *in vivo*.

Introduction

Matrix metalloproteinases (MMPs) are endopeptidases, all of which degrade components of the extracellular matrix. Based on their substrate specificities, MMPs are mainly classified into collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10), membrane-type MMPs (MMP-14, -15, -16, -17), and others (Liotta *et al.*, 1991). MMPs play important roles in the remodeling of the extracellular matrix, the movement of cells, cancer invasion, and metastasis (Stetler-Stevenson *et al.*, 1993). It is noteworthy that, among MMPs, MMP-2 induces a highly potent degradation of type-IV collagen, a major component of the basement membrane. MMPs are particularly overproduced in the tumor local environment, and it has been demonstrated that high levels of MMPs correlate with increased cancer invasion capacity, both *in vitro* and *in vivo* (Gilles *et al.*, 1994, 1996). Formerly, it was considered that tumor cells were the major source of MMPs in cancer tissue. Current evidence, however, indicates that most MMPs in cancer tissue are produced by stromal cells, including fibroblasts (Poulsom *et al.*, 1992; Guo *et al.*, 1996). Cancer cells promote the production of MMPs in stromal cells *via* soluble factors

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such as cytokines (Brown *et al.*, 1992; Marti *et al.*, 1994).

In addition to cytokines, a glycoprotein that stimulates stromal cells to synthesize MMPs has been identified (Biswas and Nugent, 1987). It is now designated as "EMMPRIN" (extra-cellular matrix metalloproteinase inducer); it is also referred to as "TCSF" (tumor collagenase stimulatory factor), "Basigin", and "CD147" (Polette *et al.*, 1997; Yan *et al.*, 2005). EMMPRIN is a highly glycosylated transmembranous molecule belonging to the immunoglobulin superfamily and is intensely expressed on the cancer-cell surface (Berditchevski *et al.*, 1997). Recent studies have indicated that purified EMMPRIN stimulates and enhances the protein synthesis of MMP-1, MMP-2, and MMP-3 in cultured fibroblasts, following elevated levels of the corresponding mRNAs (Kataoka *et al.*, 1993). EMMPRIN is enriched in a wide variety of human cancers (Polette *et al.*, 1997; Caudroy *et al.*, 1999), but soluble EMMPRIN has also been detected in non-cancerous tissues — for example, in gingival crevicular fluid (Emingil *et al.*, 2006) and ocular fluid (Määttä *et al.*, 2006). Therefore, EMMPRIN is considered to have the physiological function of modulating MMP expression during normal tissue remodeling and differentiation (Igakura *et al.*, 1998; Gabison *et al.*, 2005). It has been reported that direct cell-cell contact between cancer cells and fibroblasts is essential for EMMPRIN to induce enhanced MMP-2 production in fibroblasts (Suzuki *et al.*, 2004). On the other hand, in addition to being produced under normal physiological conditions, EMMPRIN can be released in soluble form by cancer cells, and this soluble EMMPRIN acts on fibroblasts both adjacent to and distant from the cancer site for the further induction of MMPs (Tang *et al.*, 2004). Though the influence of soluble EMMPRIN on cancer invasion has not been elucidated, it is supposed to be more effective than cell-surface EMMPRIN in promoting the invasion of cancer cells *in vivo*.

In our previous study, HEp-2 human laryngeal carcinoma cells were used to clarify the involvement of EMMPRIN in the progression of head and neck cancers. The experimental results proved that HEp-2 cells express EMMPRIN on the cell surface — just like other human cancer cells, and it is suggested that direct cell-cell interaction involving cell-surface EMMPRIN is important for pro-MMP-2 production and activation in fibroblasts (Suzuki *et al.*, 2004). To investigate further the functions of EMMPRIN involved in cancer-cell invasion and MMP-2 production, we examined in this study whether or not soluble EMMPRIN is produced by HEp-2 cells and evaluated the effects of soluble EMMPRIN on the invasion of HEp-2 cells and the induction of MMP-2 production.

Materials and Methods

Cells and cell culture

HEp-2 cells, a human laryngeal carcinoma cell line, were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai). Human fibroblasts were prepared from an outgrowth of the dermis from a healthy subject. The protocol for specimen preparation was previously approved by the Ethical Committee at the Akita University School of Medicine. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO-Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37°C.

Antibodies

Two goat anti-EMMPRIN antibodies, N-19 and C-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were used for immunoblotting. N-19 antibody has an affinity for the N-terminus of human EMMPRIN, while C-19 has an affinity for the C-terminus. A mouse anti-EMMPRIN function-blocking antibody, UM-8D6 (Research Diagnostics, Inc., Flanders, NJ, USA), was applied for the inhibition studies; its blocking activity has been described previously (Bordador *et al.*, 2000; Koga *et al.*, 2006). A negative control mouse IgG1, X0931 (Dako Cytomation, Denmark), was used as a control for the mouse monoclonal anti-EMMPRIN blocking antibody (UM-8D6) of the IgG1 isotype. Mouse IgG1 is the antibody to *Aspergillus niger* glucose oxidase, which is not present in mammalian tissues.

Immunoblotting

Immunoblotting was performed to evaluate the expression of EMMPRIN on the cell surface and in released soluble form. HEp-2 cells and fibroblasts were cultured until confluence, and the culture media were replaced by serum-free DMEM and further incubated for 48 h. The cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The proteins in the conditioned media were collected and concentrated by acetone precipitation using 3 volumes of cold acetone. The cell lysates and acetone precipitates containing 10 µg of protein were loaded and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gel according to Laemmli's method (Berditchevski *et al.*, 1997), and then blotted onto a PVDF membrane (Clear Blot Membrane-P, Atto Co., Tokyo). After blocking with

Fig. 1. Immunoblot analysis of EMMPRIN protein in the cell lysates and conditioned media. We used two goat anti-EMMPRIN antibodies: one (N-19) which reacts against the N-terminal portion (lanes 1~4), and the other (C-19) which reacts against the C-terminal portion (lanes 5~9). Samples containing 10 μ g of protein were loaded onto each lane. The loaded samples were HEp-2 cell lysate (lanes 1, 5), fibroblast lysate (lanes 2, 6), conditioned media from HEp-2 cells (lanes 3, 8), conditioned media from fibroblasts (lanes 4, 9), and concentrated conditioned medium from HEp-2 cells (lane 7). In lanes 1 to 4, the EMMPRIN expression levels in both the cell lysates and the conditioned media from fibroblasts were lower than those from HEp-2 cells, yet clear bands were observed in all lanes. In the presence of C-19 anti-EMMPRIN antibody, bands were obscured in lanes 8 and 9.

5% low-fat milk in PBS containing 0.1% Tween 20 (Calbiochem, La Jolla, CA, USA), the membrane was incubated with goat anti-EMMPRIN antibody, N-19 or C-19 (Santa Cruz Biotechnology, Inc.), diluted with 1% low-fat milk and 0.1% Tween 20 in PBS for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated anti-goat IgG diluted in 0.1% Tween 20 in PBS for 1 h at room temperature. The peroxidase activity on the membrane was visualized using ECL Immunoblotting Detection Reagents (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK).

Migration assays

The migration of HEp-2 cells was assayed using a growth-factor-reduced BD Matrigel Invasion Chamber (Becton Dickinson, Bedford, MA, USA) (Imamura *et al.*, 1994). The Matrigel invasion chamber consists of an upper and a lower chamber, separated by a polyethylene terephthalate membrane with 8- μ m pores coated with Matrigel, a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) sarcoma (Becton Dickinson). Fibroblast cell suspensions (7.5×10^5) in 0.75 ml of serum-free DMEM were added to the

lower chamber and incubated for 24 h to let the fibroblasts adhere to the bottom of the chamber. For the control, serum-free DMEM was added to the lower chamber. Then 1.2×10^6 HEp-2 cells suspended in 0.5 ml of serum-free DMEM were added to the upper chamber. After incubation at 37 $^{\circ}$ C for 48 h, those HEp-2 cells that had not migrated were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab. The HEp-2 cells that had migrated to the lower surface of the membrane were stained with Diff-Quick (International Reagents Co., Kobe) or quantified by colorimetric MTT (methylthiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich) assay (Green *et al.*, 1984). For the inhibition of EMMPRIN activity, 1.25 μ g of anti-EMMPRIN function-blocking antibody was added to the upper chamber, and, — in the same experiment — 1.25 μ g of control mouse IgG1 was applied for the negative control. For the MTT assay, 50 μ l of MTT solution (5 mg/ml in PBS) was added to the lower chamber and further incubated at 37 $^{\circ}$ C for 4 h. The cells which had migrated to the membrane formed dark blue crystals of MTT-formazan during the incubation. The membrane was transferred into another chamber containing 0.5 ml of isopropanol with 0.04 N HCl to dissolve the formazan crystals. After allowing

Fig. 2. Cross-section of the membrane which separates the invasion chamber into an upper and a lower compartment. The membrane was coated with a thin layer of reconstituted basement membrane, Matrigel, that was stained with TO-PRO-3. Using a confocal laser-scanning microscope, we examined the characteristics of the membrane. Three layers were observed: a strongly stained upper Matrigel layer, a middle unstained PET layer, and a weakly stained lower Matrigel layer.

the solution to stand at room temperature for 15 min, the absorbance of the solution containing the dissolved formazan was measured using a spectrophotometer at 570 nm. These invasion experiments were done four times and evaluated based on the mean values.

Gelatin zymography

To detect MMP-2 activity in the conditioned media derived from the invasion assay, gelatin zymography was performed (Kataoka *et al.*, 1993). The conditioned media were concentrated by cold acetone precipitation as described previously, and loaded onto SDS-PAGE under non-reducing conditions using a 7.5% polyacrylamide gel containing 0.1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were washed 3 times in 2.5% Triton X-100 (ICN Biomedicals, Inc., Aurora, OH, USA) for 30 min each time at room temperature to remove the SDS, then incubated in a reaction buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM CaCl_2 and 150 mM NaCl for 24 h at 37°C, followed by staining with 2.5% (w/v) Coomassie Brilliant Blue R-250 (Fluka Chemie AG, St.

Gallen, Switzerland) in 30% (v/v) methanol and 10% (v/v) acetic acid. The MMP-2 activity of gelatin degradation in the gels was detected as clear bands on a blue background of stained undigested gelatin.

Results

HEp-2 cells and fibroblasts produced EMMPRIN and released it in soluble form

We examined EMMPRIN expression in a human laryngeal carcinoma cell line (HEp-2) and in normal human skin fibroblasts representing stromal cells. To detect the expression of EMMPRIN, the cell lysates were subjected to immunoblot analysis. Also, concentrated conditioned media from both cell types were examined for soluble EMMPRIN. For further characterization of the soluble form of EMMPRIN, we used two anti-EMMPRIN antibodies, N-19 and C-19, reacting against the N-terminus and C-terminus, respectively, to estimate the secretion of intact full-length EMMPRIN.

Fig. 3. HEp-2 cells that have migrated to the lower surface of the membrane are shown. The migration of HEp-2 cells was observed when fibroblasts were cultured in the lower chamber (**A**). On the other hand, hardly any migration was observed in the absence of fibroblasts (**B**). The white spots are pores.

The immunoblot analysis using the N-19 antibody showed that both HEp-2 cells and fibroblasts produced EMMPRIN (Fig. 1). A previous study has shown that EMMPRIN is expressed on the cell surface of both HEp-2 cells and fibroblasts (Suzuki *et al.*, 2004). The HEp-2 cells seemed to produce EMMPRIN in larger amounts than the fibroblasts, and the difference between the two cell types was more remarkable in the conditioned media (Fig. 1). In fact, the fibroblasts secreted little soluble EMMPRIN. Broad bands corresponding to the apparent molecular weight of 35 to 60 kDa were observed in the cell lysates because of the extensive and heterogeneous glycosylation characteristic of EMMPRIN polypeptides. The unglycosylated form of EMMPRIN is 28 kDa (Biswas *et al.*, 1995). Soluble EMMPRIN was also detected, yielding a broad band of about 50 kDa (Fig. 1). The amounts of soluble EMMPRIN liberated from both cell types correlated well with the amounts obtained from the cell lysates. The amount of soluble EMMPRIN derived from HEp-2 cells seemed quite small in comparison to that in the cell lysate.

Two different antibodies, one recognizing the N-terminal and the other the C-terminal of EMMPRIN, were applied to immunoblot analysis to assess the proteolytic products. Soluble EMMPRIN lacking the C-terminal portion has previously been reported to be generated by proteolytic cleavage (Hang *et al.*, 2004; Tang *et al.*, 2004; Egawa *et al.*, 2006). In the immunoblot analysis using the C-19 antibody, broad bands were also detected in the cell lysates of HEp-2 cells and fibroblasts (Fig. 1). Soluble EMMPRIN was also detected when using a

10-fold concentrated conditioned medium from HEp-2 cells (Fig. 1). However, we failed to demonstrate the existence of soluble EMMPRIN in un-concentrated conditioned media from either cell type. The C-terminal-recognizing antibody, C-19, clearly detected C-terminal-containing soluble EMMPRIN in the HEp-2 conditioned medium. Therefore, we concluded that at least some of the molecules of soluble EMMPRIN derived from HEp-2 cells were of full length, and postulated that the soluble EMMPRIN was liberated by shedding from membrane vesicles, as in other cancer cells (Taylor *et al.*, 2002; Sidhu *et al.*, 2004; Koga *et al.* 2006; Millimaggi *et al.*, 2007).

Characterization of invasion chambers

To evaluate the effects of soluble EMMPRIN in the invasion of HEp-2 cells, we utilized growth-factor-reduced invasion chambers (Becton Dickinson). These invasion chambers consist of an upper and a lower chamber which are separated by a polyethylene terephthalate (PET) membrane with 8- μ m pores and coated with a thin layer of growth-factor-reduced reconstituted basement membrane (Matrigel). For the invasion assay, cells were applied to the upper chamber and chemoattractants were added to the lower chamber.

In order to characterize the Matrigel-coated membrane in detail, we stained the membrane with TO-PRO-3, purchased from Molecular Probes (Invitrogen, Eugene, OR, USA), and obtained images of the cross section of the membrane using a confocal laser scanning microscope.

Fig. 4. Gelatin zymography of MMP-2 in conditioned media. In the invasion chamber, HEp-2 cells and fibroblasts were separately cultured in the presence or absence of the anti-EMMPRIN antibody. The conditioned media were collected, concentrated, and analyzed for MMP-2 activity by gelatin zymography. Lane 1: control; lane 2: in the presence of 0.4 $\mu\text{g/ml}$ anti-EMMPRIN function-blocking antibody; lane 3: 1.0 $\mu\text{g/ml}$ anti-EMMPRIN function-blocking antibody; lane 4: 5.0 $\mu\text{g/ml}$ anti-EMMPRIN function-blocking antibody; lane 5: 10 $\mu\text{g/ml}$ anti-EMMPRIN function-blocking antibody; lane 6: 5.0 $\mu\text{g/ml}$ mouse negative control IgG1. Gelatin zymography demonstrated an apparent reduction of MMP-2 activity by the anti-EMMPRIN function-blocking antibody (lanes 2~5), but a slight increase in MMP-2 in the presence of mouse negative control IgG1 (lane 6) was observed.

Three distinct layers were observed in the cross section. The upper Matrigel layer was stained strongly, the middle PET layer was unstained, and the lower Matrigel layer was faintly stained (Fig. 2). It seemed that the pores in the PET layer were not plugged by Matrigel; rather, the pores traversed all the layers. To confirm this observation, we added human erythrocytes approximately 7 μm in diameter to the upper chamber. They traveled through the pores of the membrane and fell into the lower chamber (data not shown). Thus, we concluded that substances 8 μm or less in diameter can pass through the pores of the membrane freely. Using this commercially available Matrigel invasion chamber, we considered that we could evaluate the "migration" of cells *in vitro* rather than their "invasion" *in vivo*.

The soluble factors produced by fibroblasts promoted HEp-2 cell migration

Soluble factors, such as SDF-1, produced by fibroblasts have been demonstrated to promote the migration of various kinds of cancer cells (Kang *et al.*, 2005; Moskovits *et al.*, 2006; Ohira *et al.*, 2006). We hypothesized that soluble EMMPRIN would stimulate fibroblasts to produce such soluble factors, which would subsequently stimulate the migration of HEp-2. To estimate the production of soluble factors by fibroblasts, we incubated HEp-2 cells with or without fibroblasts in the invasion chamber described above. Fetal bovine serum was eliminated from the culture medium in the migration assay. A significant HEp-2 cell migration to

the lower surface of the membrane was observed when fibroblasts were cultured in the lower chamber (Fig. 3A). On the other hand, migration was hardly observed without fibroblasts (Fig. 3B). The soluble factors released by the fibroblasts were necessary for the migration of HEp-2 cells, and they probably contained chemoattractants.

Soluble EMMPRIN stimulates fibroblasts to produce MMP-2

We tried to estimate the effects of soluble EMMPRIN on HEp-2 cell migration by introducing an anti-EMMPRIN function-blocking antibody into the invasion chamber assay. Before proceeding with this experiment, the blocking activity of this antibody was evaluated. It has been reported that soluble EMMPRIN stimulates fibroblasts located distantly from cancer cells to produce MMPs, including MMP-2 (Kataoka *et al.* 1993; Tokumaru *et al.*, 2000; Kanekura *et al.*, 2002; Yan *et al.*, 2005). If this antibody can block the activity of soluble EMMPRIN, MMP-2 production by the fibroblasts is expected to be diminished.

HEp-2 cells and fibroblasts were separately cultured in the invasion chamber for 24 h in the presence or absence of the anti-EMMPRIN antibody, and we assayed the production of MMP-2 in the condition media by gelatin zymography. Two different bands of the molecular weight were detected, corresponding to pro-MMP-2 and active MMP-2 in zymography (Fig. 4). Both forms of MMP-2 coexisted in the conditioned media. MMP-2 was synthesized in a pro-form and then converted to the

Fig. 5. HEp-2 cells which had migrated in the invasion chamber, as quantified by a colorimetric MTT assay. HEp-2 cells in the upper chamber were incubated for 48 h with fibroblasts in the lower chamber under three different conditions: 1) control; 2) in the presence of 1.0 $\mu\text{g}/\text{ml}$ anti-EMMPRIN function-blocking antibody; 3) 1.0 $\mu\text{g}/\text{ml}$ mouse negative control IgG1. The migration of HEp-2 cells in the presence of anti-EMMPRIN function-blocking antibody was inhibited by more than 50 % as compared with the migration in the presence of mouse negative control IgG1. The values are means $\pm$ S.D. (n=4). The statistical difference was determined by two-sided Student's t test. * $P < 0.05$ and ** $P < 0.01$.

active form losing 80 amino acid residues from the amino terminal end (Stetler-Stevenson *et al.*, 1989). Pro-MMP-2 was activated in the process of zymography through its conformational change, and showed gelatinolytic activity as previously reported (Suzuki *et al.*, 2004). This analysis demonstrated that the anti-EMMPRIN function-blocking antibody markedly decreased the production of MMP-2, in contrast with mouse negative control IgG1 (Fig. 4). However, there was no apparent change in the production of MMP-2 in the presence of the anti-EMMPRIN antibody (Becton Dickinson) that could not block the function of EMMPRIN (data not shown). This result suggested that the anti-EMMPRIN function-blocking antibody inhibited the activity of soluble EMMPRIN to stimulate fibroblasts to produce MMP-2. The production of MMP-2 was nearly equal in the presence of different concentrations of the anti-EMMPRIN function-blocking antibody (0.4~10 $\mu\text{g}/\text{ml}$). Therefore, it is likely that this antibody sufficiently blocked the function of soluble EMMPRIN at the concentration of 0.4 $\mu\text{g}/\text{ml}$ (Fig. 4). In the case of mouse control IgG1, a slight increase in MMP-2 production was observed as compared with the medium control. Mouse control IgG1 might create favorable circumstances for HEp-2 cells and fibroblasts as a nonspecific protein, as the assayed media did not contain any proteins.

Anti-EMMPRIN function-blocking antibody inhibited the migration of HEp-2 cells.

In order to evaluate the effects of soluble EMMPRIN on the migration of HEp-2 cells, a migration assay was performed using invasion chambers with or without the anti-EMMPRIN function-blocking antibody, and the HEp-2 cells which had migrated were quantified by colorimetric assay. The migration of HEp-2 cells in the presence of the 1.0 $\mu\text{g}/\text{ml}$ function-blocking antibody was less than half that in the presence of mouse control IgG1, and approximately 70% of the medium control (Fig. 5). In contrast, the anti-EMMPRIN antibody that cannot inhibit the activity of EMMPRIN did not induce any decrease in migration (data not shown). These results clearly indicated that soluble EMMPRIN participated in the enhancement of HEp-2 cell migration, for which its activity was essential thereto. The migration of HEp-2 in the presence of mouse control IgG1 was slightly enhanced as compared with the medium control. This seemed to be due to the stabilization of the condition of the HEp-2 cells.

Discussion

Our results revealed that soluble EMMPRIN is released from human laryngeal epidermoid carcinoma cells (HEp-2) as well as from human skin fibroblasts. By immunoblot analysis, EMMPRIN was detected in the cell lysates of HEp-2 cells and fibroblasts, indicating that both cell types synthesize EMMPRIN, and the soluble form of EMMPRIN was also detected in the conditioned media of the HEp-2 cells and fibroblasts. Two anti-EMMPRIN antibodies, one reacting against the N-terminus (N-19) and the other against the C-terminus (C-19) of EMMPRIN, could recognize soluble EMMPRIN derived from HEp-2 cells. However, soluble EMMPRIN in the fibroblast conditioned medium was detected only by the N-19 antibody. We supposed that the amount of soluble EMMPRIN liberated from the fibroblasts was so small that the C-19 antibody failed to detect it in the immunoblot analysis. The N-19 antibody specifically recognized forms of soluble EMMPRIN with almost the same molecular weight derived from HEp-2 cells and fibroblasts. Based on these results described in detail above, we concluded that the molecules of soluble EMMPRIN liberated from both cells had the full-length form. Two mechanisms of soluble EMMPRIN generation have been demonstrated: 1) EMMPRIN is localized in the cell membrane and in the vesicular compartments, and vesicle shedding produces full-length soluble EMMPRIN (Taylor *et al.*, 2002; Sidhu *et al.*, 2004; Koga *et al.*, 2006; Millimaggi *et al.*, 2007); and 2) soluble EMMPRIN is liberated from membrane-associated EMMPRIN by proteolysis. MT1-MMP, MMP-2, and MMP-9 have been reported to be enzymes that cleave and release a shorter form of EMMPRIN, one which lacks the C-terminus (Hang *et al.*, 2004; Tang *et al.*, 2004; Egawa *et al.*, 2006). Thus, some of the molecules of soluble EMMPRIN in the conditioned media of the HEp-2 cells and fibroblasts in our experiments had the full-length form and were probably derived *via* microvesicle shedding.

The molecular mechanisms behind the biological activities of EMMPRIN are not fully understood — especially the precise receptor for soluble EMMPRIN remains unclear. However, it is possible that EMMPRIN itself may act as a receptor through homophilic interaction (Sun and Hemler, 2001). The soluble EMMPRIN content in the HEp-2 cell conditioned medium was much greater than that derived from fibroblasts. It is likely that the soluble EMMPRIN liberated from HEp-2 cells interacts with the EMMPRIN expressed on the fibroblast cell membrane, stimulating the release of factors which promote the migration of HEp-2 cells.

One of the prominent functions of EMMPRIN is its role in the induction of MMP production. EMMPRIN has been reported to stimulate fibroblasts to produce MMP-1, MMP-2, MMP-3, and MT1-MMP (Kataoka *et al.*, 1993; Sameshima *et al.*, 2000; Tokumaru *et al.*, 2000; Yan *et al.*, 2005). We detected MMP-2 in the conditioned media recovered from the invasion chambers by gelatin zymography, and the amount of MMP-2 was apparently reduced by the addition of the anti-EMMPRIN function-blocking antibody. MMP-2 is not a chemoattractant for HEp-2 cells; however, it has been reported that MMP-2 promoted the cell migration of normal fibroblasts (Brown *et al.*, 2007), mesenchymal stem cells (Ries *et al.*, 2007), and cancer cells (Chambers and Matrisian, 1997; Zang *et al.*, 2006; Lee *et al.*, 2007). Lee has demonstrated that the MMP-2 inhibitor, which inhibits the enzyme activity and expression of MMP-2, blocks the migration and invasion of HT-1080 human fibrosarcoma cells. We speculated that the soluble EMMPRIN derived from HEp-2 cells stimulated the fibroblasts to produce MMP-2 in our experiments, and that HEp-2 cell migration was subsequently promoted by MMP-2.

Numerous previous studies have revealed that EMMPRIN is distributed prominently in cancer tissues and participates in tissue destruction by stimulating MMP synthesis. The correlation between the malignancy, invasion, and metastasis capability and the EMMPRIN expression of cancerous specimens obtained by surgery or biopsy has been investigated. The investigators have emphasized that the intensity and pattern of EMMPRIN expression in immunohistochemical studies are related to cancer stages, and that the findings closely correlate with clinicopathological parameters; consequently, the pattern of EMMPRIN expression can be used in prognosis for breast and lung cancers (Caudroy *et al.*, 1999), in esophageal squamous cell carcinoma (Ishibashi *et al.*, 2004), in hepatocellular carcinoma (Mamori *et al.*, 2007), and in endometrial carcinoma (Ueda *et al.*, 2007). Recently, it has been demonstrated that the soluble EMMPRIN levels in the sera obtained from pancreatic cancer patients were also significantly increased as compared with those from chronic pancreatitis patients or healthy controls (Zhang *et al.*, 2007). EMMPRIN may be a potential target for cancer therapy, and RNA interference experiments to inhibit EMMPRIN gene expression have shown promising effects on cancer cell invasion and tumorigenicity (Liang *et al.*, 2005; Wang *et al.*, 2006; Zou *et al.*, 2007).

Our results clearly demonstrated that soluble full-length EMMPRIN was produced by HEp-2 cells, probably *via* membrane vesicle shedding, and that it stimulated separately cultured fibroblasts to synthesize MMP-2. The

soluble EMMPRIN subsequently showed a profound effect on the migration of HEP-2 cells, and the anti-EMMPRIN function-blocking antibody inhibited HEP-2 migration significantly. These data strongly suggest that the soluble EMMPRIN released from cancer cells should be an important therapeutic target in cancer treatment, in addition to membranous EMMPRIN.

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