Regulation of Vascular Endothelial Growth Factor Expression by EMMPRIN via the PI3K-Akt Signaling Pathway

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Abstract
Extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN) is a cell surface glycoprotein overexpressed in many solid tumors. In addition to its ability to stimulate stromal MMP expression, tumor-associated EMMPRIN also induces vascular endothelial growth factor (VEGF) expression. To explore the underlying signaling pathways used by EMMPRIN, we studied the involvement of phosphoinositide 3-kinase (PI3K)-Akt, mitogen-activated protein kinase (MAPK), JUN, and p38 kinases in EMMPRIN-mediated VEGF regulation. Overexpression of EMMPRIN in MDA-MB-231 breast cancer cells stimulated the phosphorylation of only Akt and MAPKs but not that of JUN and p38 kinases. Conversely, inhibition of EMMPRIN expression resulted in suppressed Akt and MAPK phosphorylation. Furthermore, the PI3K-specific inhibitor LY294002 inhibited VEGF production by EMMPRIN-overexpressing cells in a dose- and time-dependent manner. On the other hand, the MAPK inhibitor U0126 did not affect VEGF production. In vivo, EMMPRIN-overexpressing tumors with elevated VEGF expression had a high level of phosphorylation of Akt and MAPK. Finally, when fibroblast cells were treated with recombinant EMMPRIN, Akt kinase but not MAPK was phosphorylated concomitantly with an increase in VEGF production. Both the activation of Akt kinase and the induction of VEGF were specifically inhibited with a neutralizing antibody to EMMPRIN. Our results show that in both tumor and fibroblast cells EMMPRIN regulates VEGF production via the PI3K-Akt pathway but not via the MAPK, JUN, or p38 kinase pathways.

Introduction
Extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN) was originally purified from the plasma membrane of cancer cells as a glycoprotein of Mr 58,000 and was designated tumor collagenase-stimulating factor because of its ability to stimulate fibroblast synthesis of collagenase-I (MMP-1; ref. 1). EMMPRIN is a highly glycosylated member of the immunoglobulin superfamily expressed on the surface of most tumor cells and functions as an upstream modulator of MMP production in the local tumor environment (2, 3). EMMPRIN-positive tumor cells stimulate neighboring fibroblast cells to express MMPs and therefore facilitate tumor invasion and metastasis. Recently, we have shown that EMMPRIN not only plays an important role in cancer progression by inducing MMP production (4-11) but also stimulates tumor angiogenesis by regulating vascular endothelial growth factor (VEGF) expression (12).

Phosphoinositide 3-kinase (PI3K) is a heterodimeric enzyme composed of one 110-kDa catalytic subunit and one 85-kDa regulatory subunit (13) and serves as a major signaling component downstream of growth factor receptor tyrosine kinases (14, 15). PI3K catalyzes the production of the lipid secondary messenger phosphatidylinositol-3,4,5-triphosphate, which in turn activates a wide range of downstream targets, including the serine/threonine kinase Akt (16). The PI3K-Akt pathway regulates multiple cellular processes, including cell proliferation, survival, growth, and motility (15). In addition, Akt also transmits angiogenic signals and can directly induce tumor angiogenesis by regulating VEGF expression in endothelial cells (17).

To investigate the signaling pathway underlying EMMPRIN-mediated VEGF expression, we recombinantly engineered MDA-MB-231 human breast cancer cells to express different levels of EMMPRIN (18). The effects of EMMPRIN on PI3K-Akt, mitogen-activated protein kinase (MAPK), JUN, and p38 kinases were determined in these engineered tumor cells, in tumors derived from these tumor cells, and in fibroblast cells stimulated with recombinant EMMPRIN. Our findings show that EMMPRIN positively regulates PI3K-Akt and MAPK pathways. However, EMMPRIN stimulates VEGF production only via the PI3K-Akt pathway but not via the MAPK, JUN, or p38 kinase pathway.

Results
Effects of EMMPRIN on the Phosphorylation Status of Akt, MAPK, JUN, and p38 Kinases
To investigate the relationship between tumor cell EMMPRIN expression and the activation of key signaling pathways underlying VEGF regulation, we studied the effects of EMMPRIN expression on the phosphorylation status of Akt,
MAPK, JUN, and p38 kinases in MDA-MB-231 tumor cells expressing different levels of EMMPRIN (18). As we have shown previously, cells overexpressing EMMPRIN produce elevated VEGF expression in contrast to EMMPRIN antisense cells that have suppressed VEGF production (12). Changes in the phosphorylation status of Akt (Thr308) and p42 MAPK were correlated with EMMPRIN levels in tumor cells. The phosphorylation of Akt and p42 MAPK was augmented when EMMPRIN was overexpressed and was conversely suppressed when EMMPRIN expression was inhibited (Fig. 1A and B). In contrast, EMMPRIN expression did not affect c-Jun and p38 kinase phosphorylation (Fig. 1C and D). The total protein expression level of Akt, p42, JUN, and p38 kinases did not change as EMMPRIN expression level varied (Fig. 1A-D). The functional effects derived from stable modulation of EMMPRIN expression levels were verified in these tumor cell clones expressing different levels of EMMPRIN. Increased level of MMP-9 was detected in the conditioned medium of EMMPRIN-overexpressing cells, and MMP-9 production was suppressed in antisense clones (Fig. 1E). Taken together, these results suggest that EMMPRIN regulates the phosphorylation of Akt and MAPK but not c-Jun and p38 kinases in tumor cells.

To determine whether these EMMPRIN-mediated changes also occur in vivo, phosphorylation status of Akt and MAPK was assessed in parental MDA-MB-231 and EMMPRIN-overexpressing MDA-MB-231 tumors. Our previous studies confirmed elevated EMMPRIN expression levels in tumors derived from EMMPRIN-overexpressing tumor cells (12). In these same tumors, VEGF expression was up-regulated leading to stimulation of tumor angiogenesis and increased tumor growth rate (12). Phosphorylation of both Akt and MAPK was substantially stimulated in EMMPRIN-overexpressing tumors in comparison with that in the parental tumors (Fig. 2). The heaviest staining for both phosphorylated Akt (p-Akt) and phosphorylated MAPK (p-MAPK) was observed along the advancing margin of the tumor. Mitotic cells were much more common in EMMPRIN-overexpressing tumors, which have faster growth rate in vivo than their parental counterparts (12). In both mitotic and nonmitotic cells, p-Akt staining in the EMMPRIN-overexpressing tumors was stronger than that in the parental tumors. As shown in Table 1, the percent area stained of p-Akt in the EMMPRIN-overexpressing tumors was ~10-fold greater than that in parental tumors, and the staining intensity was ~19-fold higher (Table 1). Anti-p-MAPK stained mitotic tumor cells in both parental and EMMPRIN-expressing tumors. These results suggest that EMMPRIN regulates the phosphorylation of Akt and MAPK in vivo.

**FIGURE 1.** Effects of EMMPRIN expression on phosphorylation status of Akt, MAPK, JUN, and p38 kinases. MDA-MB-231 tumor cells engineered to express different levels of EMMPRIN were verified previously (18). Effects of EMMPRIN expression on Akt (Thr308) kinase (A), p42 MAPK (B), c-Jun kinase (C), and p38 MAPK (D) were investigated using antibodies specific to the phosphorylated forms of these kinases (top). Bottom, total levels of these kinases. Lane 1, wild-type cells; lane 2, cells transduced with vector; lane 3, cells stably transduced with sense EMMPRIN constructs to overexpress EMMPRIN; lanes 4 and 5, cells transduced with antisense EMMPRIN constructs to suppress EMMPRIN. Representative results from three independent experiments. E. Effects of EMMPRIN expression on MMP-9 production were assessed to confirm the biological activity of stable modulation of EMMPRIN expression.
EMMPRIN Regulates VEGF Expression via the PI3K-Akt Pathway but not the MAPK Pathway

Because both Akt and MAPK signaling pathways were activated in tumor cells overexpressing EMMPRIN, we hypothesized that one or both of these two signaling pathways may mediate the elevated VEGF expression in these cells. To test this hypothesis, we used Akt- or MAPK-specific inhibitors to determine which pathway is responsible for the EMMPRIN-mediated VEGF regulation.

We first tested the inhibitory effects of the PI3K-specific inhibitor LY294002 and MAPK-specific inhibitor U0126 on the phosphorylation of these two kinases, respectively. When MDA-MB-231 cells overexpressing EMMPRIN were treated with LY294002 (1-40 μmol/L) for 24 hours, Akt phosphorylation at Thr308 was dose-dependently inhibited (Fig. 3A). Complete suppression of Akt phosphorylation was seen when cells were treated with 40 μmol/L LY294002 for 24 hours. In addition, when these cells were exposed to the inhibitor at 20 μmol/L for varying times, a time-dependent suppression of Akt phosphorylation was observed (Fig. 3A). There was little change in the level of p-Akt at 6 hours after treatment. However, when cells were treated with 20 μmol/L LY294002 for 12 or 24 hours, significantly reduced Akt phosphorylation was observed. The 48-hour treatment completely blocked Akt phosphorylation (Fig. 3A). The total amount of Akt protein was not affected by inhibitor treatment, and no cell toxicity was observed at the concentrations and conditions used in these studies using the trypan blue assay (data not shown). When the Akt signaling pathway was inhibited in EMMPRIN-overexpressing cells, a concentration- and time-dependent inhibition of VEGF expression was detected (Fig. 3B and C). The trend of VEGF suppression was similar to the inhibition of Akt phosphorylation by LY294002 (Fig. 3B and C). The basal level of VEGF was 220 pg/mL in these tumor cells before treatment. This level was reduced by 15% and 40% to 190 and 140 pg/mL when cells were exposed to 1 and 10 μmol/L LY294002, respectively, for 24 hours. A further decrease in VEGF expression to 65 and 40 pg/mL was observed when Akt phosphorylation was observed (Fig. 3A).

![FIGURE 2. Immunohistochemical staining for p-Akt and p-MAPK in tumors expressing different levels of EMMPRIN. A and B, p-Akt (Ser473) staining of parental MDA-MB-231 and EMMPRIN-overexpressing MDA-MB-231 xenograft tumors. C and D, Phosphorylated p42/p44 MAPK staining of parental MDA-MB-231 and EMMPRIN-overexpressing MDA-MB-231 xenograft tumors.](image)

Table 1. Quantitative Analysis of p-Akt In vivo

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>% Stained area, median (interquartile range)</th>
<th>Integrated optical density (arbitrary units), median (interquartile range)</th>
</tr>
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<tbody>
<tr>
<td>Parental</td>
<td>0.7 (0.6-0.9)</td>
<td>1.2 (1.1-1.5)</td>
</tr>
<tr>
<td>EMMPRIN overexpressing</td>
<td>10.1* (2.9-20.0)</td>
<td>19.8* (5.3-36.4)</td>
</tr>
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*Statistically significant difference (Mann-Whitney rank-sum test).
tumor cells were treated with 20 and 40 μmol/L LY294002. When the cells were treated with LY294002 at 20 μmol/L, the suppression of VEGF expression was time dependent (Fig. 3C). At 6 hours after treatment, the VEGF level decreased by 15% to 191 pg/mL followed by further reductions to 130 and 66 pg/mL at 12 and 24 hours. The most profound suppression of VEGF expression occurred when cells were treated for 48 hours. The VEGF level of 45 pg/mL detected at this time point was similar to that found in cells treated with 40 μmol/L inhibitor for 24 hours when Akt phosphorylation was completely abolished under both conditions. As a control, MDA-MB-231 vector cells were treated with the same PI3K-specific inhibitor LY294002. Although these cells had a relatively lower level of Akt phosphorylation (Fig. 1) and VEGF production (12) compared with the EMMPRIN-overexpressing MDA-MB-231 cells, the PI3K-specific inhibitor LY294002 inhibited Akt phosphorylation and VEGF production in a dose- and time-dependent manner similar to that observed in EMMPRIN-overexpressing cells (data not shown).

Similar studies were carried out to determine the in vitro inhibitory profile of the MAPK-specific inhibitor U0126 in the EMMPRIN-overexpressing tumor cells. Whereas low concentration of U0126 (0.5 μmol/L) had very little effect on p42 MAPK phosphorylation, increasing levels of U0126 from 1 to 10 μmol/L significantly inhibited p42 MAPK phosphorylation in a dose-dependent manner (Fig. 3D). This inhibition was also time dependent, and greater decreases in p42 MAPK phosphorylation were detected when cells were incubated with U0126 at 1 μmol/L for varying times from 6 to 48 hours (Fig. 3D). However, no apparent effect on VEGF production was detected even under the conditions where MAPK phosphorylation was greatly suppressed (Fig. 3E and F).

Taken together, our findings suggest that in tumor cells EMMPRIN regulates VEGF production via the PI3K-Akt pathway but not via the MAPK, JUN, or p38 kinase pathways, although EMMPRIN also regulates MAPK phosphorylation.

Recombinant EMMPRIN Stimulates VEGF Production via the PI3K-Akt but not the MAPK Pathway in Fibroblast Cells

Exposure of fibroblasts to recombinant soluble EMMPRIN resulted not only in an induction of EMMPRIN and MMP
expression but also in release of soluble EMMPRIN (18). This self-stimulation of EMMPRIN expression has also been observed in animal models and may contribute to MMP and VEGF induction and ultimately tumor angiogenesis and progression (12). Because we have shown the effect of EMMPRIN expression on VEGF induction via the PI3K-Akt pathway in tumor cells, further investigation of this pathway in fibroblast cells would be very important given the role of EMMPRIN in mediating the tumor-stromal interaction (11, 18). The biological activity of recombinant EMMPRIN used in the study was confirmed by stimulating fibroblast cells resulting in enhanced MMP-1 production (18). When normal human lung fibroblasts were stimulated with recombinant EMMPRIN, only Akt (Thr^308) phosphorylation but not p42 MAPK was induced (Fig. 4A). Levels of p-Akt in these cells treated with EMMPRIN at 5 and 10 µg/mL were significantly elevated at 48 hours in a dose-dependent fashion. In contrast, p42 MAPK phosphorylation remained unchanged after treatment with increasing levels of EMMPRIN from 0.5 to 10 µg/mL. No changes in the total amount of Akt or MAPK kinases were detected under these conditions.

To study whether EMMPRIN-stimulated Akt phosphorylation leads to induction of VEGF production in normal human lung fibroblasts, we collected the conditioned medium after cells were treated with recombinant EMMPRIN and measured VEGF concentrations. Concomitant with an increase in Akt phosphorylation, significant induction of VEGF production was observed in cells treated with EMMPRIN (Fig. 4B). Although EMMPRIN at 0.5 and 1 µg/mL induced a slight increase in VEGF expression, a more profound effect on VEGF production was only detected at 5 and 10 µg/mL, the same doses sufficient to induce Akt phosphorylation.

Finally, the involvement of EMMPRIN in regulating Akt signaling and VEGF production was investigated using neutralizing antibodies. Both Akt phosphorylation and VEGF production induced by 10 µg/mL EMMPRIN were dose-dependently inhibited by anti-EMMPRIN antibodies (Fig. 4C and D). At 20 µg/mL, the anti-EMMPRIN antibody was able to completely block the induction of Akt phosphorylation and VEGF expression.

Taken together, these results show that EMMPRIN not only regulates VEGF expression in tumor cells but also induces VEGF production in fibroblast cells via the PI3K-Akt signaling pathway.

**Discussion**

The expression of EMMPRIN and its role in regulating stromal MMP expression have been extensively studied in various cancer types (2, 5-11, 18-20). The signaling events downstream of EMMPRIN-mediated tumor-host interactions that result in stimulation of MMP production are not yet fully established. The p38 MAPK has been implicated in inducing MMP-1 production (21); similarly, signaling through 5-lipoxygenase and phospholipase A2 may contribute to MMP-2 production (22). Recently, EMMPRIN has been shown to also participate in other activities during cancer progression not directly related to its function as a MMP stimulator (1).
For example, EMMPRIN has been shown to potentiate tumor cell anchorage-independent growth (23), a phenomenon that is characteristic of malignant cancer cells, and may be related to multidrug resistance in cancer cells (24). These effects of EMMPRIN were shown to depend on stimulation of production of hyaluronan, a pericellular polysaccharide (23, 24), and cell survival signaling pathways (24, 25).

We have recently discovered a link between the function of EMMPRIN in cancer progression to VEGF regulation and tumor angiogenesis (12). In this newly discovered system, tumor-associated EMMPRIN functionally mediates tumor-stromal interactions and directly contributes to tumor angiogenesis and growth by stimulating VEGF and MMP expression in both tumor and stromal compartments. The current study is to determine the signaling requirements for EMMPRIN-induced VEGF production. In this work, we have shown that both endogenously expressed EMMPRIN and exogenously added recombinant EMMPRIN are capable of stimulating VEGF production in tumor and fibroblast cells, respectively, via the PI3K-Akt pathway. It is likely that these two forms of EMMPRIN may exert their functions in distinct fashions. EMMPRIN expressed on tumor cell surface may activate downstream signaling pathways via homophilic interactions, which have been shown to stimulate VEGF production (26). Alternatively, it is also plausible that tumor EMMPRIN expression causes an increase in hyaluronan production to subsequently activate the PI3K-Akt pathway in tumor cells (23, 24). Both these pathways could lead to the elevated expression of VEGF in tumor cells overexpressing EMMPRIN. On the other hand, EMMPRIN expressed on tumor cells can also induce the production of a soluble form of EMMPRIN when tumor cells are in contact with fibroblast cells. This soluble EMMPRIN in turn further induces the expression of EMMPRIN in fibroblast cells through a positive feedback mechanism (12, 18). This mechanism is likely responsible for the induction of stromal EMMPRIN expression in xenograft tumor models, in which tumor EMMPRIN also stimulated VEGF and MMP expression in both tumor and host compartments (12). We have further shown in the current study that the soluble form of EMMPRIN comprising the extracellular portion of the molecule is capable of activating the PI3K-Akt pathway and subsequently inducing the VEGF expression in fibroblast cells. Therefore, via the PI3K-Akt pathway, aberrantly high levels of EMMPRIN expressed by cancer cells of multiple cancer types could directly influence the production of tumor cell–derived VEGF and also induce VEGF expression by the stromal cells at the same time. These combined effects could then lead to promotion of tumor angiogenesis and cancer progression (12, 27).

In this study, we have also discovered that elevated levels of EMMPRIN expression in tumor cell also induce the activation of MAPK in addition to Akt signaling. Although the MAPK pathway does not seem to be directly involved in EMMPRIN-dependent regulation of VEGF, it is possible that this pathway may contribute to other functions of EMMPRIN. One could speculate that EMMPRIN mediated the MAPK activation may work together with other signaling pathways, such as extracellular signal-regulated kinase and focal adhesion kinase (23, 24), to promote cancer cell proliferation, anchorage-independent growth, and drug resistance (11, 23).

Conclusion

We have identified an EMMPRIN-PI3K-Akt-VEGF signaling pathway in which tumor cell–expressed EMMPRIN and soluble EMMPRIN stimulate the production of VEGF, one of the major tumor angiogenic factors. These findings further highlight the importance of EMMPRIN in cancer formation and angiogenesis.

Materials and Methods

Materials

DMEM and fetal bovine serum were from Invitrogen (Carlsbad, CA). Antibodies to p-Akt (Thr308), Akt, phosphorylated p42 MAPK, p42 MAPK, phosphorylated p38 MAPK (Thr180/Tyr182), p38 MAPK kinases, and phosphorylated p42/p44 MAPK rabbit monoclonal antibody for immunohistochemistry studies were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-p-Akt (Ser473) for immunohistochemistry was purchased from GeneTex, Inc. (San Antonio, TX). Anti-phosphorylated c-Jun (Ser63) and anti-c-Jun antibodies were obtained from Upstate (Lake Placid, NY). Anti-EMMPRIN antibody (RDI CD-147) was purchased from Research Diagnostics, Inc. (Flanders, NJ). The bioactivity of recombinant EMMPRIN, composed of the extracellular portion of the protein (R&D Systems, Minneapolis, MN), was verified by its activity in stimulating MMP-1 expression in fibroblasts (18). LY294002 and U0126 were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture

MDA-MB-231 human breast cancer cells were purchased from American Type Culture Collection (Manassas, VA). Methods for transfection and establishment of MDA-MB-231 cells stably expressing different levels of EMMPRIN have been described previously (18). The expression levels of EMMPRIN have been routinely determined by either Western blot or flow cytometry analysis. Cells were always incubated in serum-free medium for 24 hours before treatment. Normal human lung fibroblasts were obtained from Cambrex (Walkersville, MD) and cultured in fibroblast growth medium.

ELISA

ELISA measurements of human VEGF and MMP concentrations were done using Quantikine ELISA kits (R&D Systems; ref. 12). Triplicates of each sample were analyzed using VersaMax Tunable Microplate Reader equipped with SoftMax Pro 3.1 software (Molecular Devices, Sunnyvale, CA).

Protein Electrophoresis and Western Blot Analysis

Tumor cells or fibroblast cells were lysed in cell lysis buffer containing 50 mmol/L Tris (pH 7.8), 150 mmol/L NaCl, and 1% NP40. Protein concentrations of cell lysates were determined using the MicroBCA method (Pierce, Rockford, IL). Equal amounts of protein were loaded onto 4% to 15% gradient gels and 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 TBST [10 mmol/L Tris (pH 7.2), 50 mmol/L NaCl, 0.5% Tween 20] for 1 hour at room temperature.
temperature followed by incubation with the primary antibody at 4°C overnight. Blots were extensively washed with TBST and incubated with 1:5,000 dilution of horseradish peroxidase–conjugated secondary antibody (Pierce) diluted in TBST for 1 hour at room temperature. Labeled proteins were visualized with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry
To determine if increased expression of EMMPRIN affected phosphorylation of Akt and MAPK in vivo, parental MDA-MB-231 cells and EMMPRIN-overexpressing MDA-MB-231 cells were grown as xenografts in female CD1 nu/nu mice (12). Resultant tumors were fixed with modified Beckstead’s (28) fixative [1% zinc acetate, 0.05% CaCl2, 0.5% formalin in 0.1 mol/L Tris (pH 7.4)], processed, and embedded in paraffin by routine methods. Sections for immunohistochemical staining were cut at 4 µm, deparaffinized, and rehydrated. Serial sections were stained for p-MAPK using rabbit anti-phosphorylated p42/p44 MAPK (Cell Signaling Technology, Danvers, MA) or p-Akt using rabbit anti-p-Akt (Ser473). Slides were preincubated in citrate buffer (pH 6.0) for antigen retrieval, blocked with 5% normal goat serum, incubated with primary antibody for 1 hour at room temperature, peroxidase blocked, incubated with streptavidin-horseradish peroxidase (Vector Laboratories), visualized with diaminobenzidine, and counterstained with hematoxylin. Staining was evaluated visually and morphometrically using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Three × 10 fields per tumor were captured, stained areas were segmented, and data for total percent area stained and integrated absorbance (arbitrary units) were collected. Because the data of percent area were nonnormally distributed and because the integrated absorbance data are semiquantitative, data are presented as median and interquartile range. Statistical significance was tested by a Mann-Whitney rank-sum test. \( P < 0.05 \) was accepted as significant.

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References
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