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Advances in Brief

EMMPRIN (CD147), an Inducer of Matrix Metalloproteinase Synthesis, Also Binds Interstitial Collagenase to the Tumor Cell Surface[1]

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Abstract

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells. In this study, we have found that EMMPRIN not only stimulates the production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface. Complex formation was demonstrated by phage display, affinity chromatography, and immunocytochemistry. Presentation of MMP-1 complexed to EMMPRIN at the tumor cell surface may be important in modifying the tumor cell pericellular matrix to promote invasion.

Introduction

MMPs[3] have been implicated in several aspects of tumor progression, including invasion through basement membranes and interstitial matrices, angiogenesis, and tumor cell growth (1–3). Strong support for the involvement of MMPs at some step in tumor progression comes from experiments in which tissue inhibitors of MMPs or synthetic inhibitors of metalloproteinases have been shown to reduce tumor growth and metastasis (4, 5). Over the past several years, it has become increasingly apparent that tumor cells create a pericellular environment in which MMPs and other proteases become concentrated, thus enhancing the ability of tumor cells to invade extracellular matrices (6–8). Previous studies from this laboratory have demonstrated that EMMPRIN, a member of the immunoglobulin superfamily that is enriched on the surface of most tumor cells, stimulates stromal cells to produce elevated levels of several MMPs, including MMP-1 (9–11). We have now found that tumor cell EMMPRIN not only stimulates MMP-1 production by fibroblasts but also binds MMP-1 to the surface of tumor cells, thus adding to the complement of proteases on the tumor cell surface that may promote invasion.

Materials and Methods

Phage Display Library. mRNA was prepared from human fibroblasts with the Oligotex mRNA kit (Qiagen, Valencia, CA) and used for cDNA synthesis with the Directional RH primer cDNA synthesis kit (Novagen, Madison, WI). After second-strand synthesis, the cDNA ends were flushed with T4 DNA polymerase and ligated to *Eco*RI/*Hin*dIII directional linkers. The cDNA was then digested with *Eco*RI and *Hin*dIII and ligated to T7Select1-1b vector arms (Novagen). The ligated DNA was packaged into bacteriophage T7 using the T7Select1-1 Packaging Extract (Novagen). The host strain of bacteria, BLT 5403 (Novagen), was then grown to $A_{600\ nm}$ = 0.8–1.0 and mixed with the packaged cDNA (at a ratio of 10^6 phage/10 ml cells) in LB media containing 50 μg/ml carbenicillin (Novagen). Molten top agarose at 45°C–50°C was added to the phage/host mixture (10:1) and immediately poured onto a 150-mm plate containing LB/carbenicillin medium. The plate was incubated at room temperature overnight until the plaques were nearly confluent. The phage was then eluted by covering the plate with phage extraction buffer [100 mM NaCl, 20 mM Tris, and 6 mM $MgSO_4$ (pH 8.0)] at 4°C overnight. The phage lysate was clarified with chloroform and subjected to screening by biopanning.

Screening of Phage Display Library. Twenty four-well cell culture plates were prepared for biopanning as suggested by the manufacturer (Novagen). The wells were coated with immunopurified EMMPRIN protein (Ref. 12; 1 μg/ml in Tris-buffered saline) at 4°C overnight and washed with Tris-buffered saline five times. Unreacted sites were blocked with 5% blocking reagent overnight at 4°C and washed. In the first round of screening, the phage lysate was applied to the EMMPRIN-coated plate (0.5 ml lysate/well) for 30 min at room temperature. The plate was then washed five times with Tris-buffered saline. The bound phages were eluted by adding 0.5 ml of elution buffer (1% SDS) at room temperature for 20 min. The eluted phages were then added to a culture of the host cells (BLT 5403) in LB media and incubated at 37°C with shaking for 3 h, at which time lysis was observed. The lysed culture was centrifuged, and the supernatant was collected for the next round of biopanning. A total of five rounds of screening was carried out. DNA from the phages isolated during the final round of screening was purified and sequenced using the T7 SelectUp primer (GGAGCTGTCGTATTCCAGTC) and the T7 SelectDown primer (AACCCTCAAGACCCGTTTA; Novagen).

Immunoaffinity and Ligand Affinity Chromatography. EMMPRIN was isolated from extracts of membranes from LX-1 human lung carcinoma cells by immunoaffinity chromatography using E11F4 monoclonal antibody against EMMPRIN immobilized on Sepharose beads, as described previously (12).

For manufacture of the ligand affinity medium, EMMPRIN protein (0.5 mg) was first dissolved in coupling buffer [0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3) containing 0.5% NP40]. The coupling solution was then mixed with CNBr-activated Sepharose 4B gel (Pierce; 0.25 g of dried powder swelled and washed in 1 mM HCl for 30 min) at 4°C. After overnight incubation, the gel was washed three times with 5 ml of coupling buffer, followed by incubation in 0.1 M Tris-HCl (pH 8) for 2 h to block any remaining active groups. Then the gel was washed using three cycles of 0.1 M acetate buffer, 0.5 M NaCl (pH 4), and 0.1 M Tris and 0.5 M NaCl (pH 8). After washing, the gel was resuspended in 5 ml of 10 mM Tris buffer (pH 8.3).

Extracts of human fibroblasts [10^8 cells in 5 ml of 10 mM Tris, 0.15 M NaCl, and 0.5% NP40 (pH 8.3)] were added to the EMMPRIN-coupled gel and incubated at 4°C overnight with rotation. The gel was then washed with 10 mM Tris and 0.15 M NaCl containing 30 mM octyl glucoside until the $A_{280\ nm}$ was less than 0.05. Binding proteins were eluted with 0.1 M glycine buffer (pH 2.5) containing 30 mM octyl glucoside. The eluate was neutralized to pH 7 by the addition of 1 M Tris (pH 9.5) and concentrated for further analysis.

ELISA of MMP-1. MMP-1 protein was measured in the eluates from EMMPRIN-Sepharose and in immunopurified EMMPRIN preparations using a commercial ELISA system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Briefly, 5 or 10 μl of eluate were added to microtiter plates coated with antibody to MMP-1 and incubated for 2 h at 25°C. The plates were washed with phosphate buffer and incubated with anti-MMP-1 antiserum for 2 h. After washing, the plates were incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for color development and measurement at $A_{450\ nm}$ in a microplate spectrophotometer. The concentration of MMP-1 in the eluate was estimated from a standard curve.

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[3] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; MMP-1, interstitial collagenase; MMP-2, gelatinase A; MT-MMP, membrane-type MMP; LB, Luria-Bertani.

SDS-PAGE, Silver Staining, and Western Blotting. Proteins were dissolved in SDS sample buffer containing 0.1 M DTT and heated at 95°C for 5 min. The samples were then subjected to electrophoresis on 10% SDS polyacrylamide gels. The gels were either stained using the Sterling silver staining system (National Diagnostics, Atlanta, CA) or electroblotted onto nitrocellulose membranes and incubated with antibody against EMMPRIN (E11F4; Ref. 12) or against MMP-1 (Calbiochem, La Jolla, CA) for 1 h at room temperature. The immunoreactive protein bands were detected with horseradish peroxidase-conjugated antimouse IgG and chemiluminescence reagent (New England Nuclear Life Science, Boston, MA).

Immunocytochemistry. LX-1 human lung carcinoma cells were seeded into chamber culture slides and cultured for 48 h at 37°C in 5% CO_2 air. The cells were then washed with PBS, fixed in 1% paraformaldehyde in PBS for 45 min at room temperature, quenched with 0.1 M Tris (pH 7.4), and blocked with 1% BSA, 1% goat serum, and 2% nonfat milk in PBS at room temperature for 1 h. The LX-1 cells were then incubated with monoclonal antibody against MMP-1 (Calbiochem) for 1 h at room temperature, followed by Cy3-conjugated Texas red goat antimouse IgG. The cells were washed with PBS, mounted with coverslips, and then observed and photographed using a Zeiss Axioskop-20 microscope.

Results

Phage Display Reveals MMP-1 as an EMMPRIN-binding Protein. We used the T7Select Phage Display System (Novagen) to identify EMMPRIN-binding protein(s) encoded by a cDNA library prepared from human fibroblasts, as described in "Materials and Methods." In this method, each phage becomes coated with a fusion protein comprised of the phage coat protein and a protein generated from the cDNA library used. Phages coated with putative EMMPRIN-binding protein were selected by repeated panning over 24-well plates coated with EMMPRIN. Five rounds of biopanning were carried out, and the final lysate was used for plaque assay, PCR amplification, and sequencing.

Eight clones were obtained from the procedure described above. All eight of the inserts were of identical size, *i.e.*, 0.8 kb, and were found to have identical sequences corresponding exactly to a portion of the human MMP-1 sequence (Fig. 1).

MMP-1 Binds to EMMPRIN-Sepharose. To confirm the binding of EMMPRIN to fibroblast-produced MMP-1, we performed ligand chromatography over Sepharose conjugated with immunopurified EMMPRIN. Fibroblast extracts were mixed with the EMMPRIN-Sepharose, which was then washed and eluted as described in "Materials and Methods." The eluates were subjected to SDS-PAGE, followed by silver staining. On silver staining, a prominent protein band at $\sim M_r$ 55,000 was observed, as well as a weaker band at $\sim M_r$ 67,000 (Fig. 2*A*); in some cases a $\sim M_r$ 45,000 band could also be seen.

Western blots were also performed on the eluates from EMMPRIN-Sepharose using antibody against human MMP-1. The protein band at $\sim M_r$ 55,000 (the approximate size of pro-MMP-1, which is M_r 52,000) reacted with anti-MMP-1 antibody (Fig. 2*B*), confirming our results from the phage display. ELISA measurements also revealed MMP-1 in the eluates from EMMPRIN-Sepharose (data not shown). The identities of the $\sim M_r$ 67,000 and $\sim M_r$ 45,000 proteins are not yet known.

EMMPRIN Forms a Complex with MMP-1 on the Surface of Tumor Cells. Some tumor cells themselves produce small amounts of MMP-1. Thus, we also determined whether, in addition to binding isolated EMMPRIN protein, MMP-1 forms a complex with EMMPRIN present on the surface of LX-1 human lung carcinoma cells. We immunopurified EMMPRIN from extracts of LX-1 cell membranes using monoclonal antibody E11F4 covalently bound to Sepharose beads and tested whether MMP-1 was present in the eluted EMMPRIN preparation. Fig. 3 shows a Western blot of such an

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                1                                                    55
5′ sequence:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                941                                                  995

                56                                                   110
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                996                                                  1050

                111                                                  165
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                1051                                                 1105

                1                                                    55
3′ sequence:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                343                                                  397

                56                                                   110
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                398                                                  452

                111                 131
                TGTGGACCATGCCATTGAGAA
                |||||||||||||||||||||
                TGTGGACCATGCCATTGAGAA
                453                 473
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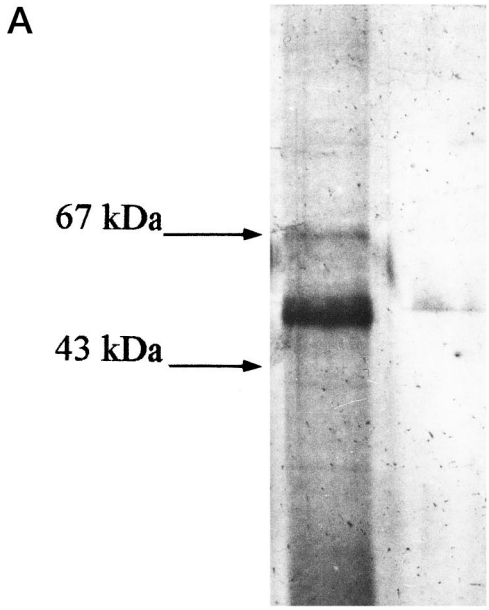
Fig. 1. Comparison of partial sequences of cDNA isolated by phage display with that of human MMP-1 cDNA. The 5′ and 3′ sequences of one of the partial cDNAs obtained are given. Eight cDNA clones were isolated after biopanning of phages on EMMPRIN; all eight clones had identical sequences.



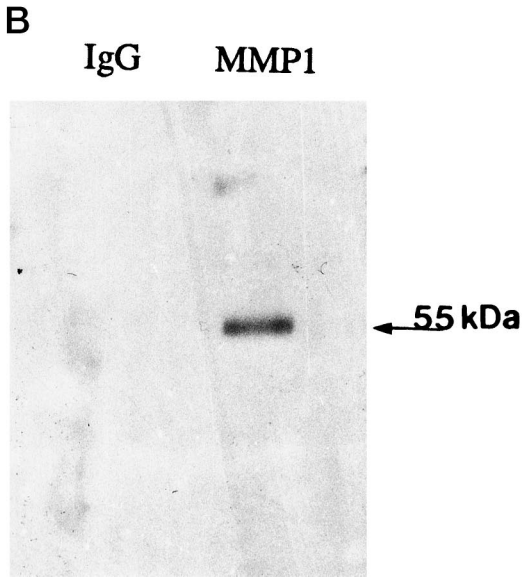


Fig. 2. EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. *A*, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at ~M_r 55,000 and ~M_r 67,000) were detected. *Arrows* indicate positions of the M_r 43,000 and M_r 67,000 markers. *B*, parallel gels to those in *A* were transblotted and reacted with antibody to MMP-1 or secondary antibody only (*IgG*); the ~M_r 55,000 band reacted with anti-MMP-1.

EMMPRIN preparation with antibody against MMP-1. A strong band at ~M_r 55,000, corresponding approximately in size to pro-MMP-1, reacted with the antibody, indicating the presence of MMP-1 in the EMMPRIN preparation. A weaker band at ~M_r 45,000, which is not seen consistently, is most likely activated MMP-1 (M_r 42,000).

Quantitation of the MMP-1 content by ELISA gave 2.1 μg of MMP-1 per 5 μg of total protein in the EMMPRIN preparation. Because EMMPRIN and pro-MMP-1 have molecular weights of ~58,000 and 52,000, respectively, this result suggests that EMMPRIN and MMP-1 are complexed in an equimolar ratio.

The presence of MMP-1 at the surface of LX-1 human lung carcinoma cells was confirmed by immunocytochemistry using antibody against MMP-1 (Fig. 4).

Discussion

Many recent studies have highlighted the importance of the pericellular milieu surrounding tumor cells in their proliferative and invasive behavior (6–8). This milieu is modified by a number of proteases, especially MMPs and tissue serine proteases, many of which are produced by tumor-associated stromal cells rather than tumor cells themselves (13, 14) and subsequently become concentrated at the tumor cell surface via interaction with specific binding

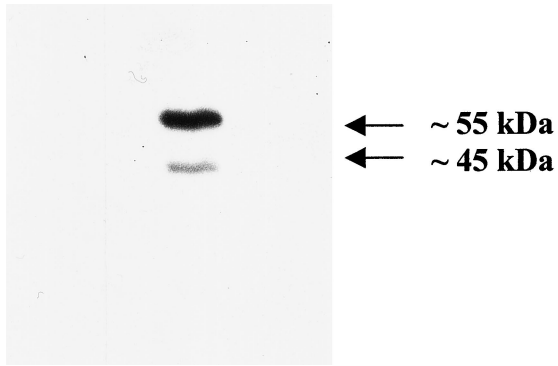


Fig. 3. Western blot of immunopurified EMMPRIN with antibody to MMP-1.

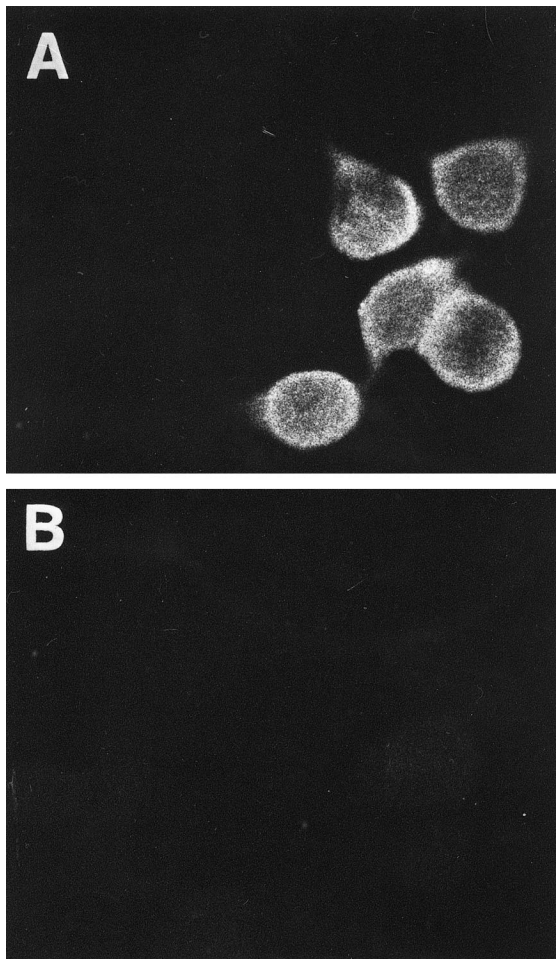


Fig. 4. Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. *A*, cells stained with antibody against MMP-1. *B*, cells stained with secondary antibody only.

sites. For example, MMP-2 binds to the tumor cell surface via a tissue inhibitor of MMPs-2-MT-MMP complex (15, 16). MMP-2 is activated by the MT-MMP, and the complex is targeted to invasive domains of the tumor cell membrane (sometimes termed "invadopodia") via specific docking of MT-MMP at these sites (17). Although MT-MMPs activate soluble MMP-2 as well as plasma membrane-retained MMP-2, membrane-bound enzyme is required for tumor cell invasion (17). A similar mechanism of activation and retention at the cell surface has been described for collagenase 3 (18). Other cell surface binding sites have been described for gelatinase B, *i.e.*, CD44 (19) and the α_2(IV) chain of collagen (20), and for MMP-2, *i.e.*, $\alpha_V\beta_3$ integrin (21). These sites also appear to be important in tumor cell invasion.

Evidence for association of MMP-1 with the surface of a human pancreatic carcinoma cell line has been published previously (22), but the mechanism whereby MMP-1 binds to these cells has not been described. In the present study, we show that MMP-1 binds to EMMPRIN, a tumor cell surface glycoprotein previously shown to induce synthesis of MMP-1 and other MMPs by fibroblasts (9–11) and endothelial cells.[4] We have also shown that an EMMPRIN-MMP-1 complex can be isolated from LX-1 human lung carcinoma cell membranes and that MMP-1 is present on the LX-1 cell surface. A preliminary report has been published suggesting that EMMPRIN becomes localized to invadopodia in human breast carcinoma cells (23). Tumor cell surface EMMPRIN may then be responsible for targeting MMP-1 to invadopodia, thus adding MMP-1 to the impressive list of proteases associated with these invasive structures (6, 17). Although other proteases have been shown to be important in tumor growth and invasion under a variety of conditions, it is likely that MMP-1 is crucial for penetration of fibrous tissues because of its ability to degrade fibrillar collagen as shown, for example, in endothelial cell invasion (24) and tumor cell invasion (25) of collagen gels. Thus localization of MMP-1 on the tumor cell surface via interaction with EMMPRIN would facilitate these invasive processes.

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[4] Unpublished observations.