Extracellular Matrix Metalloprotease Inducer–Expressing Head and Neck Squamous Cell Carcinoma Cells Promote Fibroblast-Mediated Type I Collagen Degradation In vitro

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Abstract

Until recently, tumor progression has been considered a multistep process defined by tumor cell mutations and the importance of the surrounding stroma poorly understood. It is now recognized that matrix-degrading enzymes that promote tumor cell invasion are elaborated by both tumor cells and fibroblasts in vivo. To determine the relative role of tumor cell–derived proteases compared with fibroblast-derived proteases, coculture experiments were done with each cell type using an in vitro model of type I collagen degradation. Head and neck squamous cell carcinoma cells in coculture with normal dermal fibroblasts showed matrix degradation, but neither cell type alone produced this effect. Manipulating the in vitro coculture environment showed that collagenolysis in this model was a result of fibroblast-derived matrix metalloproteases (MMP). To explore the possible role of extracellular matrix metalloprotease inducer (EMMPRIN) in this interaction, transfection of EMMPRIN into a cell line with low endogenous EMMPRIN expression was done and showed a significant increase in collagenolysis. Inhibition of collagenolysis with a tissue inhibitor of metalloprotease-2 (TIMP-2) and a synthetic furin inhibitor was observed but not with TIMP-1, which suggested a possible role for membrane type-1 MMP. These results suggest that fibroblast-derived MMPs but not those from tumor cells are important for in vitro collagenolysis and that this process is promoted by tumor cell–expressed EMMPRIN. (Mol Cancer Res 2005;3(4):195–202)

Introduction

Carcinomas develop from cells that have undergone genetic mutations resulting in the deregulation of normal growth-controlling mechanisms. Research on the origins of cancer has focused on the study of tumor cells and has considered tumorigenesis as an independent process governed by the genes carried by the tumor cells. There is growing evidence that tumor-stromal interactions promote tumor growth, invasion, and angiogenesis. Histologically, the desmoplastic response of head and neck squamous cell carcinoma (HNSCC) consists of fibroblasts, inflammatory cells, and endothelial cells (1). Although the role of fibroblasts at the leading edge of tumors remains unknown, the elaboration of growth factors and proteases by tumor cells is thought to help recruit fibroblasts to remodel the surrounding matrix and thereby promoting a favorable microenvironment for tumor growth and invasion (2).

Invasion of tumor cells across the extracellular matrix has been recognized as a protease-dependent event (3). Although proteases were initially thought to be derived primarily from tumor cells, recognition that both fibroblasts and tumor cells synthesize peri-thermal matrix metalloproteases (MMP) by in situ hybridization in breast cancer (4, 5) lead to similar observation in other tumors (6–8), including HNSCC (9–11). Despite this finding, in vitro studies of matrix degradation rarely consider the contribution of fibroblast-derived proteases. Although regulation of protease expression by tumor cells is likely to occur through multiple paracrine and autocrine factors, HNSCC tumor cells are known to express high levels of extracellular matrix metalloprotease inducer (EMMPRIN).

EMMPRIN (also called CD147 or basigin) was initially isolated by Biswas et al. (12) and is a 55-kDa molecule found on the surface of tumor cells that up-regulates MMPs in surrounding tumor cells and fibroblasts by cell-cell contact (13). The cDNA for human EMMPRIN encodes 269 amino acid residues that include a cytoplasmic domain, a transmembrane domain, and two domains that are characteristic of the immunoglobulin superfamily. Immunohistochemical studies have determined the expression of EMMPRIN is up-regulated in human tumors compared with their normal cellular counterparts (14, 15). EMMPRIN expression has been correlated with metastasis and tumor progression in multiple tumor types (16, 17), including oral cavity (18) and laryngeal HNSCC (19). EMMPRIN has been found to stimulate fibroblast expression of MMP-1, MMP-2, MMP-3, and MT1-MMP (17, 19–21). Coculture experiments of glioblastoma and melanoma cells with fibroblasts have shown that EMMPRIN stimulates MT1-MMP expression in fibroblasts that in turn converts pro–MMP-2 to its catalytically active form (16, 17). However, the importance of EMMPRIN or fibroblast-derived MMPs in collagen degradation during coculture remains unknown.
Despite high expression of EMMPRIN in HNSCC in vivo, its role in promoting tumor cell invasion in this tumor type has not been defined. In the present study, the relative functional significance of fibroblast-derived and tumor cell proteases were assessed in HNSCC tumor cell lines and fibroblasts alone and in coculture. Type I collagen degradation was found dependent on fibroblasts stimulated by cell-cell contact with tumor cells. In this in vitro model system, tumor cell–derived EMMPRIN promoted MMP-dependent collagenolysis by fibroblasts.

Results

Tumor Cell and Fibroblast Coculture Promotes Collagenolysis In vitro

Tumor cell lines (UM-SCC-1, CAL 27, and FaDu) were cocultured with normal dermal fibroblasts (NDF) atop the center of a type I collagen substratum to assess for degradation (Fig. 1A). Minimal degradation was noted with all three cell types alone; however, FaDu and UM-SCC-1 cells in coculture with NDFs showed a central area of lucency consistent with collagen degradation \((P = 0.01\) and \(P = 0.003\) compared with NDF alone, respectively). CAL 27 cells in coculture with NDFs did not degrade type I collagen in this model system. Because tumor cell–derived EMMPRIN has been known to induce type proteolytic enzymes by paracrine and autocrine stimulation, we assessed the relative expression of EMMPRIN in each cell line (Fig. 1B). Tumor cell expression of EMMPRIN correlated with the degree of collagenolysis in coculture with fibroblasts.

Type I Collagen Degradation Is Fibroblast Dependent

Increasing numbers of fibroblasts or tumor cells were added to determine the relative contribution of each cell type; the escalating numbers of fibroblasts increased collagenolysis (Fig. 2A), whereas increasing tumor cells did not alter degradation (data not shown). To determine if cell-cell contact is required for collagen degradation, conditioned medium or whole cell extracts derived from FaDu cells or NDFs were added to NDFs or FaDu cells, respectively (Fig. 2B). Conditioned medium from either cell type or NDF whole cell lysate did not result in collagen degradation; however, whole cell extract from FaDu cells added to viable NDF cells resulted in collagen degradation \((P = 0.01\). To further show the central roles of fibroblasts in collagenolysis, radiated tumor cells or fibroblasts were cocultured atop type I collagen after various doses of radiation therapy (Fig 2C). Irradiation of FaDu cells had no effect on degradation; however, irradiated fibroblasts in coculture with untreated FaDu cells at escalating doses \((2, 6, \text{or } 10 \text{ Gy})\) showed a dose-dependent decrease in collagenolysis \((10 \text{ Gy–treated compared with untreated fibroblasts, } P = 0.004\). Cell viability was assessed after 2, 6, and 10 Gy of radiation showed 90% growth, 56% growth, and 13% growth, respectively, compared with untreated controls. Taken together, these results suggest type I collagen degradation is mediated by fibroblasts and requires cell-cell contract with viable or nonviable tumor cells.

FaDu/Fibroblast Coculture Type I Collagen Degradation Is MMP Dependent

Serine, cysteine, and MMP protease inhibitors were added to determine the relative role of each protease class in type I collagen degradation in this model system. The synthetic MMP inhibitor GM6001 \((20 \mu\text{mol/L})\) but not leupeptin \((10 \mu\text{mol/L}), \text{E64} (10 \mu\text{g/mL}), \text{or epsilon-amino-caproic acid (}10 \text{mmol/L)}\) blocked collagen dissolution when FaDu cells were cocultured with NDF cells (Fig. 3). Because EMMPRIN is known to induce MMP-1, commercially available anti-MMP-1–neutralizing antibody \((20 \mu\text{g/mL)}\) were added daily to the medium over the incubation period without reduction of type I collagen degradation. To implicate MT1-MMP in this process, the synthetic furin inhibitor deonyl-Arg-Val-Lys-Arg-CMK (CMK) was added to culture medium to inhibit the intracellular conversion of pro–MT1-MMP to its active form as shown by others \((22, 23)\). CMK at concentrations of \(50 \mu\text{mol/L}\) reduced collagen degradation by 80% compared with untreated control cocultures. The endogenous MMP inhibitors, TIMP-2 \((10 \text{ nmol/L)}\) reduced collagenolysis by 90% compared with untreated control, but TIMP-1 \((10 \text{ nmol/L)}\) failed to blocked collagen degradation.

FIGURE 1. Tumor-fibroblast coculture promotes type I collagen degradation in vitro. A, CAL 27 cells, FaDu, or SCC-1 cells were cultured alone or in the presence of NDFs atop type I collagen films. Tumor cells or fibroblasts alone did not promote discrete areas of collagen lysis after a 4-day incubation period. NDF coculture with FaDu or SCC-1 cells promoted collagen degradation \((P < 0.05\) for both compared with NDF alone) but not in coculture with CAL 27 cells. B, Immunoblot analysis of tumor cell lysates atop type I collagen display differential EMMPRIN expression that correlates with collagenolysis induced upon coculture of cells with NDFs.
MMP Expression of Tumor Cell/Fibroblast Coculture

Head and neck cancers and fibroblasts in vivo and in vitro are known to express MT1-MMP, MMP-1, MMP-2, and MMP-9. EMMPRIN is known to induce expression of MMP-1, MMP-2, and MT1-MMP. To determine the expression of these enzymes in their active forms and to determine the relative up-regulation of these enzymes in coculture, serum-free medium (Fig. 4A) or cell lysates (Fig. 4B) was harvested and assessed by immunoblot and zymography. Although MMP-9 was up-regulated in coculture, it was present in its inactive form. The relative expression of active MMP-2 in the medium was not substantially different between the NDFs alone and NDFs in coculture, although the pro–MMP-2 expression levels decreased dramatically from the conditioned medium of NDF cells to the cocultured cells. Although there was a small increase in MMP-1 in coculture, MT1-MMP showed the largest up-regulation compared with either cell type alone.

Consistent with the increase in MT1-MMP, there is an increase in the percentage of active MMP-2, although the total amount of active MMP-2 remains constant. Because changes in TIMP levels can also affect the proteolytic activity in this assay as shown by the previous inhibitory assays, the levels of TIMP-2 and TIMP-3 were assessed. TIMP-3 was not identified in the conditioned medium but was found not to change in the whole cell lysate of NDF cells and NDFs in coculture. A small decrease in TIMP-2 in the culture medium was seen in coculture with the tumor cells. EMMPRIN expression seemed augmented in the coculture.

EMMPRIN Mediates Fibroblast-Dependent Collagenolysis

FIGURE 2. Viable fibroblasts are required for collagen degradation. A. When decreasing numbers of NDF cells were added to a fixed number of FaDu cells, progressively less degradation occurred. B. Whole cell extracts (lysates) or conditioned medium (CM) derived from FaDu cells or NDFs were cocultured with untreated NDFs or FaDu cells, respectively. Viable NDF cells in the presence of FaDu whole cell extracts were required to promote collagen degradation. Conditioned medium from FaDu cells failed to produce collagenolysis. C. FaDu cells or NDFs were radiated with 2, 6, or 10 Gy before coculture with untreated NDFs or FaDu cells. Escalating doses of radiation treatment in NDF cells prevented degradation but not in FaDu cells, suggesting viable NDF cells are required for degradation.
Therefore, to determine the role of EMMPRIN-mediated fibroblast collagen degradation in this model system, the CAL 27 cell line was transfected because of its low endogenous EMMPRIN expression and failure to stimulate collagen dissolution (Fig. 1). Type I collagen degradation was promoted in stably transfected CAL 27 cells (CAL 27/E) in the presence of NDFs (Fig. 5). Although EMMPRIN is known to act in an autocrine as well as a paracrine fashion, no increased degradation was seen in EMMPRIN-transfected cells in the absence of coculture with fibroblasts. Inhibitors were assessed to determine if the same MMP-dependent collagen degradation was occurring in EMMPRIN-transfected cells. The profile was the same as that identified in previously (Fig. 3), suggesting a MMP-dependent process with a possible role for MT1-MMP in fibroblast-mediated collagenolysis.

**Discussion**

The process of matrix degradation has traditionally been considered a tumor cell–mediated event (25, 26). Recently, there has been considerable interest in the role of supporting stoma in the invasive phenotype. Although tumor cells and fibroblasts are known to express matrix-degrading enzymes (10, 27), the relative importance of tumor cells compared with fibroblast-derived proteases in collagen degradation remains unclear. Consistent with epithelial-stromal interactions that occur with wound healing or organogenesis in the embryo, we have shown that type I collagen matrix degradation in this model system is a function of tumor cell–stimulated fibroblasts. Furthermore, we provide evidence that fibroblasts can be induced by tumor cell–derived EMMPRIN to degrade type I collagen in an MMP-dependent fashion.

EMMPRIN expression in tumor cells correlated with degradation in the coculture experiment (Fig. 1) and cell–cell contact was required to stimulate fibroblast-mediated degradation (Fig. 2). Although tumor cells did express MMPs, these did not seem to contribute to collagen degradation, as viable fibroblasts were required for collagenolysis (Fig. 2). These results suggested a role for the membrane-bound EMMPRIN in this phenotype; therefore, EMMPRIN was transfected into a head and neck cancer cell line with low endogenous EMMPRIN expression (CAL 27). Overexpression of EMMPRIN in CAL 27/E cells increased MMP-dependent collagenolysis in the coculture assay. This result is consistent with other investigations of EMMPRIN that has been found to stimulate fibroblast expression of MMP-1, MMP-2, MMP-3, and MT1-MMP (17, 19-21). Coculture experiments of glioblastoma and melanoma cells with fibroblasts have shown that EMMPRIN stimulates MT1-MMP expression in fibroblasts that in turn converts pro–MMP-2 to its catalytically active form (16, 17).

Although multiple proteases derived from both tumor cells and fibroblasts are likely to play a role in tumor cell invasion, other studies have suggested a central role for MT1-MMP (10, 28, 29). In HNSCC, advanced stage disease and nodal status has been correlated with immunopositivity for MT1-MMP,
MMP-1, MMP-2, and MMP-9 (28, 30-33). In our studies, the evidence suggests that MT1-MMP mediates collagenolysis rather than MMP-2. The inhibitor profile suggested that collagenolysis was MMP dependent and suggested a role for MT1-MMP. CMK inhibits furin that is required to process MT1-MMP to its active form before cell surface expression and was found to effectively block collagenolysis in our model. The failure of TIMP-1 (a poor inhibitor of MT1-MMP) to block collagenolysis and the ability of TIMP-2 (an efficient MT1-MMP inhibitor) to block collagenolysis (34), provides indirect evidence that MT1-MMP plays a central role in this phenotype. This is consistent with others who identified that head and neck cancer tumor cell lines alone could degrade type I collagen in the same assay with phorbol ester stimulation (35). This process was identified as MT1-MMP dependent; however, coculture experiments were not done by these authors. Although a role for MT1-MMP–mediated collagenolysis is suggested by the inhibitor studies, the modest increase in MT1-MMP expression under coculture conditions suggest other modulating factors are present, such as change in inhibitor levels. Furthermore, MT1-MMP is expressed by the tumor cells and the fibroblasts, although the role of tumor cell–expressed MMPs in collagenolysis is unclear in our model system. Although there is some decrease in TIMP-2 expression in the coculture, this change is relatively small. EMMPRIN has been shown in multiple cell types not to modulate TIMP-2 levels (24). In our study, TIMP-2 did not change when control vector or EMMPRIN-transfected tumor cells were cocultured with fibroblasts (data not shown).

Although multiple studies have evaluated the in vivo expression of proteases, there has been little work to show the relative importance of stromal-derived proteases. Biswas et al. identified that the collagenolytic properties of fibroblasts could be induced by poorly differentiated lung carcinoma cells and identified this factor as EMMPRIN (12, 36). Experiments using MMP-deficient mice have suggested that inflammatory cell–derived MMPs are important for squamous cell carcinoma growth (37). Tumors grown in MMP-2– and MMP-9–deficient mice show only modest reduction in growth or angiogenesis (38, 39), suggesting the relative unimportance of these host-derived MMPs in tumor cell invasion. However, these experiments have not been done in MT1-MMP knockout mice due to their limited life span.

Recognition that both carcinoma-associated fibroblasts and tumor cells synthesize peritumoral MMPs in multiple tumor types (6-8, 10) in vivo led us to investigate the functional significance of tumor cell–derived compared with fibroblast-derived proteases. Although multiple other studies have shown the importance of EMMPRIN in MMP induction, few have linked this to in vitro collagen degradation and none have done so using head and neck squamous cell carcinoma cells. In this study, we show that in vitro collagenolysis is dependent on MMPs derived from tumor cell–stimulated fibroblasts. This data prompts the question which tumor cell–related factor(s) activate fibroblast degradation. Multiple tumor-secreted growth factors and cytokines have been implicated in peritumoral MMP regulation (2, 13), including the shedding of EMMPRIN (40, 41). In our experiments,

**FIGURE 4.** Expression of MMPs and EMMPRIN in tumor cell/fibroblast coculture. A. Conditioned medium from FaDu cells, NDFs, or the two cell types in coculture on a substratum of collagen and collected after 24 hours. Top, zymography demonstrates that MMP-9 is in its inactive form and the active form of MMP-2 does not change between the NDF and coculture group. TIMP-2 expression decreases in coculture. B. Analysis of cell lysates for membrane-bound proteins. MT1-MMP is up-regulated and EMMPRIN is expressed in the FaDu tumor cell.
collagenolysis did not occur when fibroblasts were incubated with tumor cell–derived conditioned medium (Fig. 2), although EMMPRIN was detected in the conditioned media by Western blot analysis (data not shown). Although we show that tumor cell–derived EMMPRIN is capable of promoting collagenolysis through an MMP-dependent mechanism, other co-factors may up regulate other MMPs or down-regulation of protease inhibitors may be occurring in parallel with an EMMPRIN-independent mechanism.

We provide evidence that fibroblast-derived MMPs are responsible for type I collagen degradation in vitro and tumor cell–derived EMMPRIN is responsible for promoting this phenotype. This data suggests that there may be benefit in further in vivo investigation and therapeutic targeting on stromal-derived proteases in head and neck cancer.

Materials and Methods

Cells and Culture Conditions

The FaDu and CAL 27 cell lines were purchased from American Type Tissue Collection (Manassas, VA) and UM-SCC-1 was obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Cell lines were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (Mediatech, Herdon, VA), 2 mmol/L L-glutamine (Mediatech), and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin sulfate, Mediatech). NDFs were isolated from primary culture and maintained in DMEM with 20% fetal bovine serum and antibiotics. We provide evidence that fibroblast-derived MMPs are responsible for type I collagen degradation in vitro and tumor cell–derived EMMPRIN is responsible for promoting this phenotype. This data suggests that there may be benefit in further in vivo investigation and therapeutic targeting on stromal-derived proteases in head and neck cancer.
inhibitors leupeptin and epsilon-aminocaproic acid were obtained from Sigma (St Louis, MO). E64 (Sigma), deoxyl-Arg-Val-Lys-Arg-CMK (Alexis Biochemicals, San Diego, CA), GM6001 (Calbiochem), and anti–MMP-1 monoclonal neutralizing antibodies (R&D Systems) were also used in the degradation assay. Cocultures with cell extracts were done using cells that were grown to subconfluence, trypsinized and counted before resuspension in sterile water and vortexed for 10 minutes before centrifugation (13,000 rpm × 10 minutes). Cells were mechanically homogenized and cultured alone, or in combination with NDFs or FaDu cells. Cells were irradiated in subconfluent cultures using cobalt irradiation (Picker Unit, Cleveland, OH) at the prescribed dose in growth medium. Cell growth was assessed by counting cells at 4 days post-irradiation.

**Immunoblot and Zymography**

Cells were grown atop a type I collagen substratum identical to that in the collagen degradation assay and conditioned medium or lysates were obtained after a 24-hour incubation period. Cells were homogenized in radioimmunoprecipitation assay buffer with 1 mmol/L phenylmethylsulfonyl fluoride and 15 μg/mL each of aprotinin and pepstatin. After protein determination using a bicinchoninic acid protein assay (Pierce, Rockford, IL), 20 μg total protein per lane were separated by SDS-PAGE protein electrophoresis under reducing conditions on a precast 12% tris-glycine gel (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblotting was done using anti-MT1-MMP–specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti–MMP-1 monoclonal antibody (R&D Systems), TIMP-2–specific monoclonal antibody (Santa Cruz Biotechnology), TIMP-3 (R&D Systems), and anti-EMMPRIN monoclonal antibody (Zymed, San Francisco, CA). Secondary anti-mouse antibody was used followed by chemiluminescence detection. Mouse monoclonal antibody to β-actin (anti–β-actin antibody, Santa Cruz Biotechnology) was used to assess for uneven protein concentrations. Zymography was done to assess gelatinase activity. Conditioned medium was separated by 10% tris-glycine gel with 0.1% gelatin PAGE electrophoresis (Novex Precast Gel, Invitrogen) under nonreducing conditions. Gels were washed in 2.5% Triton X-100 to remove SDS and incubated for 18 hours in 50 mmol/L Tris (pH 7.5), 10 mol/L CaCl₂, 1 μmol/L ZnCl₂, and 1% Triton X-100 at 37°C. Gels were stained with 0.1% Coomassie blue G-250 for 1 hour and destained in 5% acetic acid until clear bands of collagenolysis appeared.

**Transfection of CAL 27 Cell Line with EMMPRIN**

The EMMPRIN expression vector which contains the 806-bp EMMPRIN cDNA and the 700-bp cDNA for the 29-kDa green fluorescent protein has been previously used in tumor cell lines (42) and found to express functional EMMPRIN on the cell surface of human breast cancer cell line (a kind gift of Stanley Zucker, State University of New York, Stony Brook, NY). The vector was used to transfect the CAL 27 cell line using the calcium phosphate precipitation method (43). Under G418-selective conditions (concentration, 0.1-0.8 mg/mL), resistant clones were screened by fluorescent appearance. Stably resistant clones were selected from each cell population, propagated, and analyzed for EMMPRIN protein expression by immunoblotting. All experiments were done in parallel with stably transfected pcDNA3.0 vector (Invitrogen)–only CAL 27 control cells.

**References**

has strong homology with both the immunoglobulin V domain and the β-chain of major histocompatibility complex class II antigen. J Biochem (Tokyo) 1990;107:316–23.


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