Abstract. Stromal cells are the main source of matrix metalloproteinases (MMPs) in human carcinoma tissues. Emmprin is a glycosylated transmembrane protein containing two immunoglobulin (Ig) domains that is expressed in carcinoma cells and stimulates MMP production by adjacent stromal cells. The first Ig domain (ECI) of emmprin contains the biologically active site. We investigated whether synthetic peptides carrying a partial ECI sequence could inhibit emmprin activity. Only the second peptide (emp#2), which contains a putative N-glycosylation site sequence, inhibited emmprin-stimulated production of MMP-2 in co-cultures of fibroblasts and several different human tumor cell types, including carcinoma, sarcoma, melanoma, leukemia and glioma cells. Moreover, emp#2 significantly inhibited the invasive activity of glioblastoma cells promoted by interaction with fibroblasts. Perturbation of emmprin activity by this peptide may have potential therapeutic uses in the prevention of MMP-2-dependent cancer invasion.

Introduction

Tumor cell invasion and metastasis requires degradation of extracellular matrix, which is mainly achieved by various proteolytic enzymes including zinc-dependent matrix metalloproteinases (MMPs) (1). *In situ* hybridization studies of human surgical specimens have shown that stromal fibroblasts are the predominant source of MMP-2 in most carcinomas (2-4). Carcinoma cells stimulate nearby fibroblasts to produce MMPs via soluble cytokines and growth factors, or by cell surface interactions mediated by plasma membrane proteins, such as emmprin (5-7). Emmprin, also called basigin/CD147, is expressed in significantly higher levels in tumor cells than in normal cells. Emmprin stimulates MMP production in fibroblasts, and hence the elevated expression of emmprin on the surface of tumor cells could promote tumor progression by inducing MMP production in peritumoral fibroblasts (8,9). It was also shown recently that emmprin also mediates angiogenesis via stimulation of vascular endothelial growth factor (VEGF) (10), tumor cell glycolysis (11,12), and multidrug resistance (13-16). A role for emmprin in epithelial-mesenchymal interactions was also reported (17).

Emmprin is an integral plasma membrane glycoprotein of the immunoglobulin (Ig) superfamily that contains two extracellular Ig domains (18). The activity-blocking monoclonal antibody, E11F4, recognizes the first Ig domain (ECI) of emmprin, implicating this region is involved in the various emmprin activities (18). Furthermore, recombinant and unglycosylated emmprin produced by bacteria is smaller in molecular size and in inactive, whereas emmprin isolated from Chinese hamster ovary cells is highly glycosylated with a molecular mass of approximately 58 kDa, similar to the native protein. The hamster emmprin also successfully stimulated production of MMP-1, -2, and -3 from fibroblasts (19). The presence of a putative receptor for emmprin on the fibroblast cell membrane is postulated, although not yet proven (20).

Based on the above findings, the present study tested the hypothesis that synthetic peptides carrying the ECI sequences can modify or regulate emmprin activity. The results showed that one of the ECI-derived peptides tested, emp#2, effectively inhibited emmprin-induced stimulation of MMP-2 production. This peptide contains a putative N-glycosylation...
Four peptides (emp#1: AAGTVFTTVEDLGSKIL, emp#2-1: SLNDSATEVTGHRLKGGVV, emp#2-2: GHRWLKGGGV) were synthesized. Two peptides corresponding to the first and second halves of the emp#2 peptide (emp#2-1: SLNDSATEVT, emp#2-2: GHRWLKGGGVV) were also synthesized. Emp#1 was dissolved in dimethyl sulfoxide (DMSO). Other reagents were dissolved in PBS. The final concentration of DMSO in the culture medium was less than 0.01%, and at this concentration the solvents alone had no deleterious effects on the cells or any detectable effects on MMP production.

Inhibition experiments in co-cultures using anti-emmprin blocking antibody (UM-9D6, Ancell Corporation, Bayport, MN) were performed as described previously (5). Furthermore, another inhibition experiments using TMK-1 cells, in which emmprin had been knocked down by shRNA transfections, was also performed.

For the co-culture experiments employing emp peptides (emp#1-4), tumor cells were preincubated at 37°C for 45 min with 100, 250 and 500 µg/ml of peptides before co-culturing with fibroblasts. Co-cultures were done in the presence of the peptides for 6 days with replacement of medium at 3 days, as described above.

**Immunoblotting.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of membrane extracts and conditioned medium was performed under reducing conditions using a 5-15% gradient gel (Biomate, Tokyo, Japan). After electrophoresis, the proteins were transferred electrophoretically to Immobilon membrane (Millipore, Bedford, MA). Non-specific sites were blocked with 5% non-fat dry milk in PBS. The membranes were incubated for 1 h with peroxidase-conjugated goat anti-mouse. The color was developed with chemiluminescence reagents (DuPont NEN, Boston, MA) and the glioblastoma cell line U251 was obtained from the RIKEN cell bank (Tsukuba, Japan).

**Peptides.** Four peptides (emp#1: AAGTVFTTVEDLGSKIL, emp#2: SLNDSATEVTGHRLKGGVV, emp#3: LKE DALPQQKTEFKVDSDDQWG, emp#4: EYSCVFLPEPMG TANIQLHGPPR) were synthesized, each consisting of 20-23 amino acids (aa) and corresponding to about one fourth of the total ECI of the emmprin molecule (Fig. 1). Two peptides corresponding to the first and second halves of the emp#2 peptide (emp#2-1: SLNDSATEVT, emp#2-2: GHRWLKGGGVV) were also synthesized. Emp#1 was dissolved in dimethyl sulfoxide (DMSO). Other reagents were dissolved in PBS. The final concentration of DMSO in the culture medium was less than 0.01%, and at this concentration the solvents alone had no deleterious effects on the cells or any detectable effects on MMP production.

**Zymography.** Gelatinolytic activities in conditioned media were demonstrated using gelatin as a substrate, as described...
compared with individual cultures, and this stimulation was production of pro-MMP-2 by approximately 8.0 times of U251 glioblastoma cells and fibroblasts stimulated the emmprin activity-blocking antibody (UM-8D6). Co-cultures firstly performed co-culture experiments in the presence of fibroblast interaction-stimulated upregulation of MMP-2, we appeared as a broad band ranging from approximately 43 to 58 kDa. membrane extracts prepared from these cell lines, emmprin (FUEPS-1), expressed emmprin (Fig. 2). On immunoblots of T-cells (MT-2), glioblastoma (U251), and epithelioid sarcoma (TMK-1) carcinoma, melanoma (A375), HTLV-1 transformed lines from various origins, including colon (RCM-1) and gastric induced upregulation of MMP-2.

**Involvement of emmprin in tumor cell-fibroblast interaction-induced upregulation of MMP-2.** All six human tumor cell lines from various origins, including colon (RCM-1) and gastric (TMK-1) carcinoma, melanoma (A375), HTLV-1 transformed T-cells (MT-2), glioblastoma (U251), and epithelioid sarcoma (FUEPS-1), expressed emmprin (Fig. 2). On immunoblots of membrane extracts prepared from these cell lines, emmprin appeared as a broad band ranging from approximately 43 to 58 kDa.

To determine the involvement of emmprin in tumor cell-fibroblast interaction-stimulated upregulation of MMP-2, we firstly performed co-culture experiments in the presence of emmprin activity-blocking antibody (UM-8D6). Co-cultures of U251 glioblastoma cells and fibroblasts stimulated the production of pro-MMP-2 by approximately 8.0 times compared with individual cultures, and this stimulation was inhibited by UM-8D6, i.e., approximately 41.2% inhibition at 40 µg/ml, while control non-immune IgG caused no inhibition (Fig. 3a). Similarly, stimulation of MMP-2 production in co-cultures of FUEPS-1 or MT-2 with fibroblasts was inhibited by the anti-emmprin antibody in a dose-dependent manner (6,7).

Next, we used a retrovirus-based system to deliver short-hairpin RNA (shRNA) to knock down emmprin expression in TMK-1 cells. Introduction of shBSG lowered the emmprin expression level (Fig. 3b), which was unaffected by empty retrovirus (shLacz). Downregulation of emmprin by shBSG inhibited stimulation of MMP-2 production in co-cultures by approximately 45% compared with control TMK-1 cells (shLacz, Fig. 3c).

**Effects of ECI-derived peptides on emmprin-mediated stimulation of MMP-2 production in co-cultures.** Since a previous study (18) indicated that ECI contains the structure responsible for emmprin activity, we investigated whether ECI-derived peptides might act as competitive decoys. In this study, co-culture experi-
ments of U251 glioblastoma cells and MBT-3 fibroblasts were run in the presence of four ECI-derived peptides, emp#1-4. Only the emp#2 peptide inhibited emmprin-stimulated production of MMP-2 in the co-cultures, while the other peptides showed no significant effect.

Figure 3. Involvement of emmprin in tumor cell-fibroblast interaction-stimulated upregulation of MMP-2 in co-cultures. (a) Co-culture experiments using U251 glioblastoma cells with ST353 fibroblasts were performed with emmprin activity-blocking antibody (UM-8D6) as described in Materials and methods. The cell culture media were subjected to immunoblotting using an MMP-2 mAb. The number for anti-emmprin antibody and control IgG indicates the concentrations added to co-cultures (µg/ml). (b) Knockdown of emmprin expression in gastric cancer cell line TMK-1 was analyzed by immunoblotting using E11F4. Short hairpin RNA (shRNA) sequences targeting emmprin mRNA, shBSG, was retrovirally transduced in TMK-1 cells, as shown. Cells were also infected with empty virus (shLacZ) to be used as a control. (c) The cultured media of co-cultures of shBSG or shLacZ with ST353 fibroblast were analyzed by immunoblotting using an MMP-2 mAb. Representative examples of similar results.

Figure 4. Effect of emmprin peptides on stimulated production of MMP-2 in co-cultures. Co-culture experiments with U251 glioblastoma cells and fibroblast were run in the presence of synthetic peptides emp#1-4 or emp#2-1-2 as described in Materials and methods, and their conditioned media were subjected to immunoblotting. (a) Representative immunoblots. (b) Relative protein levels of MMP-2 in co-cultures treated with emp#2 compared with those in untreated co-cultures. Data are mean ± SEM (n=3). *P<0.01, compared with the control. (c) Effect of emp#2-1 and #2-2 peptides on stimulated production of MMP-2 in co-cultures. Fb, fibroblasts alone; U251, U251 glioblastoma cells alone; Co, co-culture of U251 glioblastoma cells and fibroblasts. Blots in (a) and (c) are representative examples of three similar results.
no inhibitory effect (Fig. 4a). The inhibitory activity of emp#2 peptide was dose-dependent, with approximately 30, 45 and 85% inhibition at 100, 250 and 500 µg/ml, respectively (Fig. 4b).

To determine whether a shorter peptide was still active, the first half (10 aa) and the second half (10 aa) of the emp#2 peptide were synthesized (emp#2-1 and emp#2-2, respectively) for use in the co-culture experiments. However, neither of these two peptides inhibited emmprin-enhanced production of MMP-2 in the co-cultures (Fig. 4c).

We also confirmed the effect of the emp#2 peptide in co-cultures of other human tumor cells and fibroblasts. Fig. 5 shows representatives gelatin zymography of the conditioned media of co-cultures with emp#2 peptide. The presence of emp#2 peptide (500 µg/ml) inhibited the stimulation of MMP-2 production by 85.5±4.4% or 77.8±1.1% in co-cultures of FU-EPS-1 sarcoma cells or A375 melanoma cells and fibroblasts, respectively, while a control 20-aa peptide caused no inhibition. Table I summarizes these findings in co-cultures of five human tumor cell lines and fibroblasts. Thus, synthetic ECI-derived emp#2 peptide, containing a putative N-glycosylation site, significantly inhibited emmprin-stimulated production of MMP-2 in co-cultures of various types of tumor cells and fibroblasts.

**Effect of emp#2 on glioblastoma cell invasion through Matrigel enhanced by co-culture with fibroblasts.** Next, we investigated the inhibitory effect of emp#2 peptide on the invasive activity of tumor cells that is promoted by interacting with fibroblasts, probably due to MMP-2 upregulation. In the presence of fibroblasts, the number of invading U251 glioblastoma cells significantly increased (2 times) compared with individual cultures of U251 cells (Fig. 6). Preincubation with emp#2 peptide of U251 tumor cells suppressed the stimulated invasion of Matrigel in co-cultures by about 65% (Fig. 6).

**Discussion**

The present study demonstrated that synthetic emmprin peptides could inhibit emmprin activity. An ECI-derived peptide,(emp#2), which contains a putative N-glycosylation site sequence, significantly inhibited emmprin-stimulated production of MMP-2 in cocultures of tumor cells and fibro-

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**Table I. Effect of emp #2 peptides on stimulated production of MMP-2 in co-cultures.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tumor origin</th>
<th>Method</th>
<th>Inhibition of stimulation of MMP-2 production in co-cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>FU-EPS-1</td>
<td>Epithelioid sarcoma</td>
<td>Zymography</td>
<td>85.5±4.4%</td>
</tr>
<tr>
<td>U251</td>
<td>Glioblastoma</td>
<td>Immunoblotting</td>
<td>86.2±4.6%</td>
</tr>
<tr>
<td>A375</td>
<td>Malignant melanoma</td>
<td>Zymography</td>
<td>48.3±11.8%</td>
</tr>
<tr>
<td>MT2</td>
<td>ATL</td>
<td>Immunoblotting</td>
<td>77.8±1.1%</td>
</tr>
<tr>
<td>RCM-1</td>
<td>Colon carcinoma</td>
<td>Immunoblotting</td>
<td>64.7±12.7%</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n=3). *at 100 mg/ml. ATL, adult T-cell leukemia.
blasts. This peptide was effective on variously glycosylated emmprin molecules expressed on various tumor cells, including carcinoma, sarcoma, melanoma, leukemia and glioma cells. Moreover, the peptide significantly inhibited the invasive activity of glioblastoma cells promoted by interacting with fibroblasts.

We have mainly studied the role of emmprin in heterotypic interactions between tumor cells and fibroblasts, but emmprin is also involved in homophilic interactions between tumor cells (26). Overexpression of emmprin in tumor cells leads to increased MMP production within the same population of cells (27,28). In both types of cellular interactions, emmprin is postulated to work via homophilic interactions between emmprin molecules or through binding to a putative receptor/acceptor molecule (20,26). In either case, since competitive inhibition by decoy molecules that bind the active site but are functionally inactive could be possible, emp#2 peptide may act as a decoy. Recently, purified deglycosylated emmprin was shown to antagonize the MMP-1-inducing activity of purified native glycosylated emmprin (26).

Moreover, the deglycosylated emmprin extracellular domains including ECI, prepared as an Fc-fusion protein, markedly inhibited not only the homophilic interaction between emmprin molecules, but also the production of MMP-2 by the breast cancer cell line MDA-435 (26). In either case, since competitive inhibition by decoy molecules that bind the active site but are functionally inactive could be possible, emp#2 peptide may act as a decoy. Recently, purified deglycosylated emmprin was shown to antagonize the MMP-1-inducing activity of purified native glycosylated emmprin (26).

Figure 6. Inhibitory effects of emp#2 peptide on U251 glioblastoma cell invasive activity in co-culture condition with ST353 fibroblasts. U251 cells were labeled with D11 and preincubated with emp#2 and control peptides. Invasion assays were then performed for 72 h. The number for fibroblasts and U251 cells indicates the cell number used in co-cultures (x10^5/ml). The number for emp#2 and control peptides indicates the concentrations added to co-cultures (µg/ml). Data are mean ± SEM (n=4). *P<0.01, compared with individual cultures (Student's t-test). **P<0.01, compared with control peptide-treated cells (Student's t-test).

Another possible explanation for the emmprin-inhibitory effect of emp#2 is that emp#2 may interfere with homophilic or heterophilic cis-interactions between emmprin molecules within the plasma membrane or between emmprin and an unidentified cell surface molecule(s) on tumor cells that is required to bind a putative receptor/acceptor on fibroblasts. This possibility is based on our interesting findings that the inhibitory effect of emp#2 peptide on emmprin-mediated enhanced MMP-2 production was observed only when the peptide was preincubated with tumor cells, whereas no inhibitory activity was detected when the peptide was preincubated with fibroblasts (data not shown). A previous study associated emmprin with three major types of transporters, CD98 heavy chain (CD98hc), L-type amino acid transporter, ASCT2, and monocarboxylate transporters, in addition to a regulator of cell proliferation (epithelial cell adhesion molecule) (30).

Recently, several other molecules have also been reported to associate with emmprin in regulating cancer invasion and migration (31). One or some of these molecules or other as yet undetermined ones may be a target(s) of the emp#2 peptide. In ongoing studies, we are investigating membrane molecules that complex with emmprin in a manner that is sensitive to inhibition by the emp#2 peptide.

The third possible inhibitory mechanism could involve the lectin activity of emmprin (32). The fourth Ig-like domain of neural cell adhesion molecule (NCAM) is a lectin domain, and NCAM forms a complex with another neural adhesion molecule L1 via oligomannosidic carbohydrates on L1. The 19-aa peptide that comprises part of the C-type lectin consensus sequence in the fourth Ig-like domain of NCAM interferes with the association between L1 and NCAM (33). ECI of emmprin is homologous to this lectin domain of NCAM, and emmprin was shown to bind the high-mannose-type L3 carbohydrate epitope, leading to emmprin-dependent astrocytic out growth (32). However, it remains to be elucidated whether lectin-type binding via oligomannosidic carbohydrates plays a role in the emmprin-mediated MMP-2 upregulation observed in our tumor cell-fibroblast co-cultures.
The process of tumor cell invasion and metastasis is very complex, involving not only MMP expression, but also the activation of integrins and other adhesion receptors and the deposition of matrix molecules. The expression of emmprin in association with these factors appears to facilitate the ultimate invasive behavior of several tumors. Emmprin facilitated invasion through a reconstituted basement membrane in human oral squamous cell carcinoma (34) and hepatoma cells (35), while an in vivo study showed that emmprin cDNA-transfected breast cancer cells were considerably more tumorigenic and invasive than plasmid-transfected cancer cells when implanted into mouse (28). Furthermore, down-regulation of emmprin via RNA interference reduced tumor cell invasion (36,37), and stimulation of tumor cell invasion by co-culturing tumor cells with fibroblasts was also reported (38). In this latter study, co-cultures of SKG-II cells and fibroblasts exhibited increased in vitro invasive activity of SKG-II cells via increased activation of fibroblast-derived pro-MMP-2 on the tumor cell surface. Promotion of tumorigenesis by co-injection of emmprin-expressing tumor cells and dermal fibroblasts was also reported (39). In our study, emp#2 peptide inhibited not only emmprin-mediated MMP-2 upregulation, but also the glioblastoma cell invasion augmented by interacting with fibroblasts.

Emmprin expression is frequently detected in the vast majority of human malignancies (40). Perturbation and inhibition of emmprin-mediated paracrine and autocrine signaling networks by use of competitive peptides such as emp#2 used in this study could have potential therapeutic value in the prevention of cancer invasion and progression.

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References