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FOREWORD

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Sam Poole 5/24/00
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INTRODUCTION

Crucial steps in tumor progression and the process of metastasis, e.g. tumor growth, invasion through extracellular matrices and angiogenesis, involve proteolytic modification of the pericellular matrix surrounding tumor cells. A major class of proteases involved in these processes is the matrix metalloproteinases (MMPs), and inhibition of MMPs prevents progression and metastasis of several tumor types, including human breast carcinomas, in animal models. In vivo, tumor MMPs are usually produced by stromal cells associated with tumors rather than the tumor cells themselves. The tumor cell surface glycoprotein, EMMPRIN, stimulates MMP production by fibroblasts and endothelial cells, and may be an important regulator of MMP production during tumorigenesis in vivo. However no direct evidence for an important role in tumor progression has been published. The focus of this proposal will be to demonstrate directly whether or not EMMPRIN promotes breast cancer progression and whether a role for EMMPRIN in tumor progression may be to promote or induce angiogenesis. This study should determine definitively whether EMMPRIN-mediated regulation of MMPs may constitute a newly discovered step in breast carcinoma progression and metastasis. Interference with EMMPRIN action may then be an effective way to retard breast carcinoma progression in patients.

BODY

Task 1: To document that increased expression of EMMPRIN (extracellular matrix metalloproteinase inducer) in non-aggressive human breast carcinoma cells leads to increased tumor growth, blood vessel formation and metastasis.

As proposed in our original Statement of Work, we have tested whether tumor growth and metastasis are affected when EMMPRIN expression is increased in non-aggressive MDA-MB436 human breast carcinoma cells. In previous studies we demonstrated that EMMPRIN stimulates production of matrix metalloproteinase (MMP) production in fibroblasts, possibly explaining the finding that most MMPs are produced by tumor stromal fibroblasts rather than by tumor cells themselves. We have now shown that EMMPRIN promotes tumor progression in vivo. Human MDA-MB-436 breast cancer cells, which are slow growing in vivo, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female nu/nu mice. Green fluorescent protein (GFP) was used to visualize metastases. Breast cancer cell clones transfected with EMMPRIN/GFP cDNA were considerably more tumorigenic and invasive than GFP- or plasmid-transfected cancer cells. Increased MMP expression was also demonstrated in EMMPRIN-enhanced tumors. Details of these experiments are given in a manuscript submitted for publication and attached in Appendix 2.

Further extension of this work, as outlined in the original Statement of Work will be performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work has been approved as of March 23, 2000 (see Appendix 1).

Task 2: To document whether inhibition of EMMPRIN expression in malignant human and murine mammary carcinoma cells blocks tumor growth and/or metastasis in vivo.

As proposed, antisense cDNA and ribozyme constructs were produced for mouse and human EMMPRIN, as well as sense controls. Stable transfectants of murine TA3/St mammary carcinoma cells (an aggressive cancer cell line) were produced using these constructs. Unfortunately, complete inhibition of EMMPRIN expression has not yet been obtained. Clones were isolated that exhibited partially inhibited expression and these were used to determine whether metastasis to the lung after intravenous injection into the tail of syngeneic mice was affected. These experiments have not yet been effective. Continuation of this work will be performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work has been approved as of March 23, 2000 (see Appendix 1).

As an alternative approach to the above, we have also attempted to map the active site of the EMMPRIN molecule by testing the effect on MMP production of synthetic peptides with sequences from within the extracellular domain of the EMMPRIN molecule. The aim of this approach was to determine whether synthetic peptides from the active site would antagonize or mimic the action of EMMPRIN. Recent results suggest strongly that we have obtained an inhibitory peptide. However this work is preliminary and forms the basis of Task 1 in our newly approved Statement of Work (Appendix 1).

We have also begun to produce recombinant adenoviruses driving expression of EMMPRIN and mutated forms of EMMPRIN to further map the active site of EMMPRIN. This work has provided the basis for Task 2 of the new Statement of Work (Appendix 1), i.e. use of recombinant adenovirus technology to deliver the above inhibitory peptide *in vivo*.

Task 3: To test whether EMMPRIN stimulates endothelial morphogenesis in a 3-dimensional collagenous matrix in culture.

This objective has only recently begun. So far we have set up the methods and have performed initial experiments that support our proposal. In these experiments, human umbilical vein endothelial cells were cultured on type I collagen gels, then treated with either bFGF, a known angiogenic agent, or purified EMMPRIN. This method is a standard technique that mimics aspects of angiogenesis, i.e. invasion of a three-dimensional matrix and formation of capillary-like tubules. In our experiments we have observed that 1 μ g of EMMPRIN duplicates the effect of 5 ng of bFGF in its ability to initiate capillary-like tubule formation. We believe that the relatively high amount of EMMPRIN required is due to inactivation of most of the protein during purification. For this reason and since EMMPRIN has now been shown to bind collagenase (MMP-1) and to be present in some EMMPRIN preparations (see Appendix 3), we have switched to a new technique for studying this phenomenon.

We have found that infection of fibroblasts with recombinant EMMPRIN adenovirus is a very effective means to stimulate MMP production, due to mutual interaction between neighboring cells expressing both EMMPRIN and EMMPRIN receptor. We are now using this approach with human endothelial cells. That is, we infect endothelial cells with the adenovirus, then monitor expression of EMMPRIN and MMPs. We will determine the effect of the adenoviral-delivered EMMPRIN on capillary tubule formation. This approach will now be supported by new funds from the National Cancer Institute (CA79866). See newly approved Statement of Work (Appendix 1).

KEY RESEARCH ACCOMPLISHMENTS

- 1) Demonstration that increased expression of EMMPRIN leads to increased tumor growth and invasion in vivo
- 2) Development of an efficient recombinant adenovirus-based system for testing the cellular effects of EMMPRIN and EMMPRIN mutants
- 3) Tentative identification of the "active site" for EMMPRIN action
- 4) Demonstration of specific binding of EMMPRIN to collagenase (MMP-1)

REPORTABLE OUTCOMES

- 1) Publication:
Guo H, Li R, Zucker S, Toole BP: EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. *Cancer Res.* **60**, 888-891 (2000).
- 2) Submitted manuscript:
Zucker S, Hymowitz M, Rollo EE, Mann R, Connor CE, Cao J, Foda HD, Tompkins DC, Toole BP: Tumorigenic potential of extracellular matrix metalloproteinase inducer (EMMPRIN)
- 3) Presentation:
FASEB conference symposium on Misregulation of the Basement Membrane in Human Disease: "Regulation of tumor progression by EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases" by Bryan Toole
- 4) New funding;
National Cancer Institute (CA 79866): "Tumor cell-stromal interactions in cancer"

CONCLUSIONS

We conclude from the above work that EMMPRIN promotes mammary carcinoma progression via stimulation of MMP production in tumor stromal cells. Our preliminary data suggest that a mechanism whereby EMMPRIN may act is via stimulation of angiogenesis and also, possibly, by presentation of interstitial collagenase on the tumor cell surface. Our recent data suggest that EMMPRIN-derived peptides may be efficacious as inhibitors of EMMPRIN action and therefore may be useful therapeutically.

REFERENCES: None

APPENDIX 1

Revised Statement of Work (DAMD17-99-9413) - approved March 23, 2000

Title: Role of EMMPRIN in Tumor Progression

P.I.: Bryan P. Toole

In work supported by DAMD17-99-9413, a peptide with amino acid sequence identical to part of the outer immunoglobulin domain of EMMPRIN has been shown to inhibit stimulation of matrix metalloproteinase production by EMMPRIN in cell culture. Also, we have shown that relatively benign MDA-MB436 human mammary carcinoma cells express low levels of EMMPRIN, and that stable transfection of MDA-MBA436 cells with cDNA for EMMPRIN leads to expression of high levels of EMMPRIN; these transfectants grow much more aggressively *in vivo* than control MBA436 cells, have become invasive, and give rise to high mortality rates (S.Zucker, M.Hymowitz, E.Rollo, R.Mann, C.Conner, J.Cao, H.Foda, D.Tompkins, B.Toole: submitted for publication; **Appendix 2 of this report**).

Task 1: To test the effects of a peptide antagonist of EMMPRIN action for its potential inhibitory effect on tumor growth and invasion in animal tumor models.

- set up two animal tumor models to test effect of peptide antagonist: a) MDA-MB231 metastatic human mammary carcinoma cells; b) EMMPRIN transfectants of benign MDA-MB436 cells (see above). The tumor cells will be implanted in mammary fat pads of nude mice and monitored for tumor growth and invasion, as described in original application.

- use mini-osmotic pumps to deliver peptide antagonist or control peptide (scrambled antagonist sequence) to area of mammary carcinoma growth, using methods previously employed (C.Zeng, B.Toole, S.Kinney, J.Kuo, I.Stamenkovic, *Int. J. Cancer* 77:396-401, 1998).

Task 2: To explore use of recombinant adenoviral constructs for efficient delivery of the peptide antagonist and to determine effects on tumor growth and metastasis. We will explore this avenue in case delivery by mini-osmotic pump as proposed above does not provide sufficient concentration of peptide to be maximally effective.

- design and construct recombinant adenoviruses containing cDNA for peptide antagonist fused to carrier protein prepro-region (to ensure appropriate processing and secretion as active peptide), using methods already developed for construction of wild type and mutated EMMPRIN adenoviruses in work supported by DAMD17-99-9413.

- test efficacy of recombinant adenoviral constructs in driving expression and secretion of active peptide in cultured target cells.

- test recombinant adenovirus in tumor models above, using *ex vivo* infection in culture prior to implantation or *in situ* injection of virus into tumor *in vivo*.

- explore the effect of co-injection of recombinant virus on formation of metastatic nodules in the lung on intravenous injection of tumor cells.

APPENDIX 2

Tumorigenic Potential of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)¹

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Running Title: EMMPRIN is tumorigenic

Key Words: matrix metalloproteinases (MMPs); cancer invasion; metastasis

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Abbreviations: ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer; GFP, green fluorescent protein; MMP, matrix metalloproteinase.

ABSTRACT

EMMPRIN, a glycoprotein present on the cancer cell plasma membrane, enhances fibroblast synthesis of matrix metalloproteinases (MMPs). In this report we have demonstrated a role for EMMPRIN in cancer progression. Human MDA-MB-436 breast cancer cells, which are tumorigenic but slow growing *in vivo*, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female nu/nu mice. Green fluorescent protein (GFP) was utilized to visualize metastases. Breast cancer cell clones transfected with EMMPRIN/GFP cDNA were considerably more tumorigenic and invasive than GFP- or plasmid- transfected cancer cells. Increased MMP expression was demonstrated in EMMPRIN enhanced tumors.

INTRODUCTION

Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) was originally designated Tumor Collagenase Stimulating Factor (TCSF) by Biswas et al. following isolation and purification of the 58 kDa glycoprotein from the plasma membrane of cancer cells and demonstration of its function in stimulating fibroblast synthesis of interstitial collagenase (MMP-1) (1). The subsequent finding that EMMPRIN also induced fibroblast synthesis of gelatinase A (MMP-2) and stromelysin-1 (MMP-3) indicated a more general effect on production of MMPs (2). Recent studies have documented the capacity of recombinant EMMPRIN or EMMPRIN purified from cancer cells to stimulate fibroblast production/secretion of stromelysin-1, interstitial collagenase, and gelatinase A in vitro (2), (3). The demonstration by in situ hybridization (mRNA localization) that peritumoral fibroblasts synthesize most of the MMPs (collagenases, gelatinases, stromelysins, MT-MMPs) in human tumors rather than the cancer cells themselves has ignited interest in the role of EMMPRIN in tumor dissemination (4), (5). The association of intense EMMPRIN expression in neoplastic cells within invasive human tumors (6) further supports a role for EMMPRIN in cancer dissemination. These data are consistent with a central function for EMMPRIN in stimulating stromal cell production of MMPs which, following pericellular activation, directly degrade extracellular matrix (ECM) (1).

In the current study we have examined the function of EMMPRIN in a cancer model in immunodeficient mice. Human MDA-MB-436 breast cancer cells which are tumorigenic, estrogen independent, moderately invasive in vitro, but slow growing in vivo (7), were transfected with EMMPRIN cDNA and injected orthotopically into the mammary fat pad of nude mice. We took advantage of the observation that the 29 kDa green fluorescent protein (GFP) of the jelly fish *Aequoria victoria* retains its fluorescent properties when recombinantly expressed in eukaryotic cells (8) along with EMMPRIN cDNA and can be used as a powerful marker for gene expression and cancer dissemination in vivo. Cancer cells transfected with both EMMPRIN cDNA and GFP cDNA were compared to cancer cells transfected with GFP cDNA alone for tumorigenic behavior.

MATERIALS AND METHODS

Reagents

Restriction enzymes were purchased from Stratagene (La Jolla, CA). EMMPRIN was purified from LX-1 lung cancer cells using affinity column chromatography (9). Monoclonal antibodies to EMMPRIN (clone 1G6.2) were produced in collaboration with Dr. Dembro at Chemicon, International, Inc. (Temecula, CA).

Cell lines and Culture Conditions

Human MDA-MB-436 breast cancer cells were maintained in Richter's Improved Minimal Essential Medium supplemented with 10% donor calf serum (7). Immunostaining of MDA-MB-436 cells was performed using a primary mouse monoclonal antibody to EMMPRIN (1G6.2) and a secondary goat anti-mouse IgG (H&L) horseradish peroxidase labeled antibody.

Construction of Plasmids and Transfection into Cells

A 1.6 kb cDNA (1), representing the entire EMMPRIN sequence encoding 269 amino acid residues, was placed at an EcoR I site under the control of the CMV promoter in pcDNA3 (Invitrogen, Carlsbad, CA). To facilitate identification of transfected cells in vitro and metastases in vivo, green fluorescent protein (GFPmut1 variant) cDNA (Clontec Lab, Inc., Palo Alto, CA) was inserted into the EMMPRIN-containing plasmid. The GFP cDNA along with a separate upstream CMV promoter from pEGFP-C1 plasmid (Clontec Lab, Palo Alto, CA) was inserted into the EMMPRIN expression vector between Not I and Xho I sites as shown in Figure 1A. An additional polyadenylation (PA) signal from pSG5 (Stratagene) was placed downstream of the EMMPRIN gene to provide balanced expression of both recombinant genes under control of CMV promoters. The resulting plasmid was named EMMPRIN/GFP. As a control plasmid, GFP cDNA alone was subcloned into pcDNA3 without EMMPRIN cDNA. In experiment 2, EMMPRIN cDNA was subcloned into pcDNA3 without GFP; the control plasmid was pcDNA3 alone.

The human MDA-MB-436 breast cancer cell line was stably transfected using the calcium phosphate precipitation method (10). Selected G418-resistant clones were screened by fluorescent appearance (Figure 2A) using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set. Fluorescent positive clones were further analyzed by Northern blot analysis probed with an EMMPRIN cDNA fragment.

RNA Isolation and Northern Blot Hybridization

Total RNA was extracted from MDA-MB-436 cells stably transfected with desired plasmids by guanidine solubilization, phenol/chloroform extraction, and serial precipitation (1), (11). Approximately 20 μ g of total RNA was resolved by denaturing gel electrophoresis followed by Northern transfer to nylon membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized to 32 P-radiolabeled EMMPRIN cDNA (1.7 Kb) at 42°C as described (11) and analyzed after overnight exposure with an intensity screen at -80°C. The amount of the samples applied to the lanes was normalized by β -actin RNA.

Labeling of RNA Probes

Antisense and sense digoxigenin (DIG)-labeled RNA probes for human EMMPRIN and mouse gelatinase A, and gelatinase B were synthesized by reverse transcribing 1 μ g of cDNA from a PCR reaction that had used gene-specific primers that contain the T7 or T3 phage promoter sequence followed by 20-25 bases of the mRNA sequence (12). In vitro transcription of the amplified DNA template was performed using the digoxigenin RNA labeling kit (Boehringer-Mannheim). Labeled probes were purified and sequences were verified.

In situ hybridization

Serial sections of paraffin-embedded mouse tumors were prepared for in situ hybridization according to the method of Komminoth (13). Slides were processed for immunodetection employing anti-DIG alkaline phosphatase antibody and then incubated with substrate solution (Boehringer-Mannheim Wash and Block Set).

Cell Proliferation In Vitro

Cell proliferation assays were performed by plating MDA-MB-436 cells at 4×10^4 cells per well (Costar, Corning, NY) and then switched to serum-free media. After 48 hours, serum-enriched media was added back and cells were cultivated for 4 additional days. Cell counts were performed daily.

Tumor Formation in Mice and Preparation of Tissue Extracts

Four week old female athymic NCr nu/nu mice were obtained from Taconic Farms (Germantown, NY). Cancer cells (1×10^6) were injected into the mammary fat pad of nude mice. Tumor growth was monitored weekly. Tumor volume was calculated using the formula: $(\text{length})(\text{width})^2/2$. Tissues from autopsies were prepared for histology and stored in liquid nitrogen. The extraction procedure for tumor tissue, involved detergent and heat-extraction steps (14).

Immunoassays, Immunoblotting, Gelatin Substrate Zymography, and Protein Studies

Primary cell cultures were transferred to serum-free media for 2 days; spent media was collected and tested by gelatin zymography. Gelatinase A, stromelysin-1, and gelatinase B were measured using sandwich ELISA formats as we have described in detail previously (15). Gelatin substrate zymography was performed in 10% polyacrylamide gels that had been cast in the presence of 0.1% gelatin (NOVEX, San Diego, CA) (15), (16). Protein determinations were made using the bicinchoninic acid reagent (Pierce, Rockford, IL).

Analysis of variance and Student's t-test were employed to compare differences between groups in various experiments; $p < 0.05$ was considered significant. Survival experiences between groups were compared by the Wilcoxon chi-square test.

RESULTS

Cell Transfection and Proliferation

Northern blot analysis using EMMPRIN cDNA as a probe detected 20 fold enhanced EMMPRIN expression by EMMPRIN-transfected cells as compared to GFP or non-transfected cells (Figure 1B). Immunostaining of MDA-MB-436 cells using specific mouse monoclonal antibodies to EMMPRIN documented intense staining of EMMPRIN/GFP transfected cells and infrequent weak staining of GFP-transfected or vector transfected cells (data not shown).

There were no significant differences in cell doubling times between GFP and EMMPRIN/GFP cDNA transfected cells (~18 hours) in media with or without serum. This data is inconsistent with EMMPRIN acting as an autocrine growth factor for tumor cells in vitro.

Tumor Growth in Nude Mice

Three independent experiments, each employing a different clone of EMMPRIN-transfected MDA-MB-436 cells, were performed. In experiments 1 and 2, the GFP-alone or vector transfected clones did not form palpable tumors by the time of the experiment's termination at 12 weeks; however, ~.01 cm³ non-invasive tumors were identified at autopsy in 18/18 mice. In contrast, the EMMPRIN/GFP transfected clones formed palpable breast tumors at the site of mammary injection by week 6 in 18/18 mice which grew progressively to >1.7 cm³ in diameter by week 12 at which time the animals were sacrificed. Histologic examination revealed local cancer invasion, but no metastases. EMMPRIN/GFP and GFP expressing tumors expressed green fluorescence when examined grossly with a fluorescent light.

Experiment 3: Groups of 10 mice were injected with transfected MDA-MB-436 cells into mammary tissue. The tumors emanating from the EMMPRIN/GFP cDNA transfected MDA-MB-436 cells grew relatively rapidly and all mice expired or had to be sacrificed within 12 weeks (Figure 2B and 3A); extensive metastases to the liver, lung, pleura, spleen, lymph nodes, and mesentery were present in 3/10 mice. In contrast, injection of the GFP cDNA transfected tumor cells into mice resulted in tumors that grew much more. Tumor diameter was <0.3 cm³

and no metastases were noted at week 15. One mouse in the GFP-transfected group developed a 1.4 cm³ primary tumor by week 12.

Gelatinolytic Activity Extracted from Tumor Tissues and Cells

Gelatin zymograms of conditioned media from cultivated MDA-MB-436 cells (Figure 3B- left panel) revealed that cells transfected with GFP or EMMPRIN/GFP cDNA secreted equivalent amounts of 72 kDa gelatinase A; gelatinase B was not detected. Tumor extracts from EMMPRIN/GFP injected mice displayed intense gelatinolytic bands localized at 105 kDa, 92 kDa, 72 kDa, and 64-62 kDa (Figure 3B- right panel). The 105 kDa band is consistent with mouse latent gelatinase B; human latent gelatinase B migrates at 92 kDa (17). The 72 kDa and 62 kDa gelatinolytic bands could represent human or mouse latent and activated gelatinase A, respectively. Tumor extracts from GFP alone-injected mice revealed weaker gelatinolytic bands than EMMPRIN/GFP injected mice.

Histochemistry/In situ Hybridization

Hematoxylin and eosin staining of resected breast masses revealed extensive replacement of normal mammary tissue with carcinoma in tumors originating from mice injected with EMMPRIN/GFP or GFP transfected MDA-MB-436 cells. In situ hybridization of tumor tissue from mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining with EMMPRIN in cancer cells; surrounding benign appearing mammary ducts also expressed EMMPRIN (Figure 4). Gelatinase A mRNA was found in both cancer cells and the surrounding non malignant tissue (muscle, fat, and benign-appearing mammary ducts). There was specific staining with the gelatinase B antisense riboprobe in cancer cells, but not as widely distributed as gelatinase A; intense periductal staining was noted in surrounding normal tissue. Similar results were found on examination of metastatic tumors in the EMMPRIN/GFP treated mice. Specific staining was abolished by pretreatment of tissues with RNAase (data not shown). In the GFP alone-transfected tumors, virtually no EMMPRIN or gelatinase A staining was seen.

Focal staining for gelatinase B was noted in GFP tumor tissue, not in surrounding normal tissue. No staining was detected in any of the tumor tissues that were hybridized with EMMPRIN, gelatinase A, or gelatinase B sense probes (data not shown).

DISCUSSION

The current report describes a direct effect of EMMPRIN expression on tumorigenicity in an animal model. Transfection of EMMPRIN cDNA or EMMPRIN/GFP cDNA into human breast cancer cells resulted in marked enhancement of tumor growth in nude mice after orthotopic injection of tumor cells. High levels of EMMPRIN, gelatinase A, and gelatinase B mRNA expression within tumors was documented by in situ hybridization. Enhanced gelatinase B and gelatinase A were identified in zymograms from extracts of EMMPRIN/GFP-transfected tumors as compared to GFP tumors. It is noteworthy that MDA-MB-436 cells propagated in vitro produce gelatinase A, but not gelatinase B, whereas extracts of tumors in nude mice have higher levels of gelatinase B than gelatinase A. An association between expression of EMMPRIN and gelatinase B in benign and malignant pigment cell skin lesions has been reported (18).

Metastasis following orthotopic injection of tumor cells into nude mice was greater with EMMPRIN/GFP-transfected than with GFP-transfected cells, but the overall rate was low. EMMPRIN expression did not affect tumor cell proliferation in vitro. Based on the established role of EMMPRIN in enhancing MMP synthesis by stromal fibroblasts, it would appear that increased degradation of extracellular matrix permits more rapid tumor growth in vivo. The higher rate of tumor growth with EMMPRIN-transfected cancer cells and the associated matrix degradation may also occur by favored neoplastic cell survival in a tissue stroma environment initially not permissive for tumor growth. Enhanced extracellular matrix degradation may also release growth factor-like fragments of matrix components, resulting in an indirect effect on cell proliferation (19). These studies with EMMPRIN reinforce the notion that cancer dissemination is a multistep process and that protein degradation contributes to the process but is insufficient in itself for tumor metastasis (20).

ACKNOWLEDGMENTS

This article is dedicated to the memory of our friend and colleague, Chitra Biswas, whose career was dedicated to the discovery and exploration of EMMPRIN. Dr. Biswas died in August 1993, but her inspiration continues to guide us in our studies of EMMPRIN.

We thank Dr. Serge Lyubsky for his contribution to the histopathologic studies.

FIGURE LEGENDS

Figure 1A. Schematic illustration of the EMMPRIN/GFP plasmid. A 1.6 kb cDNA representing the entire EMMPRIN sequence was placed at an EcoR 1 site under the control of the CMV promoter in pcDNA 3. GFP cDNA was inserted along with an upstream CMV promoter into the EMMPRIN expression vector between Not I and Xho I sites. A polyadenylation (PA) signal was placed downstream.

Figure 1B. Northern blot analysis of EMMPRIN. ~20 µg of total cellular RNA from plasmid alone-transfected, GFP-transfected, and EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells was size fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 1.7 kb of ³²P-radiolabeled EMMPRIN cDNA as a probe. Blots were analyzed by autoradiography. A single 1.7 kb mRNA transcript corresponding to the known EMMPRIN band was detected at ~20 X greater intensity in EMMPRIN/GFP transfected cells as compared to plasmid alone or GFP transfected cells.

Figure 2A. Identification of green fluorescence in MDA-MB-436 cells. Left panel displays GFP-transfected cells. Middle panel displays EMMPRIN/GFP transfected cells. Right panel displays the expression of EMMPRIN-GFP as a fusion molecule (cDNA controlled by a single CMV promoter) in MDA-MB-436 cells. Fluorescence in the left and middle panels identifies GFP in the cell cytoplasm. GFP fluorescence in right panel identifies the EMMPRIN-GFP fusion protein localized to cell organelles and plasma membranes.

Figure 2B. GFP-transfected tumors are readily visible under fluorescent light. EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells were injected into a NCr nu/nu mouse. Eight weeks later, extensive green colored metastatic tumors (identified with arrows) in the pleura, peritoneum, lymph nodes, liver, and spleen are visible under fluorescent light (left photo). The middle photo demonstrates the same mouse at autopsy illuminated with bright light.

The right photo demonstrates typical tumor size of mice sacrificed at week 15. Tumors from mice injected with EMMPRIN/GFP transfected MDA-MB-436 cells are considerably larger and show prominent blood vessels on the tumor surface as compared to mice injected with GFP-transfected cells. Arrow heads point to tumors.

Figure 3A. MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA resulted in enhanced rate of tumor growth after tumor cell implantation into the mammary fat pad of nude mice as compared to GFP-transfected cells. The data represents the mean \pm standard error observed in 10 animals in each group. The numbers associated with each symbol refer to the number of mice alive at each time point.

Figure 3B. Comparison of gelatinases produced by MDA-MB-436 cells cultivated in serum-free media and extracts of nude mouse tumors. Spent conditioned media from primary cells cultivated in vitro (left panel) and tumor cell extracts (right panel) were assessed by gelatin substrate zymography. Protein concentration of samples were equalized within each group. Tumor extracts from the EMMPRIN/GFP group had more gelatinolytic activity than GFP-alone (displayed extract from GFP-alone tumor is from the largest tumor in that category).

Figure 4. In situ hybridization of primary tumors from mice injected with EMMPRIN/GFP and GFP-transfected MDA-MB-436 breast cancer cells. Serial sections from tumor tissue (panels 1-4, 9-12) and surrounding non malignant tissue (panels 5-8 and 13-16) were examined. Panels 1 and 9 represent hematoxylin and eosin staining of cancer tissues from EMMPRIN/GFP and GFP tumors, respectively; panels 5 and 13 represent H & E staining of non malignant tissues from EMMPRIN/GFP and GFP tumors, respectively. Cells in the primary tumor mass from mice injected with EMMPRIN/GFP transfected cells revealed widely distributed, specific staining with EMMPRIN, gelatinase A (GLA), and gelatinase B (GLB) antisense riboprobes (panels 2-4). Minimal cell staining was seen in cancer cells from GFP-transfected MDA-MB-436 cells for

EMMPRIN and gelatinase A (panels 10, 11), focal gelatinase B was identified in GFP tumors (panel 12). Non malignant tissues adjacent to the primary tumors from EMMPRIN/GFP mice demonstrated staining for EMMPRIN, gelatinase A, and gelatinase B in mammary ducts (D) and myocytes (M) (panels 6-8). Non malignant tissue from GFP mice revealed no staining for EMMPRIN, gelatinase A, or gelatinase B (panels 14, 15, 16).

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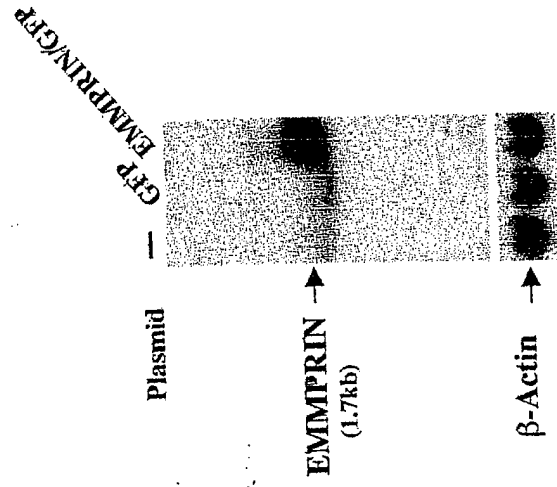


Figure 1b

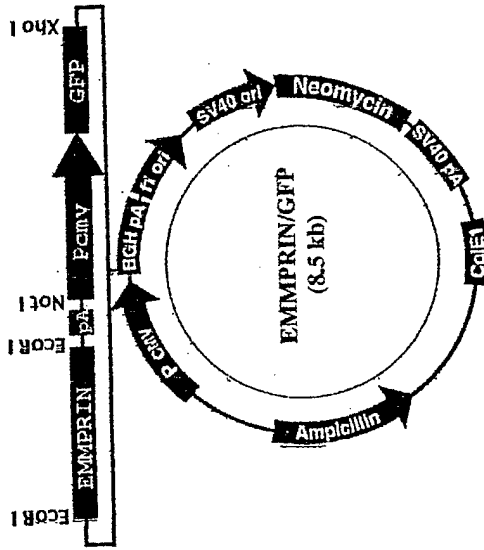


Figure 1a

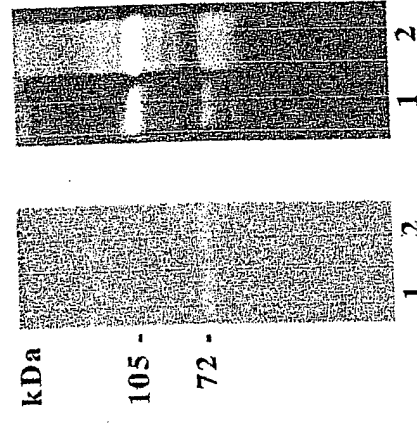


Figure 3b

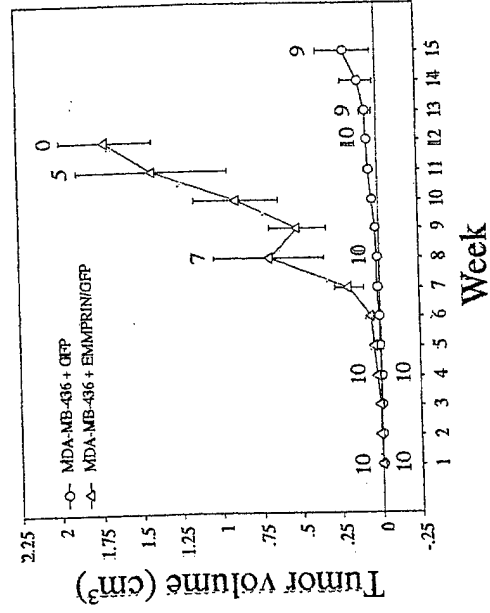


Figure 3a

Figure 2

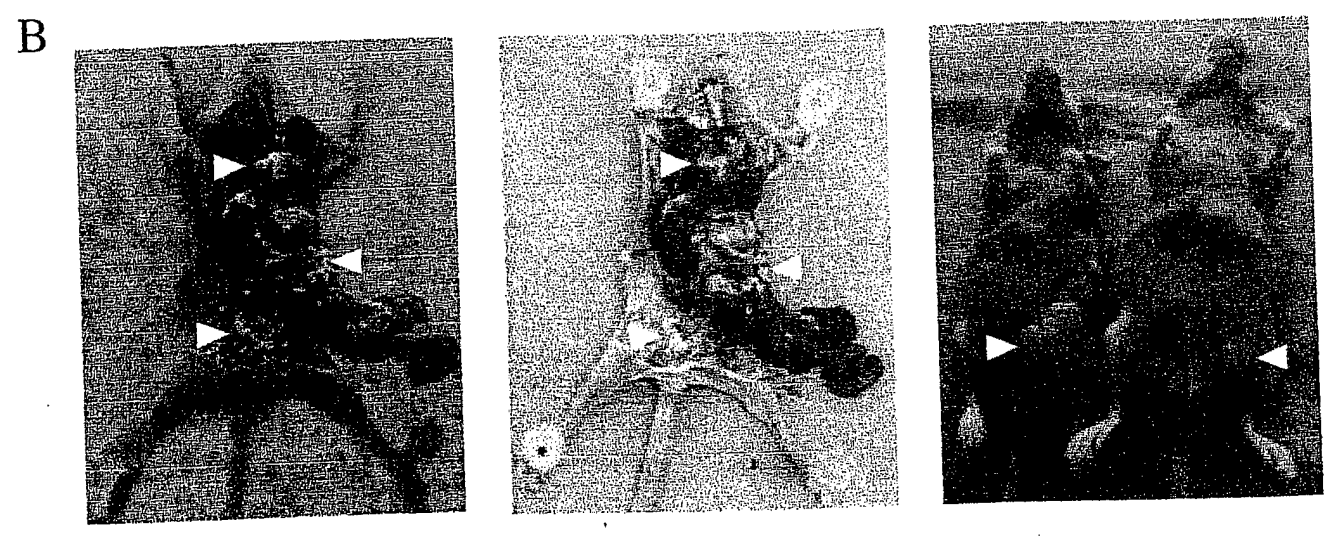
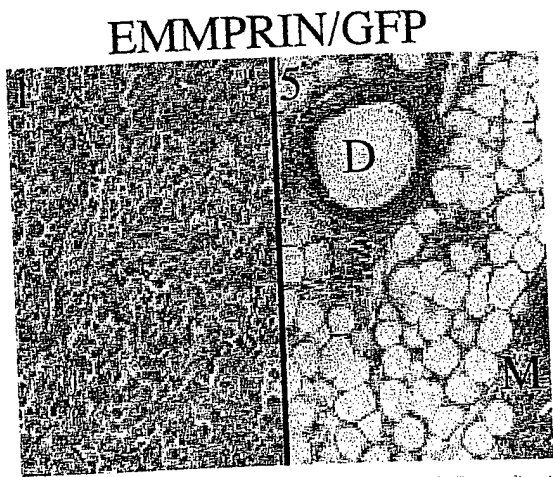
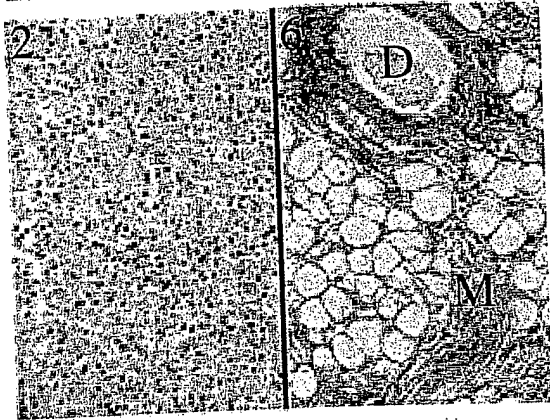
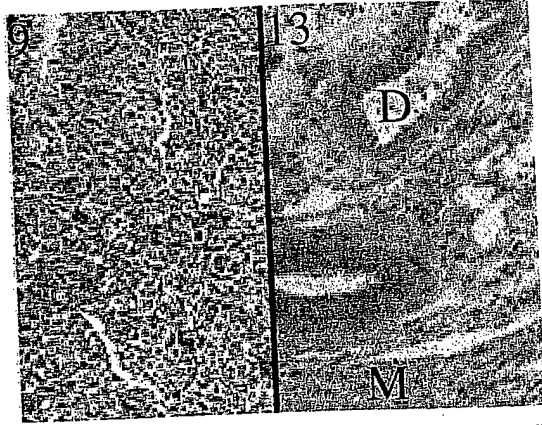


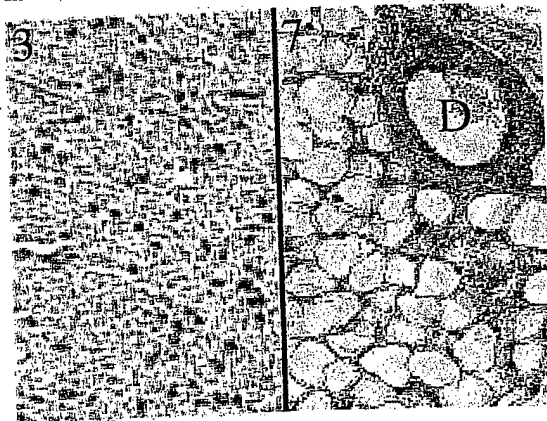
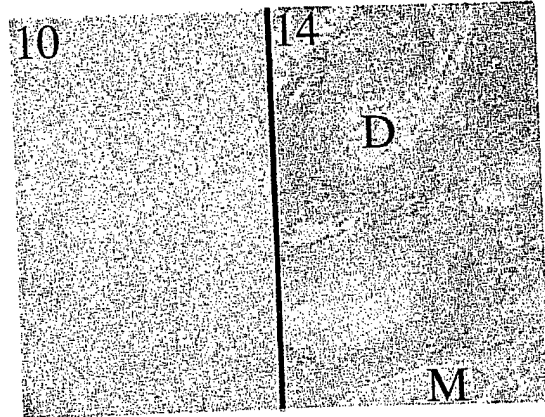
figure 4



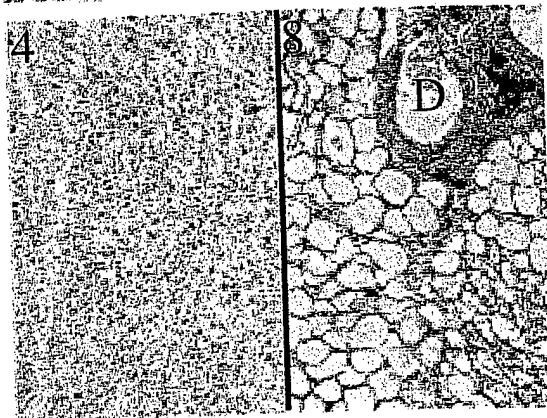
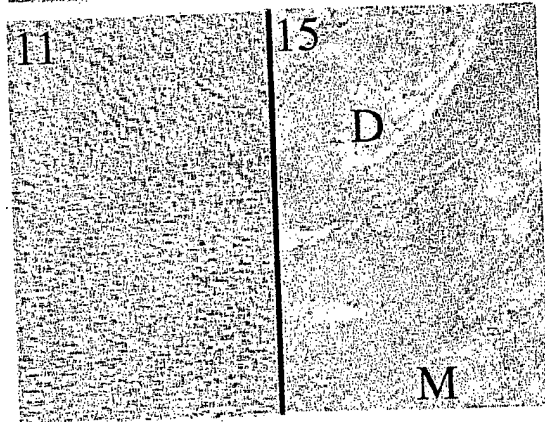
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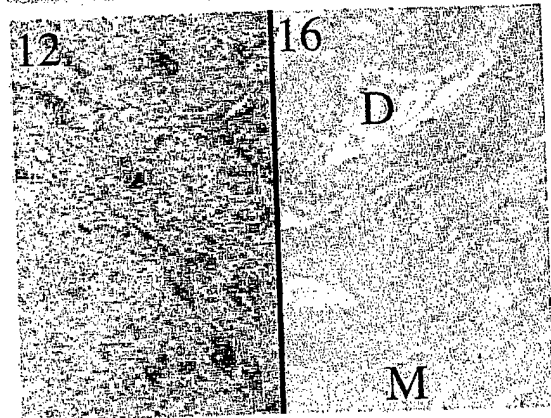
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APPENDIX 3

Guo H, Li R, Zucker S, Toole BP: EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. *Cancer Res.* **60**, 888-891 (2000).

EMMPRIN (CD147), an Inducer of Matrix Metalloproteinase Synthesis, Also Binds Interstitial Collagenase to the Tumor Cell Surface¹

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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

MMPs³ 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 *EcoRI/HindIII* directional linkers. The cDNA was then digested with *EcoRI* and *HindIII* 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 $A_{600\text{ nm}} = 0.8-1.0$ and mixed with the

packaged cDNA (at a ratio of 10^6 phage/10 ml cells) in LB media containing 50 $\mu\text{g/ml}$ carbenicillin (Novagen). Molten top agarose at $45^\circ\text{C}-50^\circ\text{C}$ 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 $\mu\text{g/ml}$ 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 Select-Down primer (AACCTCAAGACCCGTTTA; 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 $A_{280\text{ nm}}$ 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 $A_{450\text{ nm}}$ 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% CO₂ 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. 2A); 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. 2B), 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 of 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

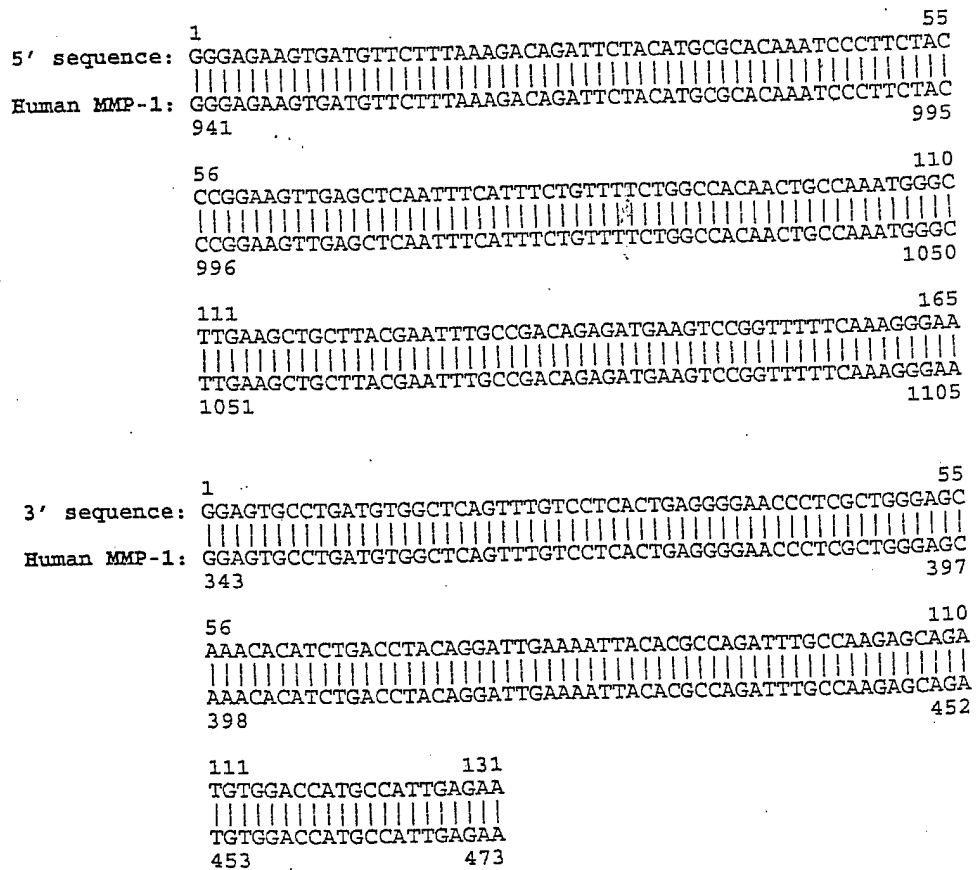
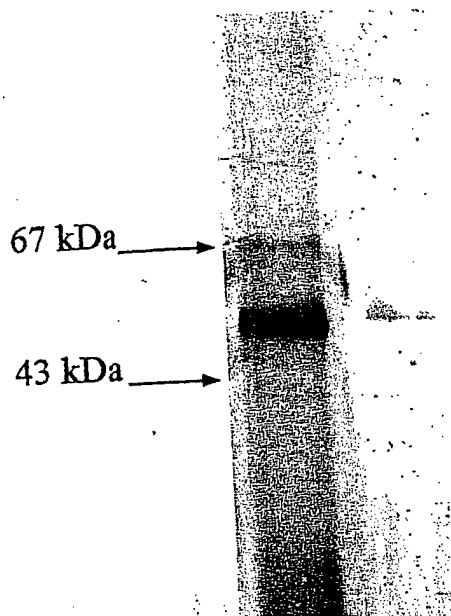


Fig. 1. Comparison of partial sequences of cDNA isolated by phage display with that of human MMP-1 cDNA. The 5' and 3' sequences of one of the partial cDNAs obtained are given. Eight cDNA clones were isolated after biopanning of phages on EMMPRIN; all eight clones had identical sequences.

A



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

B

IgG MMP1

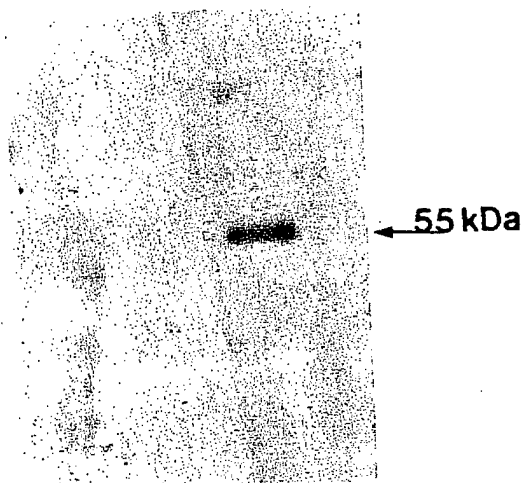


Fig. 2. EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. A, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at $\sim M_r$ 55,000 and $\sim M_r$ 67,000) were detected. Arrows indicate positions 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 $\sim M_r$ 55,000 band reacted with anti-MMP-1.

EMMPRIN preparation with antibody against MMP-1. A strong band at $\sim M_r$ 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 $\sim M_r$ 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 $\sim 58,000$ 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

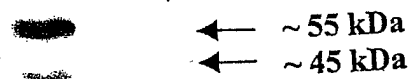


Fig. 3. Western blot of immunopurified EMMPRIN with antibody to MMP-1.

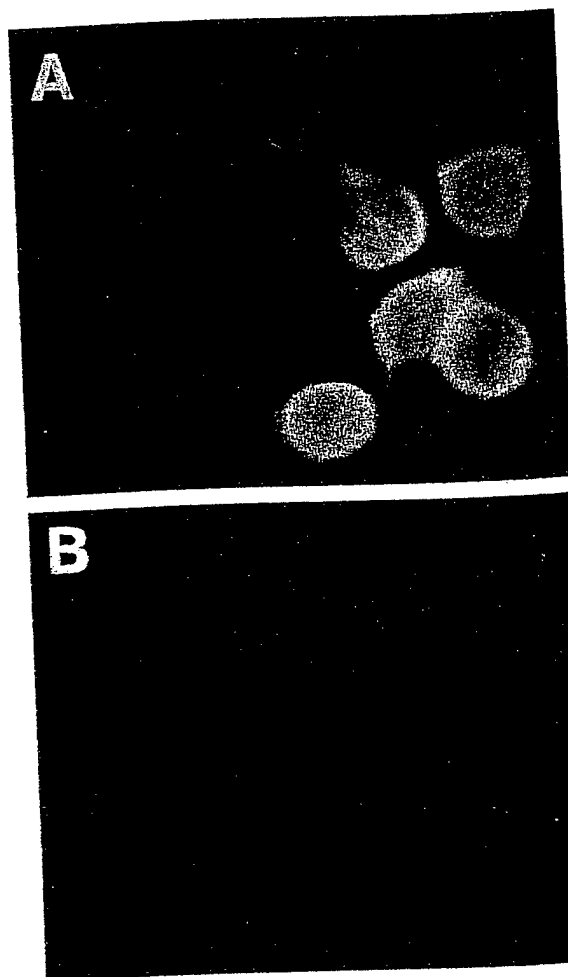


Fig. 4. Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. A, cells stained with antibody against MMP-1. B, cells stained with secondary antibody only.

ites. 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 $\alpha_2(\text{IV})$ 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.