Emmprin Promotes Anchorage-Independent Growth in Human Mammary Carcinoma Cells by Stimulating Hyaluronan Production

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Abstract

Emmprin (CD147; basigin) is a plasma membrane glycoprotein, enriched on the surface of many cancer cells, which induces matrix metalloproteinase synthesis via cell-cell interactions. Elevated emmprin production causes increased growth in vivo of human mammary carcinoma cells. In this study, we show that elevation of emmprin expression in less aggressive human carcinoma cells, which normally express low levels of emmprin levels, induces the ability to grow under anchorage-independent conditions. We also found that elevated emmprin expression stimulates hyaluronan production and that the effect of emmprin on anchorage-independent growth is dependent on hyaluronan. Furthermore, emmprin stimulates cell survival pathway signaling in a hyaluronan-dependent manner. From these and other studies we conclude that emmprin enhances several malignant properties of cancer cells, including anchorage-independent growth, invasiveness, and chemoresistance.

Introduction

Emmprin is a plasma membrane-bound glycoprotein (1) that is expressed at high levels in a variety of malignant human cancers, including melanomas, gliomas, lymphoma, and breast, lung, and kidney carcinomas (2). In accord with these correlations, experimental overexpression of emmprin in MDA MB-436 human mammary carcinoma cells, which are relatively less aggressive and express lower levels of emmprin than most malignant lines, results in their ability to form large, malignant tumors in nude mice (3).

Emmprin was originally identified as a factor on the surface of tumor cells that induces matrix metalloproteinase (MMP) production in fibroblasts and endothelial cells (1, 4–7). More recently it has been found that this is indeed the case.

in vivo of one of the most marked responses to overexpression of emmprin is enhanced tumor growth (3). Consequently we have investigated whether emmprin alters the growth properties of tumor cells in vitro. We chose MDA MB-436 and MCF-7 human mammary carcinoma cells for this study because they are relatively benign and express low levels of emmprin compared with more aggressive tumor cell lines. Using these cells, we find that increasing emmprin expression induces their ability to grow in an anchorage-independent manner, most likely due to increased activity of cell survival signaling pathways.

Hyaluronan is a very high molecular weight polysaccharide composed of repeating disaccharide units, (β1,4-GlcUA-β1,3-GlcNAc)n. It serves a structural role in many connective tissues but is also associated with the pericellular matrix surrounding proliferating and motile cells in normal and pathological systems, where it serves both structural and signaling functions (10, 11). In particular, recent work has shown that interaction of hyaluronan with receptors on the surface of tumor cells induces anchorage-independent growth and plays a critical role in cell survival (11–14). Thus, we determined whether emmprin stimulates hyaluronan production and its effects on cell survival, and found that this is indeed the case.

Materials and Methods

Cell Culture. MDA MB-436 and MCF-7 cells were obtained from American Type Culture Collection (Manassas, Virginia). MDA MB-436 cells were cultured in DMEM with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. They were stably transfected with green fluorescent protein or green fluorescent protein/EMMPRIN constructs as described previously (3). MCF-7 cells were cultured in phenol-red-free DMEM with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. The MCF-7 cells were transfected with a PC3.2 control plasmid or a PC3.2-emmprin construct and stable transfecants selected in medium supplemented with 0.5 mg/ml G418 by routine methods similar to those published previously (3). All of the cells were incubated at 37°C with 5% CO2.

Soft Agar Assay. Soft agar assays were performed as published previously (15). Cells were harvested, washed, and resuspended in 2× growth medium. Five thousand cells in 1 ml of 2× growth medium, with or without 100 µg/ml hyaluronan oligomers (Seikagaku Corp.), 100 µg/ml chitin oligomers (Seikagaku Corp.), 50 µM wortmannin (Sigma), or 20 µM LY294002 (Sigma), were mixed with 1 ml of 0.4% agarose (Life Technologies, Inc.) for plating, as described previously (15). After incubation for 13–14 days, the cultures were processed for staining. Colonies >0.15 mm in size were counted under ×10 magnification using an ocular scale lens.

Hyaluronan Assay. The hyaluronan content of conditioned medium was determined from cultures of 1×104 cells/well collected over a 24-h incubation period (after preincubation for 24 h and washing), using a competitive ELISA-like method (16).

Reverse Transcription-PCR. Semiquantitative reverse transcription-PCR was carried out by published methods (17). The primers used were identical to those used previously, except for Has1, in which case the forward primer was 5’ GTCTGTGACTCggACAC 3’, and the reverse primer was: 5’ CTAGAGGACCgGCTGAtG 3’.

Western Blot Analyses. Similar numbers of each type of cell were grown under routine monolayer conditions and harvested at 80–90% confluence. Because vector- and emmprin-transfected cells grew at approximately the same rates in monolayer culture, the numbers of cells harvested from each were similar. The cells were lysed and processed for SDS-PAGE and Western blot analyses by standard methods as described previously (15). Equal amounts of protein (15 µg) were used for the Western blot analyses.
Results

Increased Emmprin Expression Stimulates Anchorage-Independent But Not Anchorage-Dependent Growth. First we tested whether increased levels of emmprin alter the growth rate of MDA MB-436 and MCF-7 human mammary carcinoma cells in routine monolayer culture. As reported for the MDA MB-436 cells previously (3), we found no significant differences among wild-type, vector-transfected, and emmprin-transfected cells (data not shown).

We then tested the effect of increased emmprin expression on growth of colonies in soft agar. This is a measure of the ability of cells to grow in an anchorage-independent manner, a characteristic of transformed cells (18, 19). We found that wild-type and vector-transfected cells formed few colonies, whereas the emmprin-transfected cells formed numerous large colonies in soft agar (Fig. 1).

Increased Emmprin Expression Stimulates Hyaluronan Production. Recent studies have shown that increased hyaluronan production can lead to stimulation of anchorage-independent growth in tumor cells (12, 14, 20), most likely due to increased cell survival (13, 15). Thus, we tested the effect of increased expression of emmprin on accumulation of hyaluronan in conditioned medium, using an ELISA-like assay (16). In the experiment shown, increased emmprin expression in the MCF-7 cells yielded ~2-fold increase in hyaluronan levels over controls (Fig. 2A), whereas in MDA MB-436 cells hyaluronan levels increased by ~3-fold (Fig. 2B). Increases between 2-fold and 8-fold were obtained in several different experiments. We also measured the levels of cell-associated hyaluronan. Although significant increases in hyaluronan were observed, these differences were modest (~25% increase; data not shown).

To obtain additional information regarding the effect of emmprin on hyaluronan production, we examined the expression of hyaluronan synthases, because the most common mechanism of stimulating hyaluronan production is via their enhanced expression. Consequently we measured mRNA expression for all three of the hyaluronan synthases by semiquantitative reverse transcription-PCR (17), in cells with and without elevated emmprin expression. We found that emmprin stimulates all three of the hyaluronan synthases, i.e., Has1, Has2 and Has3, but its major effect appears to be on Has2 expression (Fig. 2C). Using densitometry, we estimated that, in MDA MB-436 cells, emmprin caused ~4-fold stimulation of Has1, ~10-fold stimulation of Has2, and ~3.5-fold stimulation of Has3 expression. Emmprin caused ~2-fold stimulation of Has1, ~17-fold stimulation of Has2, and ~3-fold stimulation of Has3 in MCF-7 cells (Fig. 2C).

Hyaluronan Oligosaccharides Inhibit Emmprin-Enhanced Anchorage-Independent Growth. We have shown above that emmprin stimulates both anchorage-independent growth and hyaluronan production. Next we tested whether the effect of emmprin on anchorage-independent growth is dependent on hyaluronan. We have shown previously that perturbation of endogenous hyaluronan interactions by treatment with hyaluronan oligosaccharides (oligomers) inhibits anchorage-independent growth of several tumor cell types (15, 21). These oligomers compete for interaction of endogenous hyaluronan polymer with cell surface receptors and consequently attenuate hyaluronan-dependent signaling (15). Therefore, we tested the effect of the hyaluronan oligomers on emmprin-stimulated formation of colonies in soft agar. We conducted the assay with emmprin-transfected MDA MB-436 and MCF-7 cells in the presence and absence of 100 μg/ml hyaluronan oligomers, determined previously as a reliably effective concentration (15). Chitin oligomers were used as a control because they are very similar in structure to hyaluronan oligomers but do not affect soft agar colony formation (15). Treatment with the hyaluronan oligomers reversed the effect of emmprin on colony formation in both MDA MB-436 and MCF-7 cells, whereas the chitin oligomers did not have a significant effect (Fig. 3).

Increased Emmprin Expression Stimulates Cell Survival Pathway Activity in a Hyaluronan-Dependent Manner. Anchorage-independent growth is associated with increased activity of the phosphatidylinositol 3’-kinase (PI3k)/Akt cell survival pathway (19). Thus, we treated emmprin-transfected MDA MB-436 cells with the PI3k pathway inhibitors, wortmannin and LY294002, and measured their effect on emmprin stimulation of soft agar colony formation. This treatment resulted in a reduction in soft agar colony formation back toward control levels (Fig. 3A), implying that the emmprin effect is dependent on PI3k activity.

We then measured the effect of emmprin on phosphorylation of downstream components of the PI3k cell survival pathway, e.g., Akt and BAD. Increased expression of emmprin caused elevated phosphorylation of both Akt and BAD (Fig. 4A). The latter was measured at amino acid position ser36, the site of Akt phosphorylation (22). Cotreatment with hyaluronan oligomers reversed this effect (Fig. 4A).

Fig. 2. Elevated emmprin expression stimulates hyaluronan production. A and B, the hyaluronan (HA) content (ng/ml) of conditioned medium generated from 1 × 10⁶ cells over 24 h was determined using a competitive ELISA-like method (16). A, MDA MB-436. 1, wild-type cells; 2, green fluorescent protein-transfected cells; 3, emmprin/green fluorescent protein-transfected cells. B, MCF-7. 1, wild-type cells; 2, vector-transfected cells; 3, emmprin-transfected cells. Results are expressed as the mean of triplicate measurements; bars, ±SD. Two- to 8-fold stimulation of hyaluronan production by emmprin was observed in three separate experiments. C, reverse transcription-PCR was carried out for Has1, Has2, and Has3, with GAPDH as an internal control, as described previously (17). Equal amounts of RNA from vector- (−) and emmprin-transfected (+) cells were used for each analysis.

Fig. 1. Elevated emmprin expression induces anchorage-independent growth. Anchorage-independent growth was measured by colony formation in soft agar (15). After 13–14 days of incubation, colonies ≥0.15 mm in size were counted under ×10 magnification using an ocular scale lens. A, MDA MB-436. 1, wild-type cells; 2, green fluorescent protein-transfected cells; 3, emmprin/green fluorescent protein-transfected cells. B, MCF-7. 1, wild-type cells; 2, vector-transfected cells; 3, emmprin-transfected cells. Results are expressed as the mean number of colonies per culture of triplicate measurements; bars, ±SD.
No effect on total Akt levels was observed (Fig. 4A). As noted previously (13), the level of phosphorylation of BAD at ser136 was low in MCF-7 cells. Consequently we also examined phosphorylation of extracellular signal-regulated kinase (Erk) and of BAD at ser112, the site of phosphorylation by Erk, because Erk-mediated phosphorylation of BAD also promotes cell survival (22). In both cases, stimulation due to increased emmprin expression and reversal by cotreatment with hyaluronan oligomers were clearly observed (Fig. 4B). Unlike MCF-7 cells, pBAD136 levels were high in MDA MB-436 cells and, as noted above, were inhibited by hyaluronan oligomers (Fig. 4A). Nevertheless, we also examined pErk levels in MDA MB-436 cells but found only modest stimulation as a result of increased emmprin expression (data not shown). Thus, emmprin stimulation of the Akt pathway is more prominent in MDA MB-436 cells, whereas stimulation of the Erk pathway is more prominent in MCF-7 cells, and both are reversed by hyaluronan oligomers. Finally, because anchorage-independent growth is associated with altered integrin signaling, we also examined the effect of emmprin and cotreatment with hyaluronan oligomers on phosphorylation of focal adhesion kinase (FAK), a central component in integrin-mediated cell survival signaling (23). As expected we found that emmprin stimulates FAK phosphorylation, and hyaluronan oligomers reverse this effect (Fig. 4C). No effects on total FAK expression were observed (data not shown).

Discussion

Numerous past studies have demonstrated a crucial role for emmprin in regulating expression of several MMPs (2). Emmprin acts in heterotypic cell interactions wherein tumor cell emmprin stimulates production of MMP-1, MMP-2, MMP-3, and the membrane-type MMPs by fibroblasts or endothelial cells (1, 5–7, 9, 24). These heterotypic interactions most likely explain the increased production of MMPs by stromal cells that is commonly observed within malignant tumors in vivo and that is important in some aspects of tumor malignancy (25). In addition, however, emmprin also causes increased expression of MMPs, especially MMP-2, in tumor cells themselves and consequently enhances their invasiveness (3, 6, 8, 9). The latter effect is most likely due to homophilic interaction between emmprin molecules on apposing cells (9). In this study we have presented evidence showing that elevated emmprin expression in tumor cells stimulates production of hyaluronan as well as MMPs in these cells, presumably by a similar homophilic emmprin-binding mechanism. Past studies have shown that heterotypic interactions between tumor cells and fibroblasts also enhance hyaluronan production by fibroblasts (26). It will be of interest to see whether emmprin is involved in the latter effect and whether the signaling mechanisms whereby emmprin stimulates hyaluronan production in homotypic and heterotypic systems are similar to those for MMPs.

A consequence of emmprin-induced stimulation of hyaluronan production in the less malignant human mammary carcinoma cells used herein is enhancement of their ability to grow in an anchorage-independent manner, one of the major hallmarks of transformed cells (18, 19). Our previous studies have demonstrated the importance of interactions between hyaluronan and tumor cell surface receptors, especially CD44, in regulating cell survival signaling (15–17, 21). Accordingly, we found that emmprin stimulates cell survival signaling in a hyaluronan-dependent fashion. Cell survival signaling via the PI3k and Erk pathways is critical to resistance of malignant tumor cells to apoptosis. In accord with this dependence on cell survival pathways, we found recently that emmprin enhances drug resistance and that this effect is dependent on hyaluronan (13). In addition it has been found that drug resistance in malignant cancer cells correlates with emmprin expression (8).

Our data suggest that the emmprin-induced increase in hyaluronan production is due to elevated expression of hyaluronan synthases. However, it is possible that other factors, such as decreased degradation, may contribute to the increase in hyaluronan. Future studies will focus on the precise mechanisms whereby emmprin stimulates hyaluronan synthesis expression and on whether emmprin also affects hyaluronan turnover. In preliminary studies (data not shown) we also examined the influence of emmprin on organization of hyaluronan at the cell surface.

![Fig. 3. Hyaluronan oligosaccharides inhibit emmprin-stimulated anchorage-independent growth. Anchorage-independent growth was measured as in Fig. 1.](image1)

![Fig. 4. Elevated emmprin expression stimulates cell survival pathways in a hyaluronan-dependent manner.](image2)

**A**

![Figure A](image3)

![Figure B](image4)

![Figure C](image5)
the cell surface. In several cell types, hyaluronan forms a cell-associated template for formation of a highly hydrated pericellular matrix (10). We found that increased emmprin causes enhancement of pericellular matrix assembly, but this phenomenon could only be observed at cell densities approaching confluence, not in sparse cultures. The relationship of this pericellular matrix to malignant cell properties clearly warrants further study.

It is apparent that emmprin is a major controlling factor of activities that induce malignancy in cancers, both with respect to tumor cell properties and to cell-stromal cell interactions. This conclusion is supported by the observation that experimental enhancement of emmprin expression in less malignant human breast cancer cells stimulates both growth and invasiveness in vivo (3).

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References

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