Stimulation of Matrix Metalloproteinase Production by Recombinant Extracellular Matrix Metalloproteinase Inducer from Transfected Chinese Hamster Ovary Cells*

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Many of the tumor-associated matrix metalloproteinases that are implicated in metastasis are produced by stromal fibroblasts within or surrounding the tumor in response to stimulation by factors produced by tumor cells. In this study we transfected Chinese hamster ovary cells with putative cDNA for human extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein that is attached to the surface of many types of malignant human tumor cells and that has previously been implicated in stimulation of matrix metalloproteinase production in fibroblasts. We show that these transfected cells synthesize EMMPRIN that is extensively post-translationally processed; this recombinant EMMPRIN stimulates human fibroblast production of interstitial collagenase, stromelysin-1, and gelatinase A (72-kDa type IV collagenase). We propose that EMMPRIN regulates matrix metalloproteinase production during tumor invasion and other processes involving tissue remodeling.

Successful tumor metastasis requires many steps, one of which is local proteolytic destruction of extracellular matrix at sites of tumor invasion. A major class of proteinases associated with tumor invasion is the matrix metalloproteinases (MMPs) (1, 2). Although it was initially thought that these enzymes were mainly produced by malignant tumor cells themselves, it is now clear that interstitial collagenase (MMP-1), gelatinase A (MMP-2, a 72-kDa type IV collagenase), and stromelysin-1 (MMP-3) are produced in vivo by stromal fibroblasts associated with several types of tumors (2–7). MMP-2 synthesized and secreted by these fibroblasts has been shown to adhere to the surface of tumor cells, facilitating tissue invasion (8–10). Because quiescent fibroblasts generally produce relatively low amounts of MMPs (11, 12), tumor-associated fibroblasts must be influenced in some way to give rise to the elevated levels of MMPs usually present in malignant tumors. One possibility that we have investigated is that tumor cells interact with fibroblasts via soluble cell-bound factors, stimulating fibroblast MMP production (11–15). Our studies have led to characterization of a tumor cell surface protein, extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), that stimulates fibroblast production of MMP-1, MMP-2, and MMP-3 (12–14). We recently obtained cDNAs for human EMMPRIN and verified their identity by recognition of recombinant EMMPRIN by activity-blocking monoclonal antibody and by sequence identity with amino acid sequences of peptides isolated from EMMPRIN (15). However, recombinant EMMPRIN produced by bacteria is much smaller than native EMMPRIN isolated from tumor cells because it is not post-translationally processed. This form of recombinant EMMPRIN is inactive, thus leaving some doubt regarding the identity of the cDNAs. In this study, we use the cDNAs to transfet CHO cells and show that EMMPRIN produced by these transfected cells is post-translationally processed and stimulates fibroblasts to produce elevated levels of MMP-1, MMP-2, and MMP-3.

**EXPERIMENTAL PROCEDURES**

**Stable Transfection of CHO Cells with EMMPRIN cDNA—** EMMPRIN cDNA (15) was subcloned into an expression vector, pcDNA/Neo (Invitrogen, San Diego, CA), and purified by CsCl gradient centrifugation and phenol/chloroform extraction. CHO cells (American Type Culture Collection, Bethesda, MD) were seeded at 10⁵ cells/100-mm tissue culture dish and incubated overnight, at which stage they were 50–70% confluent. The cells were then transfected in 5 ml of serum-free Ham’s F-12 medium containing lipofectamine-DNA complex (10 μl of lipofectamine [Life Technologies, Inc.] mixed with 10 μg of DNA with or without the EMMPRIN insert). After 6 h of incubation at 37 °C, 5 ml of medium containing 20% fetal bovine serum was added to the transfection mixture, which was then cultured at 37 °C for a further 72 h. The cells then were treated with trypsin-EDTA (Life Technologies, Inc.) and subcultured in medium containing 400 μg/ml of Geneticin (Life Technologies, Inc.) for 2–3 weeks. Successful transfection was assessed by immunocytochemistry using monoclonal antibody E11F4 raised against EMMPRIN, as described previously (13). Purification of EMMPRIN—EMMPRIN was purified from detergent extracts of cell membranes from LX-1 cells or stably transfected CHO cells by immunoaffinity chromatography using monoclonal antibody E11F4 against EMMPRIN as described previously (13). Briefly, the cell membranes were extracted with 10 mM Tris-HCl buffer (pH 8.2), containing 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The supernatant of the extract was then applied to a 5-ml affinity column and recirculated through the column for 12 h at 4 °C. The column was washed with buffer several times, and EMMPRIN was then eluted from the column with 50 mM diethylamine, 30 mM octylglucoside (pH 11.5). The eluted protein was neutralized with 0.5 mM Na₂HPO₄, dialyzed against 0.1 mM acetic acid, concentrated, and dissolved in 0.1 mM acetic acid.

**Assays for EMMPRIN Activity—** Human fibroblasts (isolated from...
human skin in our laboratory were cultured for 24 h in 24-well plates in 1 ml of DMEM medium supplemented with 10% fetal bovine serum, after which the medium was replaced with 0.5 ml of DMEM containing 2% fetal bovine serum in the presence or the absence of EMMPRIN or TPA, and the cultures were further incubated at 37 °C for 3 days. Media from these cultures were used for zymographic assay of MMP-3 (16) and ELISA of MMP-1, MMP-2, and MMP-3 (12, 17).

RESULTS

In initial attempts to demonstrate recombinant EMMPRIN activity we tested purified, pGEX bacterial expression protein. However, EMMPRIN produced in the pGEX system had a molecular mass of only ~29 kDa (equivalent to that expected from the cDNA open reading frame (15)), compared with native EMMPRIN from tumor cells, which is ~58 kDa (12–14). This bacterially produced recombinant EMMPRIN protein was inactive in stimulating MMP production by human fibroblasts. Next, COS and CHO cells were transfected with EMMPRIN cDNA under a variety of conditions, but in most cases the EMMPRIN produced was either of similar molecular mass to bacterial recombinant protein, i.e. ~29 kDa, or was partially post-translationally processed with molecular masses ranging from 30–45 kDa. EMMPRIN isolated from these cells was also inactive.

However we found that after stable transfection (see “Experimental Procedures”), CHO cells could be selected that synthesized high levels of EMMPRIN of similar molecular mass to that of native EMMPRIN isolated directly from LX-1 human carcinoma cells. Fig. 1A shows detection of this recombinant EMMPRIN in the transfected CHO cells by immunocytochemistry using monoclonal antibody raised against native EMMPRIN from LX-1 cells (13). Because the transfected cells are very flat, it is difficult to discern the precise cellular distribution of EMMPRIN. However, if the cells are fixed shortly after plating (~4 h), i.e. before they have flattened, it is clear that EMMPRIN is located at the surface of the transfected cells (Fig. 1B). Untransfected cells or cells that are mock-transfected with vector only show no reactivity with the antibody (Fig. 1C).

Fig. 2A shows a silver-stained SDS-PAGE gel of recombinant EMMPRIN purified by immunoaffinity chromatography from membrane extracts of the stably transfected CHO cells; this gel was deliberately overloaded to reveal potential contaminants in the preparation. A single broad band at ~58 kDa was detected, as previously obtained for tumor cell-derived EMMPRIN (13, 14). Direct comparison of purified recombinant and LX-1 carcinoma cell-derived EMMPRIN by Western blotting showed that they were identical in size (Fig. 2B), indicating that the recombinant EMMPRIN was fully or almost fully post-translationally processed. Untransfected CHO cells or cells transfected with vector only did not produce any EMMPRIN detectable by immunoaffinity chromatography and Western blotting (not shown).

We then tested purified recombinant EMMPRIN from transfected CHO cells for its ability to stimulate MMP production by human fibroblasts in culture. We first measured the effect of recombinant EMMPRIN on MMP-3 production by zymography,

**FIG. 1.** Immunofluorescent staining of CHO cells transfected with EMMPRIN cDNA. Staining was carried out using monoclonal antibody, E11F4, against EMMPRIN as described previously (13). A, CHO cells transfected with EMMPRIN cDNA, fixed under normal culture conditions. B, similar cells to those in A, but fixed 4 h after plating. C, CHO cells mock-transfected with vector. Untransfected CHO cells also show no reactivity with E11F4 (not shown).

**FIG. 2.** SDS-PAGE and Western blotting of purified recombinant EMMPRIN. A, silver-stained SDS-PAGE gel of EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA. EMMPRIN was purified from cell membranes as described under “Experimental Procedures,” dissolved in SDS sample buffer containing 0.1 M dithiothreitol, heated at 95 °C for 10 min, and subjected to 10% SDS-PAGE; the gel was deliberately overloaded to reveal potential contaminants. Lane 1, molecular mass standards (45, 66, 97, and 116 kDa); lane 2, purified recombinant EMMPRIN. B, Western blot of recombinant EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA (lane 1) and of native EMMPRIN purified from LX-1 cells (lane 2). A 10% SDS-PAGE gel was electroblotted to a nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The blot was incubated with E11F4 hybridoma supernatant (13) for 1 h at room temperature and then with horseradish peroxidase-conjugated anti-mouse IgG. The EMMPRIN protein bands were detected with ECL Western blotting detection reagents (Amersham Corp.). In both cases the anti-EMMPRIN antibody recognized a protein with a molecular mass of ~58 kDa. Some immunoreactive, aggregated protein was also present in LX-1 cells, as previously noted (14).
using two separate substrates, casein (Fig. 3A) and carboxymethylated transferrin (Fig. 3B). A clear-cut increase in active MMP-3 was observed in fibroblasts treated with the recombinant EMMPRIN (Fig. 3, A and B, lane 1 versus 2). The amount of MMP-3 was similar to that induced by TPA treatment (Fig. 3, A and B, lane 3).

To ensure that stimulation of MMP-3 production was not due to minor contaminants in the recombinant EMMPRIN preparation, we tested the effect of blocking antibody raised against native EMMPRIN (13) on the stimulation by recombinant EMMPRIN, using two different approaches. In the first approach, antibody was included in the culture medium together with EMMPRIN throughout the 3-day incubation period (Fig. 4, lane 7). In the second approach, the antibody was mixed with EMMPRIN and then removed by binding to protein A; the supernatant, after binding of antigen, was then added to the culture for the 3-day incubation (Fig. 4, lane 6). As shown in Fig. 4, the stimulation of stromelysin production in cells treated with EMMPRIN (lanes 4 and 5 versus lanes 3 and 8) was completely reversed by either of the two different treatments with antibody to EMMPRIN (lanes 6 and 7).

Finally, we measured the effect of recombinant EMMPRIN on MMP-1, MMP-2, and MMP-3 production by ELISA. In two separate experiments, treatment of fibroblasts with the EMMPRIN gave rise to significant increments in production of MMP-1 (6- and 11-fold), MMP-2 (1.5- and 16-fold), and MMP-3 (2- and 4-fold) (Table I). In most cases the degree of stimulation of MMP by recombinant EMMPRIN was similar to that caused by TPA (Table I).
Stimulation of MMP production by recombinant EMMRIN

Recombinant EMMRIN was purified from membranes of transfected CHO cells and, in two separate experiments, added at 100 μg/ml to cultures of human fibroblasts. Cultures were also incubated with TPA (0.1 μg/ml) or with no added reagent. After incubation, aliquots of culture medium were used for ELISA of MMP-1, MMP-2, and MMP-3. Amounts of MMP are expressed as μg/ml ± S.E.

| Experiment 1 | None | rEMMRIN | TPA |
|--------------|------|---------|-----|
|               | 0.03 ± 0.00 | 1.40 ± 0.01 | 0.21 ± 0.01 |
|               | 0.33 ± 0.02* | 2.12 ± 0.13* | 0.93 ± 0.13* |
|               | 0.32 ± 0.02* | 2.33 ± 0.29* | 0.42 ± 0.02* |
| Experiment 2 | None | rEMMRIN | TPA |
|               | 0.03 ± 0.00 | 0.13 ± 0.01 | 0.35 ± 0.04 |
|               | 0.17 ± 0.02* | 2.10 ± 0.37* | 0.63 ± 0.03* |
|               | 0.25 ± 0.02* | 0.46 ± 0.06* | 0.56 ± 0.07* |

* Significantly greater than control (none added), p < 0.05.

However, it is not yet clear whether processing is required to attain the appropriate conformation for activity or whether specific side groups, e.g. carbohydrate or phosphate, are involved in EMMRIN receptor recognition.

We have shown by immunocytochemistry that EMMRIN is present on the surface of tumor cells but not fibroblasts and several other normal adult cell types (13, 18, 19). Prior studies have also shown that tumor cells shed EMMRIN (13) and that several other normal adult cell types (13, 18, 19). Prior studies present on the surface of tumor cells but not fibroblasts and EMMRIN receptor recognition.

The ability of EMMRIN to stimulate MMP production has led us to propose that it may be involved in a wide range of physiological and pathological processes where tissue remodeling takes place (24). For example, its presence in the epidermis (25) and in several embryonic epithelia suggests that EMMRIN may participate in epithelial-mesenchymal interactions leading to changes in tissue architecture during development and wound healing. Also, EMMRIN on the surface of activated lymphocytes and monocytes (21) may contribute to elevated MMP levels found in arthritus. However, association of EMMRIN-like material with endothelium during formation of the blood-brain barrier and with highly organized epithelia such as retina and kidney tubules (23) suggests that it may have additional functions involving cell-cell interactions.

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