Tumor-Stroma Interaction: Positive Feedback Regulation of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) Expression and Matrix Metalloproteinase-Dependent Generation of Soluble EMMPRIN

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Abstract

Matrix metalloproteinases (MMPs) are metal-dependent endopeptidases that play pivotal roles in tumor disease progression. In many solid tumors, MMPs are indeed produced by tumor stromal cells, rather than by tumor cells. This expression pattern is, at least in part, regulated by tumor-stroma interaction via tumor cell-associated extracellular matrix metalloproteinase inducer (EMMPRIN). *In vitro*, recombinant EMMPRIN dose-dependently stimulated MMP-1 production by primary human fibroblast cells. Interestingly, in addition to stimulating MMP expression, EMMPRIN also induced its own gene expression. To further explore this potential positive feedback regulatory mechanism, we generated human breast cancer cells expressing different levels of EMMPRIN. Coculture of EMMPRIN-positive tumor cells with fibroblast cells resulted in a concomitant stimulation of MMP-2, MMP-9, and EMMPRIN production. This induction was EMMPRIN dependent, was further enhanced by overexpression, and was reduced by antisense suppression of EMMPRIN expression in tumor cells. Increased expression of membrane-associated EMMPRIN was accompanied by an MMP-dependent generation of a soluble form of EMMPRIN representing a proteolytic cleavage product lacking the carboxyl terminus. On the basis of these findings, we propose a model in which tumor cell-associated EMMPRIN stimulates MMPs, as well as EMMPRIN expression in tumor stroma. Increased MMP activity in tumor local environment results in proteolytic cleavage of membrane-associated EMMPRIN, releasing soluble EMMPRIN. Soluble EMMPRIN in turn acts in a paracrine fashion on stroma cells that are both adjacent and distant to tumor sites to further stimulate the production of MMPs and additional EMMPRIN, which consequently contributes to tumor angiogenesis, tumor growth, and metastasis.

Received 8/7/03; revised 12/12/03; accepted 1/5/04.
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Introduction

Degradation of basement membrane by matrix metalloproteinases (MMPs) is one of the most critical steps in various stages of tumor disease progression, including tumor angiogenesis, tumor growth, as well as local invasion and subsequent distant metastasis (1–3). MMPs are a family of more than 25 endopeptidases that are metal dependent and share a common modular domain structure. MMPs are overproduced in tumor local environment. Collectively, these enzymes are capable of cleaving all of the extracellular matrix components of the parenchymal and vascular basement membranes, normally mechanical barriers to tumor cell migration and invasion. High levels of MMPs have been correlated with increased tumor invasion capacity both *in vitro* and *in vivo* (4, 5). Clinically, elevated levels of MMPs detected in the body fluids of cancer patients correlate with adverse prognosis in various cancers (6, 7). Imbalances in the production of MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), result in tumor angiogenesis and metastasis (2, 3, 8–12).

Interestingly, recent studies of clinical tumor specimens provided evidence that most MMPs are generated by peritumoral fibroblasts in the stromal compartment, instead of by tumor cells themselves. Peritumor stromal cells are indeed responsible for the production of tumor-associated interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), and gelatinase A (MMP-2), in breast, colon, lung, skin, and head and neck cancers (13–16). The induction of MMP production is, at least in part, mediated by tumor-stromal cell interaction via a tumor cell surface glycoprotein, extracellular matrix metalloproteinase inducer (EMMPRIN; 17, 18).

EMMPRIN was originally purified from the plasma membrane of cancer cells as a glycoprotein of M_r 58,000, and was designated tumor collagenase stimulating factor (TCSF) because of its ability to stimulate fibroblast synthesis of collagenase-1 (MMP-1; 19, 20). Subsequent studies further demonstrated that EMMPRIN also induced fibroblast synthesis of MMP-2, MMP-3, as well as the membrane-type 1 MMP (MT1-MMP) and MT2-MMP that function as endogenous activators of MMP-2 (17, 21, 22). Therefore, EMMPRIN functions as an upstream modulator of MMP production in tumor local environment. EMMPRIN-positive tumor cells stimulate neighboring fibroblast cells to express MMPs and therefore facilitate tumor invasion and metastasis.

This rationale for the function of EMMPRIN has been supported by results from several clinical studies that

demonstrated high levels of EMMPRIN expression in tumor compartments as compared to peritumoral stromal tissues. These tumors include the lung (16), breast (16), bladder (23, 24), and glioma (25). Examination of EMMPRIN expression in these clinical samples by a variety of means, including Northern blot, *in situ* hybridization, and immunostaining, revealed that in most cases, EMMPRIN is expressed by tumor cells, but not by the neighboring stromal cells. In contrast, MMPs are expressed by peritumoral stromal cells.

The biological significance of increased expression of EMMPRIN in tumor cells was investigated by *in vitro* studies using recombinant EMMPRIN or native EMMPRIN purified from tumor cells. EMMPRIN has been shown to stimulate the expression of various MMPs produced by fibroblasts (17, 18). The induction occurred at the transcription level and is at least in part mediated by a mitogen-activated protein kinase (MAPK) p38 kinase signaling pathway (18). The role of EMMPRIN in tumor growth and metastasis was directly illustrated using EMMPRIN-overexpressing human breast cancer cells. MDA MB 463 cells are normally slow-growing cells when they are implanted into nude mice. However, when these cells were transfected with EMMPRIN, they adopted a more aggressive growth pattern, with both accelerated growth rate and metastatic phenotypes (26).

To further study the interplay between tumor cell-associated EMMPRIN and fibroblast cells, we used recombinant engineering to generate MDA MB 231 human breast cancer cells expressing different levels of EMMPRIN. We discovered a novel positive feedback regulatory mechanism controlling the interaction between tumor and fibroblast cells. Our results demonstrate that EMMPRIN positively regulates its own gene expression, in addition to stimulating MMP expression. Furthermore, we also detected generation of soluble EMMPRIN in the coculture of tumor and fibroblast cells that occurs via an MMP-dependent cleavage. On the basis of these new findings, we propose a new model in which the interplay between tumor and stromal compartments is regulated by both tumor cell surface EMMPRIN, as well as by soluble EMMPRIN, generated from MMP-dependent cleavage.

Results

Induction of MMP-1 Expression by Soluble Recombinant Human EMMPRIN in Primary Fibroblast Cells

The stimulatory effects of EMMPRIN on cultured fibroblast cells were studied using a soluble recombinant EMMPRIN protein (srEMMPRIN) corresponding to the extracellular portion of the molecule. srEMMPRIN was added at different concentrations to primary normal human fibroblast cells in culture. Serum-free media conditioned by these cells were collected at 1 or 3 days after EMMPRIN stimulation. MMP-1 production in response to EMMPRIN stimulation was determined by assaying MMP-1 activity secreted into the conditioned medium. EMMPRIN dose-dependently stimulated MMP-1 production by normal human lung fibroblast (NHLF) cells (Fig. 1). The stimulation of MMP-1 production by srEMMPRIN also occurred on the transcription level as determined by TaqMan analysis (Fig. 2A).

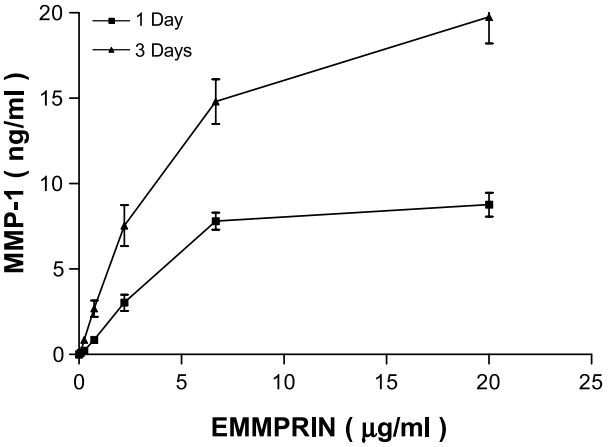

FIGURE 1. Induction of MMP-1 expression by soluble recombinant human EMMPRIN in human primary lung fibroblast cells. srEMMPRIN dose-dependently stimulated MMP-1 production by NHLF cells. Ten thousand NHLF cells (passage 1) were plated in each well of a 96-well cell culture plate. Cells were challenged with different concentrations of EMMPRIN. After 1 day and 3 days, conditioned medium was collected from each well and MMP-1 activity was determined. *Points,* mean values of duplicates from three independent experiments; *error bars,* SDs.

Positive Feedback Regulation of EMMPRIN Gene Expression in Primary Fibroblast Cells

Interestingly, when we examined the expression level of EMMPRIN in fibroblast cells after stimulation with 5 μg/ml EMMPRIN, we observed a substantial increase in EMMPRIN gene expression in these cells. As determined by quantitative TaqMan analysis, a 2.2-fold increase in EMMPRIN gene expression was induced at 4 h after cells were stimulated with EMMPRIN (Fig. 2A). EMMPRIN gene expression continuously increased to 4.6-fold at 24 h and 3.7-fold at 48 h after treatment. On EMMPRIN stimulation, MMP-1 gene expression level also increased (Fig. 2A). This induction occurred more rapidly and transiently, with a peak expression of a 3.3-fold increase at 4 h, and dropped to near pretreatment level at 48 h. When EMMPRIN protein expression in fibroblast cells treated with EMMPRIN was examined with Western blot analysis, we observed a strong increase in EMMPRIN protein expression (Fig. 2). In contrast, only minimal levels of EMMPRIN protein were detectable in unstimulated fibroblast cells. Taken together, these results suggest that in addition to increased expression of MMP-1, fibroblast cells also respond to EMMPRIN stimulation by up-regulating EMMPRIN gene expression.

Generation of MDA MB 231 Tumor Cells Expressing Different Levels of EMMPRIN

To further explore the potential regulatory mechanism of EMMPRIN gene expression in fibroblast cells, especially in the context of tumor-fibroblast cell-cell interaction, we used recombinant DNA engineering technology to generate cancer cells that express different levels of EMMPRIN. As described in "Materials and Methods," MDA MB 231 human breast cancer cells were transfected with expression vectors harboring the full-length coding region of human EMMPRIN in either sense or antisense orientations. Stably transfected cells were

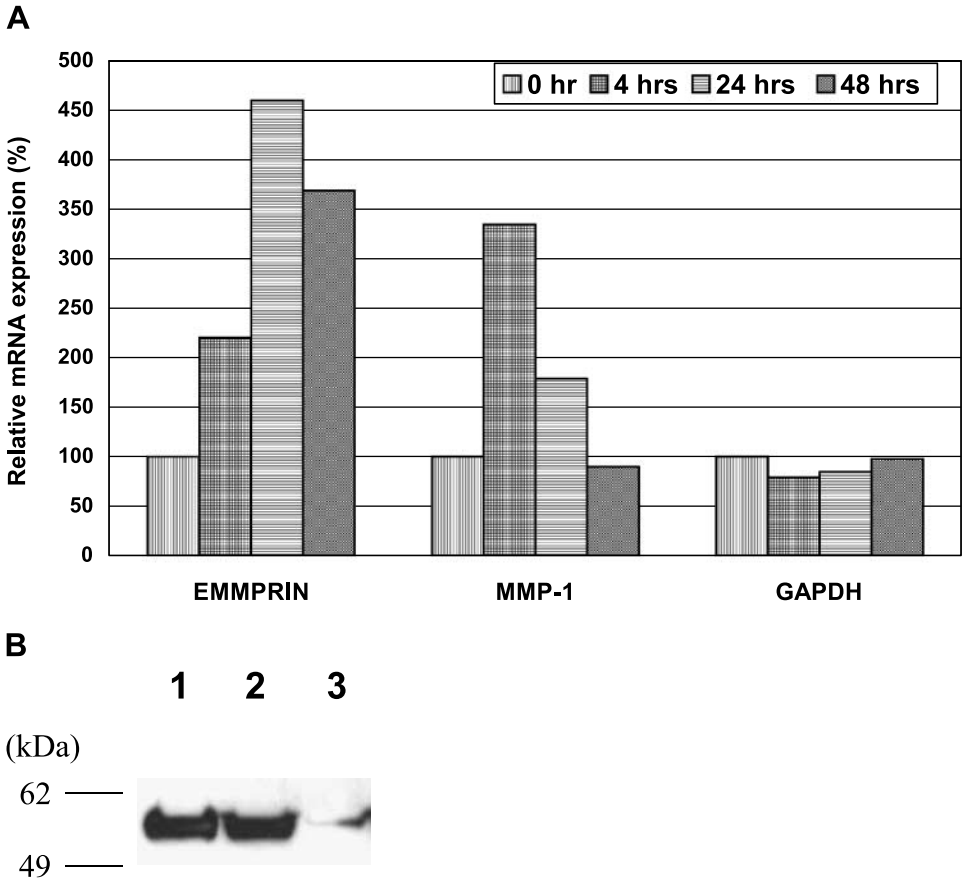

FIGURE 2. Induction of EMMPRIN expression by soluble recombinant human EMMPRIN in primary fibroblast cells. Primary normal human fibroblast cells of the lung were challenged with 5 μg/ml of srEMMPRIN. **A.** Total RNA was collected from untreated cells or at 4, 24, and 48 h after EMMPRIN treatment. EMMPRIN and MMP-1 gene expression level was determined by quantitative TaqMan analysis and was expressed as percentage to that in untreated cells, which was designated as 100%. **B.** Western blot analysis of EMMPRIN expression in fibroblast cells in response to stimulation with recombinant EMMPRIN. Cell lysate containing 50 μg of total protein was loaded into each lane, G361 high EMMPRIN-expressing melanoma cells (*lane 1*), fibroblast cells treated with EMMPRIN (*lane 2*), and unstimulated fibroblast cells (*lane 3*).

cloned and EMMPRIN expression level in these cells was determined by Western blot analysis. As shown in Fig. 3, EMMPRIN protein expression in one of sense transfected cell clones S1-3 was approximately 2-fold higher as compared to that of wild-type control cells. In contrast, EMMPRIN protein expression was substantially inhibited in two antisense transfected cell clones, AS1-5 and AS2-5, to only 47% and 62% of that in wild-type cells. Changes in EMMPRIN protein expression in these engineered cells were also independently confirmed by ELISA analysis (Fig. 4). In addition, changes in EMMPRIN protein expression also occurred on cell surface in sense and antisense transfected cells, respectively, as determined by fluorescence-activated cell (FAC) analysis (data not shown).

We next used these cells with different levels of EMMPRIN protein expression to address the role of tumor cell-derived EMMPRIN in regulating its own expression during tumor-fibroblast cell interaction.

Stimulation of Both Cell-Associated and Soluble EMMPRIN Expression in Coculture of Tumor Cells and Fibroblast Cells

In monoculture of tumor cells or fibroblast cells, only low levels of EMMPRIN protein were detected in cell pellets or conditioned medium by ELISA analysis. Transfection with sense or antisense expression constructs resulted in substantial changes in cell-associated EMMPRIN expression, with an increase from 3.32 ng/ml in wild-type to 7.2 ng/ml in sense transfected S1-3 cells, and a decrease to 0.85 and 1.43 ng/ml in antisense transfected AS1-5 and AS2-5 cells, respectively (Fig. 4A). Only low levels of soluble EMMPRIN were detected in conditioned medium of these cells in monoculture. Transfection of tumor cells with sense and antisense expression constructs resulted in only minor changes in soluble EMMPRIN protein levels (Fig. 4B).

The expression of cell-associated EMMPRIN was readily induced when tumor cells were cultured together with fibroblast cells. In coculture, EMMPRIN was detected at 10 ng/ml as compared to 0.8 ng/ml in fibroblast cells and 3.32 ng/ml in wild-type tumor cells in monoculture (Fig. 4A). This increase in EMMPRIN expression in coculture was mediated by tumor cell-associated EMMPRIN. When EMMPRIN expression in tumor cells was inhibited by antisense transfection in AS1-5 and AS2-5 cells, the increase in EMMPRIN expression stimulated by coculture was concomitantly reduced to minimal levels (Fig. 4A).

It is known that coculture of EMMPRIN-positive tumor cells with fibroblast cells stimulates MMP expression (22, 27). These MMPs can in turn cleave cell surface proteins to generate soluble proteins. We therefore postulated that there may be an increase in soluble EMMPRIN protein level in conditioned

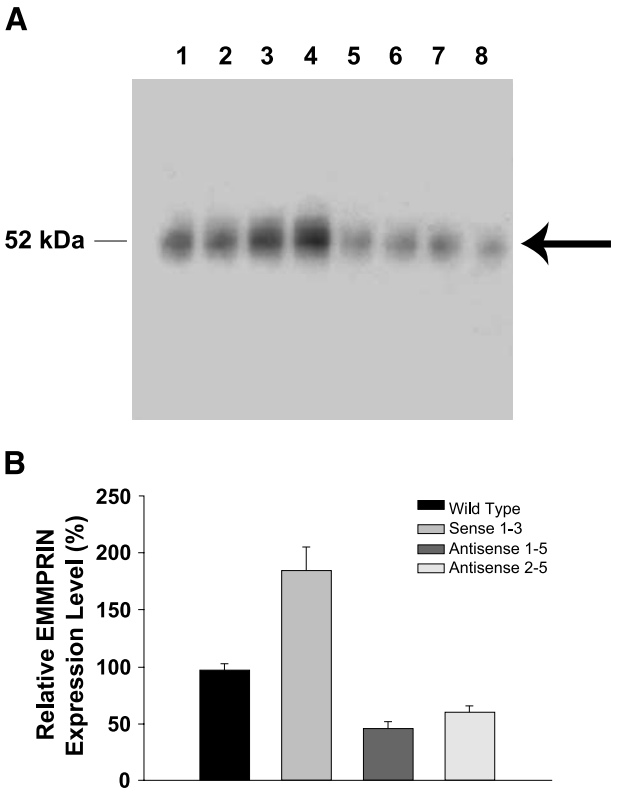

FIGURE 3. Generation of MDA MB 231 tumor cells expressing different levels of EMMPRIN. **A.** Western blot analysis of EMMPRIN protein in cell lysates. Samples containing 20 μg of total protein from each cell type were loaded in duplicates, MDA MB 231 wild-type cells (*lanes 1* and *2*), S1-3 cells (*lanes 3* and *4*), AS1-5 cells (*lanes 5* and *6*), and AS2-5 cells (*lanes 7* and *8*). A single EMMPRIN protein band of approximately M_r 52,000 was detected in all of the samples. **B.** Quantitative determination of EMMPRIN expression by scanning densitometry. EMMPRIN Western blot was scanned and the pixel density of each band was determined by scanning densitometry. EMMPRIN expression level in each cell type was expressed as the percentage of density of that in wild-type cells (*lane 1*), which was designated as 100%; *error bars,* SDs.

medium of these cells in coculture. In monoculture, the levels of soluble EMMPRIN were extremely low in conditioned medium of fibroblast cells (0.31 ng/ml) and wild-type tumor cells (0.53 ng/ml; Fig. 4B). However, coculture of tumor cells with fibroblast cells induced the release of substantial amounts of soluble EMMPRIN, 7.48 ng/ml by wild-type and fibroblast coculture and 10.39 ng/ml by S1-3 and fibroblast coculture (Fig. 4B). Similar to cell-associated EMMPRIN, the increase in soluble EMMPRIN expression was also correlated with the degree of EMMPRIN expression in tumor cells. Inhibition of EMMPRIN expression by antisense transfection virtually abolished the generation of soluble EMMPRIN in coculture (Fig. 4B).

Taken together, these results demonstrate a positive feedback regulatory mechanism of EMMPRIN gene expression during tumor-fibroblast cell-cell interaction. In addition to stimulating EMMPRIN expression and MMP expression, tumor cell-associated EMMPRIN also mediates the release of soluble EMMPRIN during tumor-fibroblast cell interactions.

MMP-Dependent Generation of Soluble EMMPRIN in Coculture of EMMPRIN-Positive Tumor Cells and Fibroblast Cells

Western blot analyses using antibodies against different regions of EMMPRIN were performed to further characterize soluble EMMPRIN generated in the coculture of tumor cells and fibroblast cells. Antibodies against the extracellular portion of EMMPRIN molecule detected a single band of approximately M_r 52,000, consistent with the size of intact protein, in total cell lysates. Meanwhile, this same antibody detected a slightly smaller molecule in the conditioned medium from coculture, indicating a loss of a portion of EMMPRIN protein (Fig. 5A). Interestingly, antibodies specific to the carboxyl terminus of EMMPRIN only detected EMMPRIN in total cell lysates, but failed to detect any EMMPRIN protein in conditioned medium (Fig. 5A). Taken together, these data suggest that soluble EMMPRIN secreted into coculture medium of tumor and fibroblast cells is likely a cleaved product of the full-length transpanning form of membrane EMMPRIN.

Because the generation of soluble EMMPRIN occurred concomitantly with increased production of MMP in coculture of tumor and fibroblast cells, we speculated that the release of soluble EMMPRIN might be mediated by MMP-dependent proteolytic cleavage. To further explore the mechanism of soluble EMMPRIN generation, 1,10 phenanthroline (1,10 PA) a general metalloproteinase inhibitor, was used at noncytotoxic

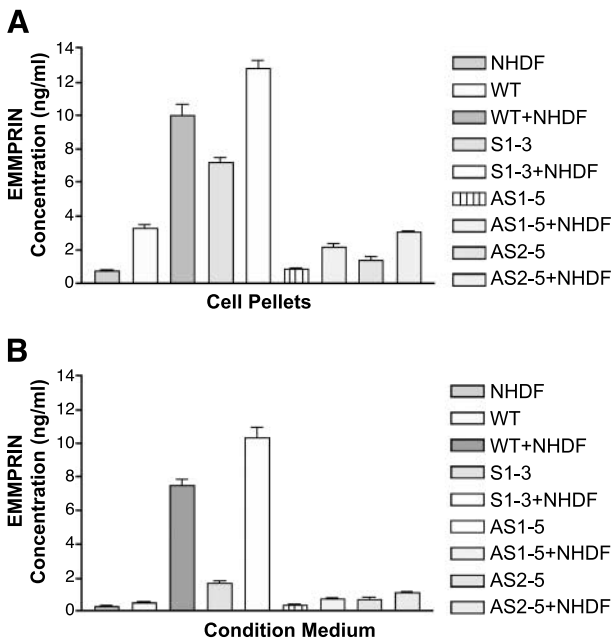

FIGURE 4. Stimulation of both cell-associated and soluble EMMPRIN expression in coculture of tumor cells and fibroblast cells. EMMPRIN protein concentration was determined by ELISA analysis in total cell lysate (**A**) or in conditioned medium (**B**) of breast cancer cells expressing different levels of EMMPRIN either in monoculture or in coculture with NHDF cells. All concentrations were normalized to total protein amounts. *WT,* wild-type MDA MB 231 human breast cancer cells; *S1-3,* sense transfected EMMPRIN-overexpressing cells; *AS1-5* and *AS2-5,* antisense transfected cells. *Columns,* representative of two independent experiments; *error bars,* SDs.

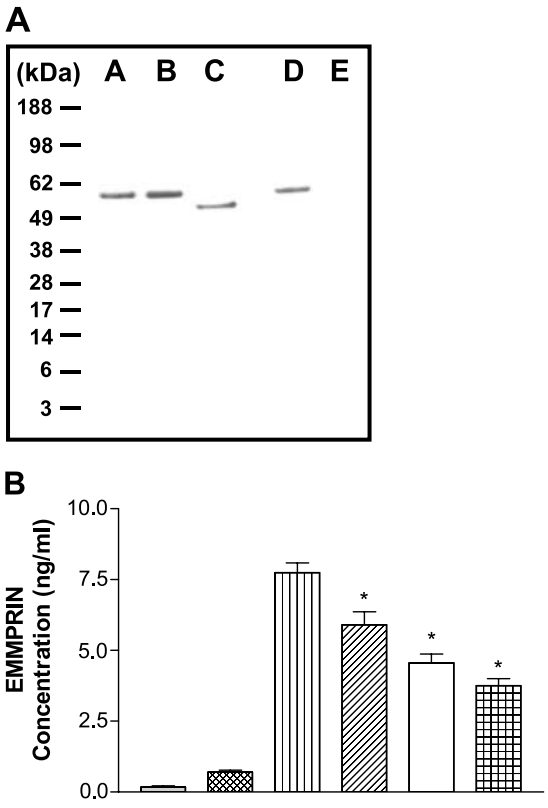

FIGURE 5. Soluble EMMPRIN in coculture of EMMPRIN-positive tumor cells and fibroblast cells—proteolytic cleavage products generated in an MMP-dependent fashion. **A.** Western blot analyses of cell lysates or conditioned medium of coculture of wild-type MDA MB 231 human breast cancer cells and fibroblast cells. Membranes with 50 ng recombinant EMMPRIN extracellular domain (*lane A*), cell lysates of 50 μg total protein (*lanes B* and *D*), and conditioned medium of 50 μg total protein (*lanes C* and *E*) were blotted with antibodies that recognize extracellular domain (*lanes A–C*) or intracellular carboxyl terminus (*lanes D* and *E*). **B.** Soluble EMMPRIN concentration in conditioned medium from coculture of tumor cells and fibroblast cells was determined using ELISA analysis. 1,10 phenanthroline was included in the study from 0.1, 0.5 to 1.0 μM to inhibit MMP enzymatic activity. *WT,* wild-type MDA MB 231 human breast cancer cells. *Columns,* representative of two independent experiments; *error bars,* SDs. ▭, NHDF; ▩, WT; ▥, WT + NHDF; ▨, WT + NHDF + 0.1 μM 1,10 PA; ▭, WT + NHDF + 0.5 μM 1,10 PA; ▦, WT + NHDF + 1.0 μM 1,10 PA; *, $P < .05$ compared to WT + NHDF.

concentrations to inhibit the enzymatic activity of MMPs. As shown in Fig. 5B, inhibition of MMP activity by 1,10 PA resulted in significant decreases in the level of soluble EMMPRIN in a dose-dependent fashion.

These results demonstrate that the generation of soluble EMMPRIN stimulated by tumor fibroblast cell-cell interactions is the result of proteolytic cleavage of membrane-associated EMMPRIN by increased MMP activities in coculture.

Discussion

It is now known that the microenvironment of the tumor-host interface plays a proactive role during malignant disease progression, including the transition from carcinoma *in situ* to invasive cancer, tumor cell proliferation, and tumor cell dissemination and metastasis (28). On the host side, the stromal compartment is comprised of a variety of cell types, including immune cells, inflammatory cells, muscle and myofibroblast cells, and vascular cells. Tumor cells express molecules, either secreted or presented on cell surface, to interact with surrounding stromal cells. In the current study, we have demonstrated that EMMPRIN exists in both soluble and membrane-bound forms. Generation of soluble EMMPRIN resulted from tumor-fibroblast cell interaction in an EMMPRIN-dependent fashion, and was mediated by MMP proteolytic activities. These novel findings suggest a positive feedback regulatory mechanism of EMMPRIN expression that occurs at tumor-host interface.

EMMPRIN is normally expressed as a glycoprotein on tumor cell surface that migrates at an apparent molecular size of M_r 58,000 (19). The domain structure of EMMPRIN consists of an extracellular portion which contains two immunoglobulin-like domains, a transmembrane domain, and a short cytoplasmic domain (19). It has been suggested that tumor cell-associated EMMPRIN exerts its MMP stimulatory effects on fibroblast cells via close cell-to-cell contact. However, the production of soluble EMMPRIN by tumor cells has recently been indicated in several studies (29, 30). In these studies, tumor cells affect fibroblast cells in the absence of physical contact in an EMMPRIN-dependent fashion, resulting in increased MMP-2 expression and activation (29), or enhanced cell migration or invasion (30). In addition, soluble full-length EMMPRIN has been purified from concentrated culturing medium of breast cancer cells, presumably secreted in shed vesicles (31). However, secreted soluble EMMPRIN in cancer cell monoculture is estimated to account for only 2–3% of EMMPRIN produced by these cells (31). In the present study, we have demonstrated the release of a substantial amount of soluble EMMPRIN into cell culture medium of cocultured tumor and fibroblast cells. Because fibroblast cells respond to EMMPRIN stimulation in producing increased levels of EMMPRIN, it is likely that the soluble EMMPRIN detected in coculture is derived from both tumor and fibroblast cells. In addition, the soluble EMMPRIN generated in coculture lacks the intracellular carboxyl terminus as determined using an antibody against the intracellular region of the molecule. In contrast, this carboxyl terminal region was preserved in cell-associated EMMPRIN. More interestingly, generation of soluble EMMPRIN in coculture could be efficiently blocked via inhibiting MMP activity. Therefore, this soluble EMMPRIN likely represents the proteolytic product of membrane-associated EMMPRIN.

The generation of soluble EMMPRIN at the interface of tumor-host interaction may have profound biological significance. The soluble EMMPRIN could diffuse away from local tumor invasion site and stimulate stromal cells at distant sites. We and others have shown that soluble EMMPRIN consisting of only the extracellular portion of the molecular is functional in stimulating MMP expression in fibroblast and endothelial cells (17, 32). Increased MMP activity in the stromal compartment in turn promotes tumor invasion and angiogenesis.

The receptor on fibroblast cells that is responsible for EMMPRIN-mediated stimulation of MMP production has remained elusive (17, 21). It has been suggested that EMMPRIN may serve as its own counter-receptor in cancer cells, thus stimulating MMP via a homophilic interaction (33).

One could speculate that EMMPRIN on fibroblast cells could also serve as counter-receptors for tumor cell-associated EMMPRIN in stimulating MMP expression. However, this hypothesis is not supported by the fact that only extremely low levels of EMMPRIN expression could be detected in normal fibroblast cells. Our finding of a novel positive feedback regulatory mechanism of EMMPRIN expression provides an alternative explanation for the potential role of EMMPRIN as its own counter-receptor in fibroblast cells. When fibroblast cells were exposed to EMMPRIN stimulus, EMMPRIN expression is up-regulated in these cells. Newly synthesized EMMPRIN could then be presented on the cell surface and serves as the counter-receptor for EMMPRIN-dependent signaling between tumor and fibroblast cells. This novel regulatory mechanism is supported by recent findings of EMMPRIN mRNA expression in peritumoral fibroblasts in ovarian carcinoma (34). However, this homophilic interaction likely only accounts for EMMPRIN-mediated tumor-host interaction in some tumors because EMMPRIN expression has not been detected in the stroma of many other tumors including melanoma (35), or lung and breast cancer (16). In these samples, despite of a lack of EMMPRIN expression in stromal tissue, MMP expression is stimulated in stromal tissues adjacent to EMMPRIN-positive tumor cells, suggesting that there might be an unknown receptor(s) on stromal cell surface mediating these interactions.

In conclusion, we have identified a novel positive feedback regulatory mechanism of EMMPRIN expression in fibroblast cells and discovered a soluble form of EMMPRIN. These findings suggest an amplification of EMMPRIN-mediated, MMP-dependent signaling events at the tumor and host interface, as well as at distant sites (Fig. 6).

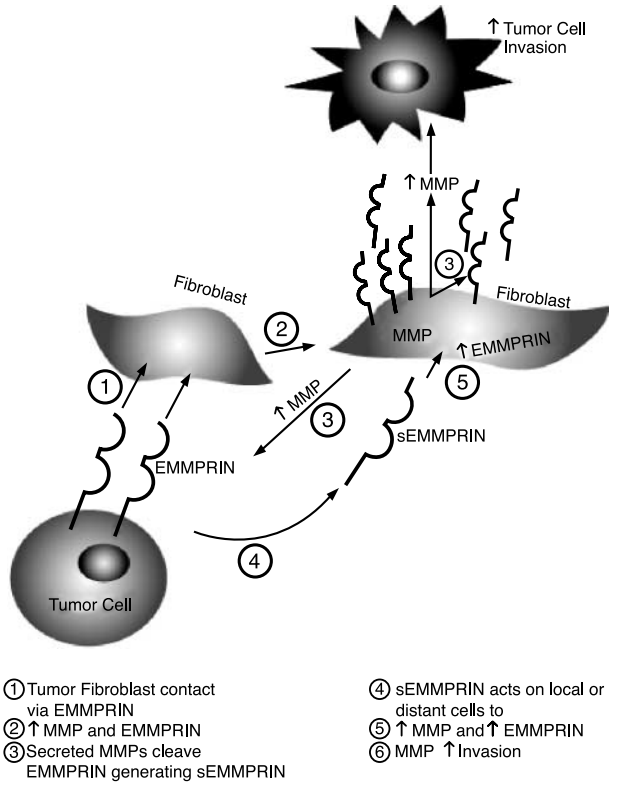

FIGURE 6. Proposed model for signal loops between EMMPRIN and MMP and tumor cells and host cells. Tumor cells use cell surface EMMPRIN to initiate contact with surrounding fibroblast cells (*1*), signaling fibroblast cells to synthesize MMPs (*2*). MMPs secreted by fibroblast cells subsequently cleave cell surface EMMPRIN and generate soluble EMMPRIN (*sEMMPRIN*; *3*). These sEMMPRIN molecules, in turn, act on cells either in local tumor environment or diffuse away to act on distant cells (*4*) to further stimulate MMP and EMMPRIN expression (*5*) and augment the migration and invasion potential of tumor cells (*6*).

Materials and Methods

Cell Culture

MDA MB 231 human breast carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured under recommended conditions. Briefly, cells were cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). NHLF or normal human dermal fibroblast (NHDF) cells were obtained from Clonetics (Walkersville, MD) and were cultured in fibroblast growth medium containing 1 μg/ml human recombinant fibroblast growth factor (hFGF), 5 mg/ml insulin, 50 mg/ml gentamicin, and 50 mg/ml amphotericin B, at 37°C, 5% CO_2. For coculture experiments, 100,000 cancer cells (MDA MB 231, MDA MB 231 S1-3, MDA MB 231 AS1-5, or AS2-5) were cultured together with 200,000 NHDF cells in a six-well cell culture plate in complete DMEM culturing medium. After 24 h, culturing medium was replaced with fresh serum-free DMEM and cells were cultured for 2 days. Culture medium was then replaced with 1.0 ml of fresh serum-free DMEM. Conditioned medium was collected 3 days later and secreted EMMPRIN protein concentration was determined using ELISA. Cells were lysed with 200 μl lysis buffer [50 mM Tris (pH 7.8), 150 mM NaCl, and 1% NP40] to determine cell-associated EMMPRIN concentrations.

Cell Treatment and MMP-1 Activity Assay

Recombinant EMMPRIN corresponding to the extracellular domain of human EMMPRIN protein was produced in NSO cells (R&D Systems, Minneapolis, MN). MMP-1 activity in serum-free medium conditioned by fibroblast cells treated with different amounts of recombinant EMMPRIN protein was quantitatively determined using an MMP-1 Activity Assay Kit according to product manual (R&D Systems). Briefly, MMP-1 contained in 150 μl of standards or samples was captured by anti-MMP-1 antibodies immobilized on the bottom of assay wells. Captured MMP-1 was subsequently activated by 4-aminophenylmercuric acetate (APMA). MMP substrate added into each well was cleaved by activated MMP-1 and the resulting fluorescence was determined using SpectraFluor Plus Plate Reader (TECAN, Research Triangle Park, NC) with the following parameters: excitation wavelength at 320 nm and emission wavelength at 405 nm.

Generation of Sense and Antisense Expression Constructs and Establishment of Stable Transfectants

To study the function of EMMPRIN in mediating tumor and stromal fibroblast cell interaction, the cDNA sequence

corresponding to human EMMPRIN open reading frame (ORF) was PCR amplified and subcloned into pcDNA3.1TOPO vectors (Invitrogen, Carlsbad, CA). The EMMPRIN open reading frame was cloned in both sense and antisense orientations for cytomegalovirus promoter-driven expression in mammalian cells. Transfection of MDA MB 231 human breast carcinoma cells was performed using Effectene transfection reagents according to manufacturer's recommendations (Qiagen, Valencia, CA). Stably transfected cells were selected using Genicitin at 1000 μg/ml (Life Technologies). Individual transfectant colonies were established by cloning antibiotic-resistant cell colonies. Effects of sense and antisense transfection on EMMPRIN protein expression were determined using Western blot analysis and ELISA.

RNA Sample Preparation and TaqMan Quantitative Reverse Transcription-PCR

Total RNA was extracted from cells using RNeasy Kit (Qiagen) following manufacturer's instructions. Five micrograms of total RNA were used in the reverse transcription (RT) reactions which were carried out in a 50-μl total volume using Superscript First-Strand Synthesis System for RT-PCR kit following manufacturer's instructions (Invitrogen). Briefly, RNA samples were mixed with 0.5 μg/μl Oligo(dT), 0.4 mM dNTP mix in a total volume of 25 μl and were incubated at 65°C for 5 min, followed by quick cooling on ice for 2 min. The tubes were spun briefly and added with 5 μl 10× RT buffer, 10 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNaseOUT, 1 μl (50 units) of Superscript II RT, and 6 μl nuclease-free water. The tubes were gently mixed and incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min followed by quick cooling on ice for 2 min. The tubes were spun briefly and 1 μl of RNase H was added and incubated for 20 min at 37°C before proceeding to amplification of the target gene.

Real-time PCR analyses were performed in 50 μl volume containing 2× TaqMan Universal PCR Master mix (Perkin-Elmer, Norwalk, CT), appropriate amounts of cDNA or genomic DNA, and 200 nM of TaqMan primers and 400 nM of TaqMan probes. Primer pairs and probes used in the study were designed using Primer Express software (Perkin-Elmer), with sequences listed in Table 1. Probes were labeled at the 5′-end with fluorescent reporter dye Fam and at the 3′-end with fluorescent quencher dye Tamra by Synthegen (Houston, TX) to allow direct detection of PCR products. Real-time PCR amplification and detection were performed in 96-well optical plates using ABI 7900HT sequence detector (PE Biosystems, Norwalk, CT). PCR conditions included thermal cycles of 30 s of 95°C for denaturing, 30 s of 60°C for annealing, and 60 s of 68°C for elongation. Relative gene copy numbers were calculated using a standard curve generated using PCR standards, serially diluted human genomic DNA (Clontech, Palo Alto, CA).

Table 1. TaqMan Probes and Primers

Human EMMPRIN	(GenBank Accession Number AB072923)
TaqMan probe:	5′-TGGCTCCAAGATACTCCTCACCTGCTCCT-3′
Sense primer:	5′-CGGCACAGTCTTCACTACCGTA-3′
Antisense primer:	5′-GTGCCCTGTGACCTCTGTGG-3′
Human MMP-1	(GenBank Accession Number AF219624)
TaqMan probe:	5′-TGGTCCCTGAGCCGCCGCCG-3′
Sense primer:	5′-GCACTTCGACCAAGATGAGCG -3′
Antisense primer:	5′-CCAAGCGTGTGACCGATCTC-3′
Human GAPDH	(GenBank Accession Number BC014085)
TaqMan probe:	5′-ACCACAGTCCATGCCATCACTGCCA-3′
Sense primer:	5′-CAAGGTCATCCATGACAACTTTG-3′
Antisense primer:	5′-GGCCATCCACAGTCTTCTGG-3′

Protein Electrophoresis and Western Blot Analysis

Stably transfected cells were lysed in cell lysis buffer containing 50 mM Tris (pH 7.8), 150 mM NaCl, and 1% NP40. Protein concentration of cell lysate or conditioned medium was determined using the MicroBCA method (Pierce, Rockford, IL). Equal amount of proteins was loaded onto 4–15% gradient gels and was separated by SDS-PAGE under reducing conditions. Resolved proteins were electrophoretically transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% low fat dry milk in TBS-T [10 mM Tris (pH 7.2), 50 mM NaCl, 0.5% Tween 20] for 1 h at room temperature, followed by incubating with primary antibody at 4°C overnight. Anti-EMMPRIN monoclonal antibody purchased from Research Diagnostics Inc. (Flanders, NJ) recognizes the extracellular portion of the protein. C-19 polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was raised against peptides of the carboxyl terminus of EMMPRIN protein. Blots were extensively washed with TBS-T and incubated with 1:5000 dilution of horseradish peroxidase (HRP) conjugated secondary antibody (Vector Laboratories, Burlingame, CA) diluted in TBS-T for 1 h at room temperature. Labeled proteins were visualized with Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Pixel density of protein bands on Western blot was determined using Un-Scan-It software (Silk Scientific Corporation, Orem, UT).

EMMPRIN ELISA

EMMPRIN protein concentration in conditioned medium was determined using EMMPRIN ELISA. Briefly, 96-well Nunc Immuno Plates (Nunc, Denmark) were coated overnight at 4°C with 50 μl of anti-EMMPRIN antibody (R&D Systems) at 5 μg/ml in buffer A (pH 7.4, 20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μM $MnCl_2$). After one wash in buffer A, nonspecific binding sites were blocked with 150 μl of blocking buffer [3% bovine serum albumin (BSA) in buffer A] for 1 h at 37°C. Wells were then rinsed two times with binding buffer (buffer A with 0.3% BSA and 0.2% Tween 20). Either recombinant human EMMPRIN or conditioned medium was added into each well and was incubated at 37°C for 2 h. After three washes with binding buffer at RT, plates were blocked with 150 μl of blocking buffer (3% BSA in buffer A) at 37°C for 1 h. Fifty microliters of 1:5000 diluted biotin-conjugated anti-EMMPRIN antibodies (RDI-147, Research Diagnostics) were added into each well and incubated at 37°C for 2 h. After three washes with binding buffer, 50 μl of horseradish peroxidase-conjugated streptavidin 1:10,000

diluted in binding buffer were added into each well, and were incubated at 37°C for 1 h. After three washes with binding buffer at RT, 50 μl of developing buffer (1 mg/ml *O*-phenylenediamine, 0.1 M citric acid, 0.2 M sodium phosphate, 0.01% H_2O_2) were added to each well and incubated at RT until color develops. Colorimetric reactions were stopped by adding 25 μl of 4 N H_2SO_4. ELISA data acquisition was performed using VersaMax Tunable MicroPlate Reader (Molecular Devices, Sunnyvale, CA) at 490 nm. Data were analyzed using Softmax Pro 3.1 software (Molecular Devices).

Acknowledgments

Authors thank Dr. Bob Jordan for his critical review of the manuscript and the Research Visual Communication Department at Centocor, Inc. for assistance in graphic illustrations.

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Mol Cancer Res 2004;2:73-80.