Overexpression of Extracellular Matrix Metalloproteinase Inducer in Multidrug Resistant Cancer Cells

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Abstract
Multidrug resistant (MDR) cancer cells overexpressing P-glycoprotein (P-gp) display variations in invasive and metastatic behavior. We previously reported that these properties of MDR cancer cell lines overexpressing P-gp could be altered by chemotherapeutic drugs or MDR modulators (R. S. Kerbel et al., Cancer Surv., 7: 597–629, 1988). To attempt to clarify the mechanism(s) underlying these observations, we studied the expression of extracellular matrix metalloproteinase inducer (EMMPRIN), a glycoprotein enriched on the surface of tumor cells that can stimulate the production of matrix metalloproteinases (MMPs), in sensitive and MDR cancer cells. Using immunofluorescence staining and fluorescence-activated cell sorting analysis, we found that EMMPRIN expression was increased in MDR carcinoma cell lines, MCF-7/AdrR, KBV-1, and A2780Dx5, as compared to their parental counterparts. The MDR cell lines produced more matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-9 (MMP-9), as determined by zymography, Western blot, and reverse transcription-PCR. Treatment of MDR cells with an anti-EMMPRIN antibody inhibited the activity of MMP-1, MMP-2, and MMP-9. In MDR cell line MCF-7/AdrR, an increased in vitro invasive ability was observed as compared with the sensitive line MCF-7, and EMMPRIN antibody could inhibit the in vitro invasion of drug-resistant cells. In addition, the expression and activity of MMP-1, MMP-2, and MMP-9 in MDR cells were decreased by treatment with U-0126, an inhibitor of mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/Erk). Our results suggest that during the development of MDR, the expression of EMMPRIN is responsible for the increased activity of MMP in MDR cell lines.

Introduction
Drug resistance and tumor metastasis are the main causes of treatment failure and mortality in cancer patients. Although these two properties of malignant tumors have been studied extensively, most investigations have proceeded along separate pathways. There is also evidence, however, suggesting a functional linkage between the two phenotypes. For example, Kerbel et al. (1–3) reported that the expression of an array of diverse genes was switched on in metastatic cancer cells, including genes encoding proteinases, adhesion molecules, growth factors, and motility factors. Further studies found that some of these genes could also affect sensitivity to chemotherapeutic drugs (4–6). However, the relationship between drug resistance and metastatic behavior remains unclear. Conflicting results were reported by several groups (7–10). While some studies demonstrated enhanced invasive or metastatic ability of drug-resistant cancer cells (7, 8), others found the opposite results (9, 10).

Overexpression of P-glycoprotein (P-gp), a transmembrane, ATP-dependent transporter encoded by the MDR1 gene, is associated with a poor clinical outcome in cancer patients often attributed to resistance to treatment (11, 12). We observed increased motility, invasion, and metastasis of certain P-gp-overexpressing multidrug resistant (MDR) cancer cells treated with P-gp transportable drugs (13). In addition, chemotherapy-induced progression of disease and loss of response to chemotherapy was noticed earlier by others (14). The purpose of the current study was to determine the mechanism(s) by which changes in invasive/metastatic potential of MDR cancer cells occur. We began by comparing the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and the activity of matrix metalloproteinase (MMP) in parental, sensitive cancer cells and their MDR counterparts. EMMPRIN (also known as CD147, basigin, M6 and tumor cell-derived collagenase stimulatory factor) is a glycoprotein belonging to the immunoglobulin superfamily that is enriched in the plasma membrane of most tumor cells (15). EMMPRIN stimulates the production of several MMPs, including MMP-1, MMP-2, MMP-3, and membrane-type MMP. Recently, increased expression of EMMPRIN was found in invasive human cancers (16, 17). MMPs include a class of at least 20 human zinc-dependent endopeptidases, which can be classified into subgroups of collagenases, gelatinases,stromelysins, membrane-type MMP, and others
The expression or activity of distinct MMP is increased in invasive or metastatic tumors as compared to non-invasive tumors (19–21). The purpose of this study was to determine whether the increase in MMPs observed in MDR cancer cells could be attributed to the expression of EMMPRIN. Our results provide further evidence that drug resistance and invasive/metastatic potential might be linked during the progression of malignant disease.

Results

We compared the expression of EMMPRIN in three pairs of sensitive and MDR cancer cell lines. MCF-7/AdrR, KBV-1, and A2780Dx5 expressed higher level of EMMPRIN than the parental sensitive cell lines (MCF-7, KB3-1, and A2780), as determined by immunofluorescence staining (Fig. 1A) and fluorescence-activated cell sorting (FACS) (Fig. 1B). Quantification of FACS-generated histograms estimated that the expression of EMMPRIN was 3- to 4-fold higher in MDR cells than in sensitive cells.

Because EMMPRIN stimulates the production of MMP, we next compared MMP activity and expression in sensitive and MDR cancer cell lines. Fig. 2 demonstrates that the activity of MMP-9, MMP-2, and MMP-1 present in the media from cultures of MDR cell lines was greater than the activity present in the media from the parental, sensitive cell lines, as measured by zymography. Fig. 3 shows that this increased enzymic activity was due to increased mRNA expression (Fig. 3A) and secretion of the enzymes into the media (Fig. 3B). The content of MMP-9, MMP-2, and MMP-1 was 4- to 7-fold, 2- to 8-fold, and 2- to 10-fold higher in the media from cultures of various MDR cell lines than in the media from cultures of parental, sensitive cells, as determined by Western blots. MMP-3, MMP-7, MMP-13, and MMP-14 were unchanged (data not shown).

To analyze the role of EMMPRIN in the up-regulation of MMP in MDR cells, we treated sensitive and MDR cells with an anti-EMMPRIN antibody or control antibody (mouse IgG1) for 24 h, then measured MMP activity in the culture medium. Fig. 4 demonstrates that treatment with EMMPRIN antibody (50 μg/ml) decreased the activity of MMP-9, MMP-2, and MMP-1 in MDR cell lines. Anti-EMMPRIN antibody (50 μg/ml) had no effect on cell viability (data not shown).

EMMPRIN can stimulate MMP production through the mitogen-activated protein kinase (MAPK) pathway (22). To
determine whether MAPK signaling also mediates the EMMPRIN-stimulated production of MMP-9, MMP-2, and MMP-1 in MDR cancer cells, we tested the effects MAPK inhibitors on MMP activity and expression. U-0126, an inhibitor of MAPK/extracellular signal regulated kinase (Erk), inhibited the activity (Fig. 5A) and expression (Fig. 5B) of MMP-9, MMP-2, and MMP-1 in MDR cells in a dose-dependent manner (1–10 μM). Over the same concentration range, SB-203580, an inhibitor of MAPK/p38, had no effect (data not shown).

We next measured the invasive capacity of the sensitive and MDR cell lines using an in vitro assay. MCF-7/AdrR cells were more invasive, as demonstrated by the greater number of MCF-7/AdrR cells penetrating the Matrigel than the sensitive MCF-7 cells (Fig. 6). However, drug-resistant cell lines, KBV-1 and A2780Dx5, were less invasive than the parental, sensitive lines (Fig. 6). To determine whether or not a linkage exists between increased EMMPRIN expression and invasive ability, we treated MCF-7/AdrR cells with an anti-EMMPRIN antibody or control IgG1, then assayed in vitro invasion. Fig. 7 shows that the antibody against EMMPRIN significantly inhibited the in vitro invasive ability of MCF-7/AdrR line as compared with the control antibody (P < 0.05).

**Discussion**

In the current study, we found that EMMPRIN is overexpressed in several MDR cancer cell lines compared to the parental controls (Fig. 1). EMMPRIN is a cell surface glycoprotein that stimulates the production of multiple MMP by tumor cells and fibroblasts (23–26). In addition, EMMPRIN can interact with lactate transporters and facilitate their cell surface expression (27), serve as a receptor for extracellular cyclophilins (28), and regulate store-operated calcium entry (29). EMMPRIN was reported to be expressed in several types of cancers, including oral squamous cell carcinoma, lung, breast, bladder, and brain cancers (17, 30–32). In addition, expression of EMMPRIN was found to correlate with
invasiveness of tumor cells (16, 33). Because one of the major functions of EMMPRIN is to regulate the production of certain MMP (24–26), we determined the activity and expression of MMP in MDR cell lines.

The activity and expression of MMP-9, MMP-2, and MMP-1 are increased in the MDR cell lines as compared to the parental, sensitive lines (Figs. 2 and 3), suggesting a causal relationship between overexpression of EMMPRIN and increased production of MMP in MDR cells. In addition, we found that an anti-EMMPRIN antibody decreases MMP-9, MMP-2, and MMP-1 activity in MDR cells (Fig. 4), confirming a role for EMMPRIN in the overproduction of MMPs. This result is consistent with the observations that EMMPRIN can serve as a receptor for interactions with extracellular molecules that stimulate signal transduction pathways culminating in the transcriptional activation of MMPs (26, 29). For example, Sameshima et al. (26) demonstrated that EMMPRIN-stimulated production of MMP-2, MT1-MMP, and MT2-MMP in cocultures with brain-derived fibroblasts was inhibited by an anti-EMMPRIN monoclonal antibody.

These results are consistent with other work indicating alterations in cellular invasiveness in drug-resistant tumors. For example, the expressions of integrins (34), tissue inhibitor of metalloproteinases and plasminogen activator inhibitor (35), and adhesion molecules such as CD44 and very late activation antigens (36), were reported to be altered in drug-resistant cancer cells. Our results provide additional evidence that with the acquisition of the MDR phenotype, other cellular or biochemical changes occur that favor cancer cell dissemination.

An important question raised by these results is whether or not a link exists between MDR1 expression and acquisition of a pro-metastatic phenotype. Several laboratories (37–39), including our own (39), found that transcriptional activation of MDR1 was regulated by the MAPK pathway. For example, we demonstrated that transient transfection of v-Raf increased MDR1 promoter activity, whereas transfection with dominant-negative mutant, Raf-C4, had the opposite effect (39). In addition, the activation of MDR1 was blocked by U-0126, a MAPK/Erk inhibitor. Stimulation of MMP production by EMMPRIN was also reported to involve MAPK signaling (22). For example, Lim et al. (22) found that EMMPRIN derived from lung cancer cells stimulated fibroblast MMP-1 expression via MAPK/p38. In our hands, the MAPK/p38 inhibitor, SB-203580, did not affect MMP activity in the MDR cell lines tested (data not shown). We found that inhibition of
MAPK/Erk by U-0126 decreases the activity and expression of MMP-9, MMP-2, and MMP-1 (Fig. 5), suggesting that MAPK/Erk is involved in the regulation of MMP in MDR cells, whereas MAPK/p38 does not appear to be essential. This difference may reflect the distinct signaling pathways involved in the regulation of MMP production by fibroblasts. MAPK/Erk also was reported to activate MMPs in keratinocytes and vascular smooth muscle cells (40, 41). Therefore, the induction of MDR1 and EMMPRIN-regulated MMP expression appear to use a common signaling pathway, and could be part of cellular response to cytotoxic agents. However, whether EMMPRIN expression is up-regulated in parallel with the induction of MDR1 during the development of drug resistance, and the mechanism by which EMMPRIN is up-regulated, remain unknown. We found that knockdown of MDR1 expression by RNA interference did not affect EMMPRIN expression (data not shown), suggesting that EMMPRIN does not lie directly downstream of MDR1. Work in progress is designed to determine whether EMMPRIN can up-regulate MDR1.

MMPs can degrade components of the extracellular matrix, including fibrillar and non-fibrillar collagens, fibronectin, laminin, and basement membrane glycoproteins. Numerous studies have demonstrated a close association between the expression or activity of distinct MMPs and tumor cell invasion and metastasis (42). MDR cells have been reported to show paradoxical metastatic behavior. We and others found that untreated MDR cells were less metastatic than their parental counterparts (9, 10, 13); yet, others have shown increased invasive potential (7, 8). Our current results indicate that MMP expression alone does not fully explain the invasive behavior of MDR cells. Although all MDR cell lines tested express greater amounts and activity of EMMPRIN and MMPs (Figs. 1–3), their invasive ability varied in a Matrigel invasion assay (Fig. 6). Whereas MDR MCF-7 cells displayed increased invasiveness, MDR KB and A2780 cells showed decreased invasiveness as compared to the sensitive cell lines (Fig. 6). Invasion is a multi-step process that involves proteolytic degradation of extracellular matrix and basement membranes, altered cellular adhesion, and locomotion (43). Thus, the ultimate metastatic behavior will depend on each of these factors. In fact, MCF-7/AdrR cells showed increased
motility, whereas KBV-1 and A2780Dx5 cells had decreased cellular motility as compared to their sensitive counterparts (data not shown). In addition, the existence of anti-invasion factors such as tissue inhibitors of metalloproteinases provides another level of control. Therefore, despite the increased MMP expression and activity in MDR cells, overall invasiveness reflects a complex array of cellular and extracellular factors. In MDR MCF-7 cells in which a correlation exists between EMMPRIN and MMPs expression and invasive capacity, treatment with an anti-EMMPRIN antibody significantly inhibited the in vitro invasion (Fig. 7), suggesting that the increased expression of EMMPRIN and MMPs can play a role in invasion in some drug-resistant cancers.

In summary, these studies demonstrate that overexpression of EMMPRIN may underlie the up-regulation of MMP activity in MDR cancer cells, and provide further insight into the relationship between drug resistance, invasion, and metastasis.

**Materials and Methods**

**Cell Lines and Culture**

The MDR human breast cancer cell line, MCF-7/AdrR (44), and its sensitive parental line, MCF-7, were kindly supplied by Dr. Kenneth Cowan of the Eppey Institute for Research in Cancer (Omaha, NE) and were maintained in RPMI 1640 containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂/95% air. The MDR human oral carcinoma line, KBV-1 (45), and the sensitive parental line, KB3-1, were grown in DMEM containing 10% FBS under the identical conditions described above. For KBV-1, 1 µg/ml of vinblastine was added in the medium for the maintenance of the MDR phenotype. A2780 and A2780Dx5 cells (46) were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY). All cultures were checked routinely for contaminants of Mycoplasma or fungi. All cell lines were discarded after 3 months and new lines obtained from frozen stocks.

**Immunofluorescence Staining**

Cells grown on four-chamber, polystyrene vessel tissue culture glass slide (Becton Dickinson/Collaborative Biomedical Products, Bedford, MA) were washed three times with PBS and fixed with 95% ethanol for 10 min. Cells were then incubated with 95% ethanol for 10 min. Cells were then incubated with blocking solution consisting of 3% milk in PBS containing 2% FBS. Cells were then incubated with monoclonal anti-CD147 (EMMPRIN) antibody (BD PharMingen) for 60 min, followed by incubation with FITC-conjugated anti-mouse IgG or anti-rabbit IgG for 30 min. After washing twice with PBS, the cells were analyzed on the FACSScan (Becton Dickinson Cytometry System, Mansfield, MA).

**Zymographic Analysis**

MMP activity was assayed using zymography. Conditioned media from cells cultured in the absence of serum for 72 h were collected and concentrated 10-fold using Centriprep YM-30 (Millipore, Bedford, MA). Concentrated conditioned media (10 µg of protein) were mixed with Laemmli loading buffer (without reducing agent) and subjected to gelatin (Difco Laboratories, Detroit, MI) or casein (Sigma) zymography as previously described (47). Briefly, samples were electrophoresed at 4°C on 8% SDS-polyacrylamide gel containing 0.1% gelatin or on 10% SDS-polyacrylamide gel containing 0.1% casein. Gels were then washed in 2.5% Triton X-100 at room temperature to remove SDS, followed by incubation at 37°C for 40 h in 50 mM Tris-HCl (pH 7.6), 5 mM CaCl₂, 1 mM ZnCl₂, 0.02% Brij-35, and 150 mM NaCl. MMP activity was visualized as clear bands with Coomassie Brilliant Blue R-250 staining.

**Western Blot Analysis**

Conditioned cell-culture media (10 µg proteins) or mouse ascites (50 µg proteins) were separated by 10% SDS-PAGE under reducing conditions. Transfer of proteins to nitrocellulose was carried out by the method of Towbin et al. (48). The blots were incubated in blocking solution consisting of 3% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 at room temperature for 1 h, then immunoblotted with rabbit anti-MMP-1, anti-MMP-2, anti-MMP-9, or other MMP antibodies (Sigma). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). β-Actin was used as a loading control. Protein expression was quantified by Molecular Analyst software (Bio-Rad Laboratories).

**Reverse Transcription-PCR**

Total RNA was extracted from cells with TRIzol reagent (Life Technologies) using the protocol provided by the manufacturer, and quantified by UV absorbance. The RT reaction was performed by using the SUPERScript First-Strand Synthesis System (Life Technologies) in a final volume of 20 µl containing 5 µg total RNA, 200 ng of random hexamers, 1 × RT buffer, 2.5 mM MgCl₂, 1 mM dNTP mixture, 10 mM DTT, RNaseOUT recombinant ribonuclease inhibitor, 50 units of Superscript reverse transcriptase, and diethylpyrocarbonate-treated water. After incubation at 42°C for 80 min, the RT reaction was terminated by heating at 70°C for 15 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 µl of cDNA template, 1.5 mM MgCl₂, 2.5 units of Tag polymerase, and 0.5 µM primers of MMP-1 (ATTGGAGCGACGAGGGGC; GTCCACATCTGCTTGTGC) that generate a 459-bp fragment, of MMP-2 (GGCAACTGGAATACCTTGAGAC; CAAAGCTCATAGCTCATGTC) that generate a 500-bp fragment, and of MMP-9
(GAGGAATACCGTACCCGCTATG; CAAACCAGTTTG-GAACACCAC) that generate a 531-bp fragment. GAPDH primers (GCCAAGAGGTCCATCCTAC; GTTGGCCAGGGA-GTGTTGTT) (49), which amplify a 358-bp fragment, were used as internal control. Amplification cycles were: 94 °C for 3 min, then 33 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min, followed by 72 °C for 15 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

**Invasion Assay**

Invasive ability of tumor cells was measured using a method similar to that as described by Albini et al. (50). Briefly, 13-mm polyvinylpyrrolidone-free polycarbonate filters of 8-µm pore size were coated with commercial Matrigel® (Becton Dickinson/ Collaborative Biomedical Products), dried overnight at room temperature, and reconstituted with serum-free medium for 30 min at 37 °C. Sensitive and MDR cells in log phase of growth were suspended in RPMI 1640 supplemented with 0.1% BSA and plated on Matrigel-covered filters in a Boyden chamber (5 × 10^5 cells/well). After incubation at 37 °C for 24 or 48 h, the cells that reached the underside of the Matrigel-coated membrane were stained with H&E and counted under a microscope in four predetermined fields at a magnification of 200×.

**References**


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