Interference with the Complement System by Tumor Cell Membrane Type-1 Matrix Metalloproteinase Plays a Significant Role in Promoting Metastasis in Mice

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Abstract
Neoplasms have developed strategies to protect themselves against the complement-mediated host immunity. Invasion- and metastasis-promoting membrane type-1 (MT1) matrix metalloproteinase (MMP) is strongly associated with many metastatic cancer types. The relative importance of the individual functions of MT1-MMP in metastasis was, however, unknown. We have now determined that the expression of murine MT1-MMP in murine melanoma B16F1 cells strongly increased the number of metastatic loci in the lungs of syngeneic C57BL/6 mice. In contrast, MT1-MMP did not affect the number of metastatic loci in complement-deficient C57BL/6-C3−/− mice. Our results indicated, for the first time, that the anticomplement activity of MT1-MMP played a significant role in promoting metastasis in vivo and determined the relative importance of the anticomplement activity in the total metastatic effect of this multifunctional proteolytic enzyme. We believe that our results shed additional light on the functions of MT1-MMP in cancer and clearly make this protease a promising drug target in metastatic malignancies.

Introduction
Controlled activation of the complement system is a critical component of host immunity (1, 2). Complement activation products stimulate a localized protective inflammation, which is involved in both inductive and effector phases of an immune response (3). Complement can be activated via three pathways: (a) antibody-dependent classic, (b) alternative, and (c) lectin (4). In the classic pathway, immunoglobulin-coated targets bind and then activate the first complement component C1. This event initiates the complement activation cascade and results in the generation of anaphylatoxins (C3a, C4a, and C5a) and cytolytic C5b-9 membrane attack complex (5). Tumor cells frequently overexpress the specialized cell surface–associated proteins, including the proteases, to inhibit the complement propagation and thus to protect themselves against the complement-mediated cytolytic (6–13).

Membrane type-1 (MT1) matrix metalloproteinase (MMP) is the most common membrane-tethered protease of the MMP family (14). MT1-MMP plays an important, albeit insufficiently characterized, role in tissue remodeling and cell motility and especially in tumor progression, angiogenesis, and metastasis (14–17). Our recent data suggested that MT1-MMP, in addition to its pericellular proteolytic function (18, 19), protects malignant cells from complement-mediated cytotoxicity (20). We have also determined that the His171-Glu-Lys-Gln-Ala-Asp176 and Val223-Arg-Asn224 peptide sequences of the catalytic domain of MT1-MMP are directly involved in binding with the complement component C1q (21). Both sequence regions are spatially distant from the active site of MT1-MMP; therefore, whether active or inactive, the protease species efficiently bind but do not cleave C1q. We have also shown that MT1-MMP efficiently cleaves C3b, a central protein of all complement propagation pathways (22), and that this cleavage liberates the deposited C3 activation fragments from the cell surface. The C3b cleavage fragment (iC3b) serves as a receptor of natural killer (NK) cells, which are the most potent, tumor-eliminating, immune weapon of the host. MT1-MMP, by shedding iC3b from the cell surface, interferes with anticancer cellular immunity (23). Consequently, MT1-MMP inhibits the complement cascade and protects tumor cells from complement-mediated injury. Based on these results, we hypothesized that in vivo MT1-MMP empowers tumors with a potent mechanism to escape the complement system and promote the survival of malignant cells in the bloodstream and, through this mechanism, facilitate overall metastasis.

To determine both precise role and relative importance of the anticomplement activity of MT1-MMP in the metastatic process, we used metastatic murine melanoma B16F1 cells (24, 25) and syngeneic C3-deficient mice (26). B16F1 cells were stably transfected with murine MT1-MMP and, as a result, expressed the levels of MT1-MMP that were comparable with fibrosarcoma HT1080 cells and other human cells, which express MT1-MMP naturally (20). Using this in vivo cell and animal model, we determined that MT1-MMP efficiently protected metastasizing cells from complement-mediated injury and that the anticomplement function of MT1-MMP plays a highly significant, albeit unrecognized previously, role in the metastatic process. Overall, our results extend our previous, in vitro observations and show that, after induction of MT1-MMP, malignant cells acquire metastatic potential.

Materials and Methods
Proteins, antibodies, and general reagents. Biochemical and cell culture reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise. Human fibrosarcoma HT1080 cells were obtained from American Type Culture Collection (Manassas, VA). Murine breast carcinoma 4T1 and melanoma B16F1 cells were kindly provided by Dr. Ralph A. Reisfeld (The Scripps Research Institute, La Jolla, CA). Pro-MMP-2, free from
tissue inhibitor of metalloproteinases-2, was isolated from the medium conditioned by p2AH2A72 cells (a derivative of HT1080 fibrosarcoma cell line doubly transfected with the E1A adenoviral gene and MMP-2; ref. 27). Antiserum against murine MT1-MMP was raised in rabbits against the individual catalytic domain of murine MT1-MMP (mu-MT1-CAT), and the polyclonal antibody was purified from the immune serum by using a Protein G column. A murine 5D1 monoclonal antibody was raised against the catalytic domain of human MT1-MMP in our laboratory. According to our tests, the 5D1 antibody efficiently cross-reacts with murine MT1-MMP.

MT1-MMP constructs and cell transfection. The wild-type, full-length, murine MT1-MMP gene was cloned by PCR from the FVB/N mouse cDNA gene library. The 5'-ATGTGCTCCGGCCCTCCCTGGA-3' and 5'-TCAGACCTTGTCAGCAAGCCTG-3' oligonucleotides were used as direct and reverse primers, respectively. The identity of the MT1-MMP gene was confirmed by DNA sequencing. The oligonucleotide sequence of murine MT1-MMP has been submitted to GenBank (accession no. DQ249870). The MT1-MMP construct was recloned into the pCDNA3-seoz plasmid (Invitrogen, Carlsbad, CA) under the control of the cytomegalovirus promoter. Murine melanoma B16F1 cells were stably transfected with the MT1-MMP construct using LipoFectAMINE (Invitrogen). Cell clones resistant to 0.6 to 0.8 mg/mL zeocin were further selected by Western blotting with the mu-MT1-CAT antibody. To avoid clonal effects, transfected cell lines were generated as a pool of positive cell clones (five clones for a cell line; B16F1-MT1 cells). Control cells transfected with the original pcDNA3-seoz plasmid were generated as a pool of zeocin-resistant cells (B16F1-zeo cells). The levels of expression of MT1-MMP were additionally confirmed by fluorescence-activated cell sorting (FACS) analysis, gelatin zymography, and immunostaining of the MT1-MMP-transfected cells. Transfected cells were routinely grown in DMEM supplemented with 10% FCS and 0.2 mg/mL zeocin.

Cloning of mur-MT1-MMP. The primers, 5'-CCGCTGGAGAAAGAAGAGGTCGGAGCTGCGCTGCCCTCTGGGTCCTG-3' (direct primer) and 5'-TAAGAATGCGGCCGCTTATCCATAAAGTTGCTGGATGCC-3' (reverse primer), were used to isolate the DNA fragment encoding for the Ala21-Gly284 mu-MT1-CAT. The cDNA fragment was further recloned into the pPIC9 plasmid (Invitrogen), and its structure was confirmed by sequencing. The pPIC9 plasmid carrying the mu-MT1-CAT insert was expressed in Pichia pastoris as described earlier (28). The medium was used to purify mur-MT1-CAT by ammonium sulfate precipitation (80% saturation) followed by fast protein liquid chromatography of the precipitated material. The fractions containing mu-MT1-CAT were pooled and used to raise antibodies in rabbits.

Immunocapture and Western blotting. Cells were surface biotinylated for 1 hour on ice with 0.1 mg/mL sulfo-N-hydroxysuccinimide-LC-biotin (Pierce, Rockford, IL). Labeled cells (5 × 10^5 to 10^6) were lysed in TBS (pH 7.4) containing 50 mM L-octyl-p-D-glucopyranoside, 1 mM L-CaCl2, 1 mM L-MgCl2, 1 mM L-phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin for 1 hour on ice. Insoluble material was removed by centrifugation. Aliquots (each containing 1 mg of total protein) were incubated overnight at 4°C with 50% streptavidin-agarose slurry (Sigma). After several washings, the beads were boiled for 5 minutes in 2× SDS sample buffer containing 50 mM L-DTT to release and reduce the captured proteins. Eluted proteins were separated by electrophoresis on 10% acrylamide gels and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). Bands containing biotin-labeled proteins were visualized by using the mu-MT1-CAT antibody followed by goat anti-rabbit IgG conjugated with horseradish peroxidase and a TMB/M substrate (Chemicon, Temecula, CA).

Flow cytometry. Cells were incubated at 37°C with the mu-MT1-CAT antibody (5 μg/mL) in Dulbecco's PBS (DPBS) supplemented with 1 mM L-CaCl2, 1 mM L-MgCl2, 1% bovine serum albumin (BSA), and 0.02% sodium azide (DPBS/BSA; pH 7.2). Following 1 hour of incubation, cells were washed with DPBS/BSA and incubated with a 1:200 dilution of F(ab')2 fragment of donkey anti-rabbit IgG conjugated to FITC (Chemicon) for 30 minutes. After removal of unbound antibodies, cells were resuspended in DPBS/BSA supplemented with 3 μg/mL propidium iodide (Sigma), and viable cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Population gates were set by using cells incubated with normal rabbit IgG.

Activation of MMP-2. B16F1, 4T1, and HT1080 cells (1 × 10^5 per well) were each plated in wells of a 48-well plate. After incubation overnight, DMEM-10% FBS was replaced with serum-free DMEM (0.2 mL). Where indicated, B16F1 and 4T1 cells were supplemented with purified pro-MMP-2 (10 ng/mL), whereas HT1080 cells synthesized pro-MMP-2 naturally. After incubation for 16 hours, medium aliquots were analyzed by gelatin zymography on 10% acrylamide gels containing 0.1% gelatin to detect proenzyme, activation intermediate, and mature enzyme of MMP-2 (68, 64, and 62 kDa, respectively; ref. 27).

In vitro cell invasion. Cell invasion assays were done in wells of a 24-well Transwell plate with an 8-μm pore size (Costar, Cambridge, MA; refs. 29, 30). Transwell inserts were each coated with 100 μL Matrigel (0.15 mg/mL) and dried overnight. Inserts were rehydrated in 500 μL DMEM for 2 hours immediately before the experiments. Cells (5 × 10^5/mL; 0.1 mL/well) were loaded in wells of the Transwell plate. The bottom chamber of each well contained 600 μL DMEM-10% FCS. Cells were allowed to invade for 12 hours. The medium was then aspirated, and 300 μL 0.2% crystal violet in a 20% methanol/water solution was added to the bottom of each well for 10 minutes (31). Inserts were washed thrice with water. The colored cells were detached and lysed for 30 minutes in 1% SDS (250 μL). The A_570 nm value of the lysates was measured using a plate reader.

Immunostaining. Cells were plated onto Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL). Following incubation for 48 hours, cells were fixed for 20 minutes with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 for 5 minutes. Cells were stained with the MT1-MMP antibody followed by goat anti-rabbit IgG conjugated with Alexa Fluor 568 (Molecular Probes, Eugene, OR). Following several washes with PBS, the slides were mounted in SlowFade Light Antifade solution (Molecular Probes). Images were acquired at ×600 original magnification on an Olympus BX51 fluorescent microscope equipped with a cooled Magnafire camera (Olympus, San Diego, CA).

Cytotoxicity assay. B16F1-zeo and B16F1-MT1 cells (1 × 10^5 each) were grown in DMEM-10% FCS in wells of a 48-well plate. The cells were then resuspended in serum-free DMEM and coincubated for 2 to 3 hours at 37°C in 20% to 40% normal murine serum to induce the activation of the complement pathway and the lysis of the cells by the resulting membrane attack complex (MAC). The cells were then stained with propidium iodide and counted on a FACS.

Animal experiments. Normal 6-week-old female C57BL/6 mice were purchased from Harlan Laboratories (Houston, TX). The generation of C57BL/6-ICS/-C3-deficient mice has been described earlier (26, 32). C57BL/6 and C57BL/6-ICS/-C3/- mice were injected i.v. with B16F1-zeo and B16F1-MT1 cells each (1 × 10^5 per animal in 0.1 mL PBS). All mice were maintained with the Burnham Institute for Medical Research Animal Care Facility (La Jolla, CA) according to the Institutional Animal Care and Use Committee procedures. In 25 days, mice were sacrificed. The heart, lungs, trachea, and thymus were excised and weighed as described (33). The lungs were inflated intratracheally with 1 to 2 mL of 15% India ink solution using a 31-gauge needle. The lungs were then fixed and bleached with Fekete's solution (3.7% formaldehyde, 70% ethanol, and 5% glacial acetic acid) for 24 hours. Metastases in the lungs were counted using a stereoscope. All data were analyzed using the StatMost software (DataMost, Sandy, UT) and the Fisher's Least Significant Difference Test.

Results

B16F1-MT1 cells produce physiologically relevant levels of MT1-MMP. To determine the role of MT1-MMP in metastasis, the parental murine melanoma B16F1 cells were transfected with the pcDNA3.1-zeo plasmid carrying the cDNA sequence of the full-length murine MT1-MMP (B16F1-MT1 cells). Transfection of parental B16F1 cells with the original plasmid was used to generate

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the control B16F1-zeo cells. High levels of surface expression of MT1-MMP in B16F1-MT1 cells, when compared with that in B16F1-zeo cells, were confirmed by immunoprecipitation followed by Western blotting and flow cytometry (Fig. 1). At the same time, the levels of cell surface–associated, recombinant, MT1-MMP in B16F1-MT1 cells were comparable with those in murine breast carcinoma 4T1 cells, which express the protease naturally (Fig. 1). Immunostaining of the permeabilized B16F1-zeo and B16F1-MT1 cells also confirmed that, after transfection with the MT1-MMP gene, the concentrations of cellular MT1-MMP significantly increased (Fig. 2). This immunostaining pattern is normal for MT1-MMP in permeabilized cells. It is well known that the significant amounts MT1-MMP are sorted to a transient storage compartment (trans-Golgi network/endosomes), where the protease is available for rapid trafficking to the plasma membrane and cell surface proteolytic activity (34).

To exclude possible artifacts caused by MT1-MMP overexpression and to show that the transfected cells exhibited physiologically relevant rather than aberrantly high levels MT1-MMP, we compared the levels of the MT1-MMP proteolytic activity in melanoma B16F1-MT1, breast carcinoma 4T1, and fibrosarcoma HT1080 cells. Because of the distinct antigenic properties of human and murine MT1-MMP, the use of Western blotting and flow cytometry was not expected to produce the quantitative assessment of MT1-MMP in human HT1080 and murine 4T1 and B16F1 cells. We overcame this problem and then used gelatin zymography to determine the level of MMP-2 activation by cellular MT1-MMP. HT1080 cells produce MT1-MMP and MMP-2 naturally (35). 4T1 cells synthesize MT1-MMP, but not MMP-2, naturally (Fig. 1). The efficiency of the MT1-MMP-mediated activation of MMP-2 and the rate of conversion of the 68-kDa proenzyme into the 64-kDa intermediate and then into the active, mature 62-kDa enzyme were used as measures of the cell surface–associated MT1-MMP activity. Gelatin zymography was used to visualize the levels of MMP-2 activation. HT1080, B16F1, and 4T1 cells were each incubated for 16 hours in a serum-free medium. Where indicated, B16F1 and 4T1 cells were supplemented in these assays with external proMMP-2 in amounts that were similar to those naturally synthesized by HT1080 cells. Our data indicate that, in the transfected B16F1-MT1 cells, the efficiency of the MT1-MMP-dependent step of the MMP-2 activation pathway (the conversion of the 68-kDa zymogen into the 64-kDa activation intermediate of MMP-2; refs. 27, 36) was similar to that in HT1080 cells and in 4T1 cells (Fig. 1). These results suggest the presence of similar, physiologically relevant levels of the functional activity of cell surface–associated, mature MT1-MMP in these three cell types.

Consistent with many other cell types (14, 19), B16F1 cells, following transfection with the invasion-promoting MT1-MMP, acquired a migratory phenotype. B16F1-MT1 cells invaded through Matrigel-coated permeable filters with rates significantly greater than those observed in B16F1-zeo control (Fig. 1).

**Cellular MT1-MMP inhibits the complement system and promotes metastasis.** To determine the precise role of MT1-MMP in promoting the survival of tumor cells in the bloodstream and follow-on metastasis, we used syngeneic, complement-deficient C57BL/6-C3−/− homozygote mice. Normal C57BL/6-C3+/+ animals were used as a control.

We specifically selected low metastatic melanoma B16F1 cells for our experiments because we hypothesized that stimulatory effects of MT1-MMP would be more evident on a low metastatic background. B16F1-zeo and B16F1-MT1 cells were each injected i.v. into C57BL/6 and C57BL/6-C3−/− mice. In 25 days, mice were sacrificed, and metastatic loci were counted in the lungs. The lungs of C57BL/6 and C57BL/6-C3−/− mice were also weighed. The number of metastatic loci was significantly higher in C57BL/6-C3−/− mice when compared with control animals, thus confirming an important antimetastatic function of the complement system. The metastatic efficiency of B16F1-zeo and B16F1-MT1 cells was similar in the complement-deficient C57BL/6-C3−/− animals. In contrast, B16F1-MT1 and B16F1-zeo cells were highly and poorly metastatic in C57BL/6 mice, respectively (Fig. 3). These findings clearly showed that the complement-mediated cytotoxicity and/or the complement-dependent host immune response played a significant role in suppressing the metastatic process and that cellular MT1-MMP is highly efficient in inhibiting the complement-induced cytotoxic responses. Figure 3 shows that the anticomplement activity of MT1-MMP constitutes at least 50% of the total metastasis-promoting effect of this multifunctional protease.

As judged by the increased weight of the lungs, MT1-MMP, after the establishment of the B16F1-MT1 micrometastasis, seemed to play a role in supporting the subsequent tumor growth in normal
mice. Our observations agreed with the earlier results of other investigators and emphasized the important role of MT1-MMP in the tumor growth of the established malignant loci (15, 19, 37, 38). There was, however, no statistically significant difference between the weight of the lungs with B16F1-zeo and B16F1-MT1 micrometastasis in C57BL/6-C3/C0 mice. The reduced initial weight of the C3 knockout mice when compared with that of normal animals (data not shown) complicated an interpretation of the data.

MT1-MMP protects melanoma B16F1 cells from complement-mediated injury. Earlier, we showed that MT1-MMP protects breast carcinoma MCF7 cells from complement-mediated cytolysis (20). To validate these results in our current cell system, we coincubated B16F1-zeo and B16F1-MT1 cells for 2 to 3 hours in the serum isolated from C57BL/6 and C57BL/6-C3/C0/C0 mice (Fig. 4). This short incubation time is insufficient to induce anoikis of the detached malignant cells; therefore, anoikis did not interfere with the complement-mediated lysis. After incubation, the concentrations of dying cells were identified by the FACS analyses of the cell samples. Both cell types were stable during their incubation in 10% FCS. Neither 20% nor 40% serum prepared from C57BL/6-C3/C0/C0 mice significantly affected the viability of B16F1-zeo and B16F1-MT1 cells. In contrast, the C57BL/6 mouse serum was highly damaging to B16F1-zeo cells, whereas B16F1-MT1 cells were resistant to the serum complement action. Because the levels of antimelanoma immunoglobulins were definitely low in the syngeneic unchallenged C57BL/6 mice, the cytolytic effect of the C57BL/6 serum was likely mediated via the alternative complement activation pathway rather than the classic pathway.

Discussion

The most deadly aspect of cancer is its ability to metastasize. The metastatic process consists of a series of sequential, interrelated steps that are not as yet completely understood (39). To metastasize, malignant cells must break away from the primary tumor, invade the circulatory system, and establish themselves as micrometastases in a new location (40). It is important to understand what happens to cancer cells between the time of their entry into the circulation and the formation of metastases (41). In the bloodstream, cancer cells are attacked by macrophages, phagocytes, NK cells, and the complement (14, 38, 42). To avoid complement-mediated cytolysis, metastatic cells developed multiple strategies, including expression of the cell surface–associated complement regulatory proteins, including CD46/MCP, CD55/DAF, and CD59/protectin (43–45), and proteases that clear the deposited C3b protein from the cell surface (10, 11).

MT1-MMP functions as a membrane activator of soluble MMPs. In addition, MT1-MMP cleaves extracellular matrix and cell adhesion signaling receptors and tumor suppressor proteins, including type I collagen, lumican, laminin, tissue transglutaminase, CD44, integrins, LRP, gC1Q-R/p33, and KiSS 1 (18, 42, 46–51). We have also determined that MT1-MMP interacts with the C1q and C3b components of the complement system and inactivates the complement propagation cascade. Our cell-based tests showed that MT1-MMP expression protected tumor cells from the complement-mediated cytolysis (20). Consequently, we hypothesized that...
MT1-MMP was capable of defending the individual metastasizing tumor cells from the complement system and the follow-on antitumor immunity of the host, thus extending the survival of tumor cells in the bloodstream. The increased survival of tumor cells in the bloodstream likely results in an increased efficiency of metastasis.

To determine the relative importance of the anticomplement function of MT1-MMP in the total metastatic effect of the protease, we used murine melanoma B16F1 cells stably transfected with the murine MT1-MMP gene and syngeneic, complement-deficient C57BL/6-C3−/− homozygote mice. B16F1 cells stably transfected with the original vector and normal C57BL/6 mice were used as controls. The concentrations of functionally active cell surface MT1-MMP in B16F1-MT1 cells were significantly higher than in the B16F1-zeo control, but they were comparable with those in murine breast carcinoma 4T1 cells and human fibrosarcoma HT1080 cells, which express MT1-MMP naturally. Based on these data, we concluded that B16F1-MT1 cells exhibited physiologically relevant rather than aberrantly high levels of MT1-MMP activity.

The metastatic efficiency of B16F1-MT1 cells was ~2-fold higher in C57BL/6 mice when compared with B16F1-zeo cells, whereas, in C57BL/6-C3−/− mice, the metastatic efficiencies of both cells were similar. These results indicate, for the first time, that the anticomplement activity of MT1-MMP plays a highly significant role in promoting the metastatic process in vivo. These data correlate well with our previous results, which determined that MT1-MMP protected tumor cells from direct complement-mediated injury (20). In agreement, our current work determined that the C57BL/6-C3−/− serum was inefficient in inducing cytolyis of both B16F1-zeo and B16F1-MT1 cells. In contrast, the serum from normal C57BL/6 mice caused damage of B16F1-zeo cells, whereas B16F1-MT1 cells were resistant to the complement.

We conclude that the functional link associating MT1-MMP with the host immune system, heretofore unrecognized, empowers metastatic cells with an escape mechanism. This mechanism we believe contributes to the protection of neoplasms against host antitumor immunity and results in the survival of invading and metastatic malignant cells in the bloodstream. Excitingly, the anticomplement activity of MT1-MMP is responsible for at least one-half of the total metastatic effect of the protease. In addition, the cytolytic activity of the complement system in mice is significantly less powerful than that in humans (52). Accordingly, we expect that anticomplement activity of MT1-MMP plays a more significant role in humans when compared with that we observed in our mouse model.

Overall, our data are the first to determine the relative importance of the individual function of this multifunctional protease in the complex biological process of metastasis. We believe that our studies shed additional light on the exceptionally important role that MT1-MMP plays in connection with invading metastatic malignant cells.

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Figure 4. MT1-MMP protects melanoma cells from direct complement-mediated injury. B16F1-zeo and B16F1-MT1 cells were incubated with 10% FCS or with 20% and 40% serum prepared from C57BL/6 and C57BL/6-C3−/− mice. Cells were next stained with 3 μg/mL propidium iodide. The number of the dead, propidium iodide–stained cells in the samples was determined by flow cytometry of the cell samples. Histograms, percentage of dead cells in each sample was calculated. RFI, relative fluorescence intensity.


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