Cellular Membrane Type-1 Matrix Metalloproteinase (MT1-MMP) Cleaves C3b, an Essential Component of the Complement System*

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Neoplasms have developed numerous strategies to protect themselves against the host immune system. Membrane type-1 matrix metalloproteinase (MT1-MMP) is strongly associated with many cancer types and is up-regulated in the aggressive, metastatic neoplasms. During the past few years, there has been an increasing appreciation of the important, albeit incompletely understood, role of MT1-MMP in cancer. We have discovered, using cell-free and cell-based assays in vitro, that MT1-MMP proteolysis specifically targets C3b, an essential component of the complement propagation pathway. MT1-MMP proteolysis liberates the deposited C3 activation fragments from the cell surface. The shedding of these cell-deposited opsonins by MT1-MMP inhibits the complement cascade and protects breast carcinoma MCF7 cells from direct complement-mediated injury in the in vitro tests. The functional link associating MT1-MMP with the host immune system, heretofore unrecognized, may empower tumors with an escape mechanism that contributes to the protection against the host anti-tumor immunity as well as to the survival of invading and metastatic malignant cells in the bloodstream.

It is well established that the progression of metastatic cancer involves the interplay of the host environment with the malignant cells (1). Neoplasms employ multiple means to sustain themselves and to proliferate in vivo. Evidence has emerged that tumor immunity is an important defense mechanism protecting malignant cells from the host immune surveillance. The host immune system is an apparatus directed against foreign invading organisms and tumor cells (2–4). Controlled activation of the complement system is a critical component of host immunity. Complement activation products stimulate a localized protective inflammation and are involved in both the inductive and effector phases of an immune response (5). In many cases malignant cells exhibit antigens that are not typically associated with normal cells. These antigens can be identified by the complement system via antibody recognition and, as a result, the recognized cells are attacked by the immune system (6).

The complement system is comprised of soluble proteins that interact in a stepwise manner. Complement can be activated via three different pathways: the classical pathway that is usually antibody-dependent, and the alternative and lectin pathways. In the classical pathway, immunoglobulin-coated targets bind and subsequently activate the complement component C1. This event starts the complement cascade, the propagation of which results in the generation of anaphylatoxins (C3a, C4a, and C5a) and ultimately the cytolytic C5b-9 membrane attack complex (MAC) (7).

In the process of complement propagation, proteolytic cleavage of serum C3 and C4 creates transient soluble C3b and C4b products with an exposed reactive thioester group. Once exposed, the thioester group forms amide and ester bonds with the target cell surface molecules. This binding of C3b and C4b is critical for amplification of the cascade and for MAC formation. Subsequent proteolytic cleavage transforms the cell-bound C3b into the cleavage fragments iC3b, C3dg, and C3d, which remain covalently attached to the cell surface. These C3 fragments serve as ligands for receptors on phagocytic and NK cells. Opsonization of target cells with these C3 fragments promotes and enhances both antibody-dependent and complement-dependent cell cytotoxicity, two additional effector systems that play an important role in the elimination of neoplastic cells (8, 9). The deposition of C3b and C4b and the follow-up amplification of the complement cascade also results in the generation of soluble bioactive C3a and C5a peptides that may potentiate anti-tumor responses via their chemoattractant and proinflammatory activities. Thus, the inactivation of C3b and C4b and their removal from the cell surface represents an important immune evasion mechanism of tumor cells.

An important role for complement resistance of tumor cells is indicated by the fact that many tumor cells overexpress one or more of the cell surface-associated complement regulatory proteins: CD46/MCP, CD55/DAP, and CD59/protectin. These regulatory proteins act at different stages of the complement propagation (10–13). In addition to the cell-associated regulatory proteins, some tumor cells bind serum complement inhibitory proteins (14, 15), express proteases to clear the C3b protein from the cell surface, and inhibit the complement cascade (16–18).

Membrane type-1 matrix metalloproteinase (MT1-MMP) is the most common protease from the membrane-tethered enzyme subfamily of MMPs (19). MT1-MMP plays an important, albeit insufficiently characterized, role in tissue remodeling and cell motility, and especially in tumor progression, meta-

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1 The abbreviations used are: MAC, membrane attack complex; FBS, fetal bovine serum; HRP, horseradish peroxidase; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type-1 matrix metalloproteinase; TIMP-2, tissue inhibitor of metalloproteinases-2; DMEM, Dulbecco’s modified Eagle’s medium; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter.
tasis, and angiogenesis (20, 21). MT1-MMP is a cell surface activator of soluble pro-MMP-2 and pro-MMP-13 and has also been implicated in matrix proteolysis and turnover as well as in the proteolytic processing of cell surface-associated adhesion and signaling receptors (19, 22, 23). Although MT1-MMP is detectable in normal tissue, the expression of this protease is strongly associated with aggressive, invasive malignant cells (19, 24).

Here, we report a novel, unexpected and highly significant function of MT1-MMP in the proteolysis of the opsonic complement components C3b and C4b. This proteolysis efficiently inhibits complement activation in cell-based models. We suspect that MT1-MMP is likely to make malignant cells more resistant to complement-mediated cytotoxicity in vivo. Our data indicate that this novel function of MT1-MMP is involved in the release of C3b from the tumor cell surface, and is likely to contribute to the survival and propagation of malignant cells. We believe that the proteolysis of the complement components by MT1-MMP is a powerful and efficient mechanism employed by aggressive malignant cells to protect themselves against host complement, immune surveillance, and destruction.

**MATERIALS AND METHODS**

**Antibodies and Reagents**—All reagents were purchased from Sigma unless otherwise indicated. Purified human C3b, iC3b, and C4b, goat anti-human C3, and antibodies, normal human serum, and C5-depleted human serum were from Advanced Research Technologies. Monoclonal murine antibody H206 against human C3b α chain was purchased from Research Diagnostics. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and HRP-conjugated F(ab')2 fragment goat anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories. Rabbit antibody AB515 against the hinge domain of C3b was purchased from Zymed. Rabbit anti-human C4d antibody and anti-human C3d antibody, and TM/E substrates, and GM6001 (a broad-range hydroxamate inhibitor of MMPs) were from Chemicon. Monoclonal anti-human CD59 monoclonal antibody BRC229 and murine anti-human CD55 monoclonal antibody 1A10 are as described earlier (25, 26). Murine anti-human CD46 monoclonal antibody M75 was obtained from BD Pharmingen. The recombinant catalytic domain of human MT1-MMP (MT1-CAT) was expressed in Escherichia coli. The recombinant catalytic domain of human MT1-MMP, HRP-conjugated rabbit antibodies, and TM/E substrates, and GM6001 (a broad-range hydroxamate inhibitor of MMPs) were from Chemicon. Monoclonal anti-human CD59 monoclonal antibody BRC229 and murine anti-human CD55 monoclonal antibody 1A10 are as described earlier (25, 26). Murine anti-human CD46 monoclonal antibody M75 was obtained from BD Pharmingen. The recombinant catalytic domain of human MT1-MMP (MT1-CAT) was expressed in Escherichia coli.

**Cell Lines**—Human breast carcinoma MCF7 cells (ATCC) stably transfected with the empty pcDNA3-ezoe vector (Invitrogen) (control MCF-zeo cells) and the full-length wild type MT1-MMP (MCF-MT cells) were constructed and extensively characterized in our prior work (28). Human fibrosarcoma HT1080 cells, which synthesize MT1-MMP and MMP-2 naturally, were obtained from ATCC. Transfected cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Irvin Scientific) supplemented with 10% fetal bovine serum (FBS) (Tissue Culture Biologicals) and 0.2 mg/ml mecon. Human breast carcinoma BT549, MCF7, MDA-MB-231, T47D, and SK-Br-3 cells (ATCC) were propagated in DMEM supplemented with 5% FBS and penicillin-streptomycin (100 IU/ml and 100 μg/ml).

**MMP-2 Activation by MT1-MMP and Gelatin Zymography**—TIMP-2 free-pro-MMP-2 was isolated from a conditioned medium of p2HA2T2AG cells derived from an HT1080 fibrosarcoma cell line sequentially transfected with the E1A and MMP-2 cDNAs (29). MCF7-zeo and -MT cells (1 × 105 cells each) were incubated for 12 h in DMEM supplemented with pro-MMP-2 (20 ng/200 μl of medium). HT1080 cells (1 × 105 cells) were incubated 12 h in DMEM (200 μl) alone. Where indicated, PMA (5 μg/ml) was added to the cells. Aliquote (10 μl) of medium conditioned by the cells were analyzed by gelatin zymography.

**Immunoprecipitation and Western Blotting**—HT1080, MCF, and MCF-MT cells were grown in DMEM/FCS. Where indicated, PMA (5 μg/ml) was added to the cells. After incubation for 12 h, cells were washed with PBS and surface biotinylated with sulfo-NHS-LC-biotin (Flcro) according to the manufacturer's instructions. Next, cells were washed with ice-cold PBS and lysed with 50 mM N-octyl-p-o-glucopyranoside (Amresco) in PBS supplemented with 1 mM CaCl2, 1 mM MgCl2, and protease inhibitor mixture containing 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of aprotonin, pepstatin, and leupeptin. The lysates were pre-cleared with Protein G-agarose beads (Calbiochem). The samples of cell lysates each containing 1.0 mg of protein were mixed with an MT1-MMP antibody (1 μg) and Protein G-agarose, and incubated at 4 °C overnight. After extensive washings, immune complexes were released by boiling the beads for 5 min in 2× SDS sample buffer containing 50 mM dithiothreitol. Solubilized proteins were subjected to SDS-PAGE and Western blotting (28).

**MT1-MMP Proteolysis of C3b and C4b in Vitro**—Purified human C3b and C4b (200 ng each) were co-incubated with MT1-CAT (0.2–20 ng) at 37 °C in 15 μl of 50 mM HEPES, 10 mM CaCl2, 0.5 mM MgCl2, 50 μM ZnCl2, 0.01% Brij-35, pH 6.8. Where indicated, GM6001 (5 μM) was added to the reactions to block MT1-MMP activity. The cleavage samples were separated by SDS-PAGE in 10% gels followed by Western blotting with goat anti-human C3 and C4 antibodies (1 μg/ml each in PBS supplemented with 1% BSA and 0.1% Tween 20), HRP-conjugated rabbit anti-goat secondary antibodies (1:5,000 dilution), and the TMB/M substrate.

**MT1-MMP Proteolysis of the Cell Surface-bound C3b and C4b in MCF7 Cells—MCF-zeo and -MT cells (105 cells per well) were each grown for 48 h in DMEM/FBS in wells of a 48-well plate. The cells were then washed and incubated for 30 min at 37 °C in 20% rabbit anti-MCF7 heat-inactivated serum (56 °C, 30 min). The cell samples, in which sensitization with anti-MCF7 serum was omitted, were used as controls. Sensitized cells were additionally incubated for 5–60 min at 37 °C in 10% or 20% C5-depleted human serum to induce the deposition of the C3b and C4b onto the cell surface. Following extensive washing, cells were subjected for flow cytometry or additionally incubated at 37 °C for 2 h in DMEM/FBS (heat-inactivated) to induce the proteolytic shedding and the release of the cell-bound C3b and C4b into the extracellular milieu. Where indicated, GM6001 (50 μM) was added to DMEM/FBS (heat-inactivated) to block cellular MT1-MMP. The residual levels of cell surface-associated C3b and C4b were measured by Western blotting and flow cytometry.

For flow cytometry analyses the cells were detached by an enzymefree buffer (200 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotonin) to DSMS-FBS (heat-inactivated) to induce the proteolytic shedding and the release of the cell-bound C3b and C4b into the extracellular milieu. Where indicated, GM6001 (50 μM) was co-incubated with the cells for 24 h.

For Western blotting cells were lysed for 1 h at 0 °C in 100 μl of 5 mM Tris-HCl buffer, pH 8.0, containing 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotonin to DSMS-FBS (heat-inactivated) to induce the proteolytic shedding and the release of the cell-bound C3b and C4b into the extracellular milieu. Where indicated, GM6001 (50 μM) was added to PBS supplemented with 1% BSA and 0.1% Tween 20), HRP-conjugated rabbit anti-goat secondary antibodies (1:5,000 dilution), and the TMB/M substrate.

C3b liberated from the cell surface by proteolytic shedding and released into the extracellular milieu was identified by ELISA of the medium samples. For these purposes, MCF-zeo and -MT cells (105 cells each) were incubated for 1 h at 37 °C in DMEM supplemented with 20% C5-depleted human serum to induce deposition of C3b on cell surfaces. Next, unbound material was removed by washing cells with PBS. A mixture of DMEM/FBS (200 μl) was added to the cells. Following incubation for 30–120 min to release the cell-bound C3b, the aliquots of medium were withdrawn for a subsequent analysis of the liberated C3b. Where indicated, GM6001 (50 μM) was co-incubated with the cells to block activity of the cellular MT1-MMP.

**ELISA of Soluble C3b**—Wells of a 96-well plate (Corning) were coated with goat anti-human C3 antibody (5 μg/ml) and then blocked with 1% BSA. Medium aliquots (100 μl) were allowed to bind for 1 h at 37 °C with the antibody-coated wells. The bound C3b was detected with HRP-conjugated rabbit anti-human C3 antibody (1 μg/ml) followed by streptavidin-HRP and the TMB/E substrate. The absorbance of the samples was measured at 450 nm.

**Flow Cytometry of the Complement Regulatory Proteins**—Flow cytometry was used to assess the cell surface expression of complement regulatory proteins CD46, CD55, and C5d9 in MCF-zeo and -MT cells. For these purposes, cells were detached by the enzyme-free buffer (Specialty Media) and co-incubated with the respective primary anti-
body (5 μg/ml each) followed by incubation with fluorescein isothiocyanate-labeled secondary antibody (1:500 dilution). Population gates were set by using cells incubated with normal murine IgG. Cells were analyzed on a FACStar flow cytometer (BD Biosciences).

Cytotoxicity Assay—MCF-zeo and -MT cells (1 × 10^6 each) were grown in DMEM/FBS in wells of an 8-well Lab-Tek chambered glass slide (Nalge Nunc). Heat-inactivated 20% rabbit anti-MCF7 serum was co-incubated with the cells for 30 min at 37 °C. The sensitized cells were then placed for 1 h at 37 °C in 20% normal human serum and then fixed with 4% glutaraldehyde for 1 h at ambient temperature and photographed. Dead cells were made visible with propidium iodide using a LIVE/DEAD RedBiohazard Viability/Cytotoxicity L-7013 Kit (Molecular Probes) and the images were taken by a Nikon Eclipse TE300 fluorescence microscope equipped with a SPOT Real-Time SP402–115 digital camera (Diagnostic Instruments).

Purification and Iodination of TIMP-2—Human TIMP-2 was produced by Chinese hamster ovary cells stably transfected with the C-terminal His-tagged human TIMP-2. TIMP-2 was purified from conditioned medium by eluting chromatography and ion-exchange chromatography using the slightly modified protocol described in Ref. 30. TIMP-2 (10 μg) was labeled with Na^125I (Amersham Biosciences) using the IODO-GEN iodination reagent (Pierce) and separated from unincorporated radioactivity by gel-filtration. Normally, the specific radioactivity of labeled TIMP-2 was 4–5 μCi/μg.

Cell Surface Binding of Radiolabeled TIMP-2—To remove cell-bound endogenous TIMP-2, cells (1.5 × 10^6 cells per well of a 12-well plate) were washed with 50 mM glycine-HCl buffer, pH 3.0, containing 100 mM NaCl, then neutralized with 0.5 M HEPES buffer, pH 7.5, containing 100 mM NaCl and, finally, equilibrated with DMEM supplemented with 20 mM HEPES, pH 7.5, and 0.2% BSA. Increasing concentrations of 125I-TIMP-2 (0.03–14 nM) were added to the cells. Following a 3-h incubation at 4 °C, cells were washed twice with ice-cold 200-fold excess of unlabeled over highest concentration of 125I ligand. Following a 3-h incubation at 4 °C, cells were washed twice with ice-cold 200-fold excess of unlabeled over highest concentration of 125I ligand.

Scatchard plot analysis of binding data demonstrated the existence of high affinity binding sites with KD value. Similarly, BT549 cells exhibited 136,000 MT1-MMP sites/cell counted in cell lysates. Scatchard plot analysis of binding data demonstrated the existence of high affinity binding sites with KD value. Similarly, BT549 cells exhibited 136,000 MT1-MMP sites/cell counted in cell lysates.

RESULTS

Transfected Breast Carcinoma Cells Express Physiologically Relevant Levels of MT1-MMP—We selected breast carcinoma MCF7 cells for our studies because the parental cells are deficient in MT1-MMP and MMP-2 (28). Here, we used MCF7 cells transfected with the wild type MT1-MMP cDNA (MCF-MT cells) as control cells transfected with the original pcDNA3-zeo plasmid (MCF-zeo cells).

To exclude possible artifacts caused by MT1-MMP overexpression and to demonstrate that the transfected cells exhibit physiologically relevant, rather than aberrantly high levels of MT1-MMP, we compared MT1-MMP protease activity in MCF-MT and fibrosarcoma HT1080 cells. HT1080 cells produce MT1-MMP and MMP-2 naturally. 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 employed to visualize the levels of MMP-2 activation. MCF7 cells were supplemented in these assays with external pro-MMP-2 in amounts that were similar to those naturally synthesized by HT1080 cells. To promote the activation of MMP-2, HT1080 cells were stimulated with PMA (5 ng/ml) (31, 32). Our data indicate that the efficiency of MMP-2 activation by the transfected MCF-MT cells is highly similar to that of HT1080 cells (Fig. 1A), suggesting similar levels of cell surface-associated mature MT1-MMP in these two cell types.

To confirm these observations, we directly identified the levels of cell surface-associated MT1-MMP in MCF-MT and HT1080 cells. For these purposes, cells were surface-labeled with membrane-impermeable biotin and then analyzed. Biotin-labeled, cell surface-associated MT1-MMP was immunoprecipitated from cell lysates and detected by Western blotting with streptavidin-HRP (Fig. 1B). These studies demonstrated that cell surface-associated, full-length 60-kDa MT1-MMP was equally represented in HT1080 and MCF-MT cells. In addition, MCF-MT cells exhibit significant amounts of the 43–45-kDa catalytically inactive ectodomain forms of the protease. PMA treatment increased the levels of the degraded MT1-MMP in HT1080 cells. The existence of these full-length and degraded species of MT1-MMP is in agreement with our earlier findings (28) and the results published by others (32). Importantly, these data, which agree well with those of the MMP-2 activation studies, indicate similar levels of the full-length, catalytically potent MT1-MMP in HT1080 and MCF-MT cells.

To extend these comparison studies further, we compared Northern blotting the levels of the MT1-MMP mRNA in fibrosarcoma HT1080 cells with those in breast carcinoma BT549, MDA-MD-231, and MCF7 cells (Fig. 1C). In agreement with the data of Western blotting that did not detect the expression of the MT1-MMP protein in MCF7-zeo cells (Fig. 1B), Northern blotting did not identify any detectable amounts of the MT1-MMP mRNA in this cell type. In turn, the levels of the MT1-MMP mRNA were relatively low in MDA-MD-231 cells but significantly higher in BT549 cells and comparable with the levels of the messenger identified in HT1080 cells.

To substantiate these observations even further, we identified the number of the TIMP-2-binding MT1-MMP cell surface sites in HT1080 and BT549 cells. For these purposes, we incubated the cells with increasing concentrations of radiolabeled TIMP-2. An excess of unlabeled TIMP-2 fully blocked the binding of the labeled inhibitor with the cells. TIMP-1 (a poor inhibitor of MT1-MMP) was incapable of interfering with the binding of the labeled TIMP-2. TIMP-2 (a poor inhibitor of MT1-MMP) was incapable of interfering with the binding of the labeled TIMP-2. PMA treatment with membrane-impermeable biotin and then analyzed. Biotin-labeled, cell surface-associated MT1-MMP was immunoprecipitated from cell lysates and detected by Western blotting with streptavidin-HRP (Fig. 1B). These studies demonstrated that cell surface-associated, full-length 60-kDa MT1-MMP was equally represented in HT1080 and MCF-MT cells. In addition, MCF-MT cells exhibit significant amounts of the 43–45-kDa catalytically inactive ectodomain forms of the protease.
well as in numerous other cell types including breast carcinoma BT549 cells, human umbilical vein endothelial cells, and melanoma G361 cells. Therefore, the effects of MT1-MMP on the complement system reported below with the transfected MCF7 cells are directly applicable to many other cell systems in which this membrane protease is synthesized and activated naturally.

MT1-MMP Efficiently Cleaves C3b in Both Cell-free and Cell-based Systems—Previous studies have demonstrated the susceptibility of cell surface-bound opsonic C3b to several distinct proteases (16–18). To extend these observations and to examine if the complement components C3b, iC3b, and C4b are susceptible to MT1-MMP proteolysis, we co-incubated these purified human components of complement with increasing concentrations of the proteolytically potent catalytic domain of MT1-MMP (MT1-CAT) and analyzed the digest samples by Western blotting. C4b and, especially C3b and iC3b, were cleaved by catalytic amounts of MT1-CAT. Multiple 30–50-kDa cleavage fragments were observed in the C3b/iC3b cleavage reactions. The α chain of both C3 and C4 was especially sensitive to MT1-MMP proteolysis in vitro (Fig. 2). A potent broad-range hydroxamate inhibitor of MMP activity, GM6001 (5 μM), blocked MT1-MMP proteolysis of C3b, iC3b, and C4b.

To investigate if MT1-MMP cleaves the complement components when deposited on a tumor cell surface, we performed proteolytic assays using complement opsonized MCF-7 and MCF-MT cells. To activate complement and deposit C3b and C4b on the cell surface, the cells were preincubated with anti-MCF7 cell lysates with the AB15 antibody. The precipitates were analyzed by SDS-PAGE, followed by Western blotting with streptavidin-HRP and the TMB/M substrate. Positions of molecular mass markers are indicated on the right. C, the levels of MT1-MMP mRNA are similar in HT1080 cells and breast carcinoma BT549 cells. RNA was isolated from HT1080, BT549, MDA-MB-231, and MCF7 cells and analyzed by Northern blotting with a MT1-MMP probe (upper panel). Northern blot analysis of 28S ribosomal RNA (rRNA) was used to ascertain the equal amounts of RNA in each sample (lower panel).

D, Scatchard plot analysis of TIMP-2 binding data for HT1080 and BT549 cells.

FIG. 1. The levels of functionally active MT1-MMP are similar in HT1080 fibrosarcoma, which naturally synthesize the protease, and in MT1-MMP-transfected breast carcinoma MCF-MT cells. A, activation of MMP-2. HT1080, MCF-7eo, and -MT cells (1 × 10⁶ cells each) were incubated for 12 h in 200 μl of DMEM. MCF7 cells were supplemented with the purified TIMP-2-free pro-MMP-2 (100 ng/ml). The amounts of the external MMP-2 added to MCF-7eo and -MT cells were similar to the levels of MMP-2, which are naturally synthesized by HT1080 cells during a 12-h incubation time. Where indicated, PMA (5 ng/ml) was added to the cells. Following incubation, aliquots (10 μl each) of media conditioned by the cells were analyzed by gelatin zymography to determine the conversion of the 68-kDa MMP-2 proenzyme into the 64-kDa activation intermediate and the 62-kDa mature enzyme of MMP-2. Note the existence of equal amounts of the active and inactive forms of MMP-2 in HT1080-PMA and MCF-MT cells. B, cell surface expression of MT1-MMP. Cells were surface biotinylated and lysed. MT1-MMP was immunoprecipitated from the cell lysates with the AB15 antibody. The precipitates were analyzed by SDS-PAGE, followed by Western blotting with streptavidin-HRP and the TMB/M substrate. Positions of molecular mass markers are indicated on the right.
The data were statistically significant (Figs. 4A and 5). The results of three additional experiments were highly similar. The results of a representative experiment performed in triplicate are shown. The results of several additional experiments were highly similar. Sensitized cells, bold line; unsensitized control, thin line, gray shading. M2 region, C3b-positive cells; M1 region, C3b-negative cells.

FIG. 3. Western blotting of cell-bound C3b. Deposition of C3b and C4b on the cell surface was induced by incubating the sensitized MCF-zeo and -MT cells in 10% C5-depleted human serum. In control samples the sensitization with MCF7 antiserum was omitted. The clearance of the deposited C3b and C4b was induced by incubating the cells for 12 h at 37 °C in DMEM/FBS (heat-inactivated). Following cell lysis, the residual levels of the cell surface-associated C3b and C4b were measured by Western blotting of the cell lysate samples.

FIG. 4. MT1-MMP accelerates the release of the cell-bound C3b. MCF7 cells were sensitized by incubation with anti-MCF7 serum or left untreated. Cells were then incubated for 1 h with 10% human C5-depleted serum to stimulate deposition of C3b. Next, incubation of cells for the indicated times in DMEM/FBS was used to stimulate C3b shedding. The levels of the cell surface-associated C3b was identified by FACS, whereas the liberated C3b was determined in the medium aliquots by ELISA. A, histograms of the FACS analysis of the cells. Where indicated, GM6001 (50 μM) was added to the cells. Sensitized cells, bold line; unsensitized control, thin line, gray shading. B, ELISA of the soluble C3b liberated from the surface of MCF7 cells. The medium samples were analyzed as described under "Materials and Methods." The results of a representative experiment performed in triplicate are shown. The results of three additional experiments were highly similar. The data were statistically significant (p < 0.05). A star indicates a statistically significant difference between the groups.

FACS analysis (Fig. 4A) demonstrated that the functionally competent MT1-MMP induced the clearance of the cell surface-bound C3b α subunit in MCF-MT cells. The hydroxamate inhibitor, GM6001, blocked this effect of MT1-MMP. Reciprocally, the concentrations of liberated soluble C3 fragments were higher in the medium conditioned by MCF-MT cells relative to those of MCF-zeo cells. As expected, GM6001 repressed the release of C3 by MCF-MT cells (Fig. 4B). Because treatment of MCF-MT cells with GM6001 resulted in a lower clearance of C3 from the cell surfaces than was observed for untreated MCF-zeo cells, our data also indicate that other proteinases (most probably, ADAM and ADAMTS enzymes, which are sensitive to inhibition by hydroxamate inhibitors) are potentially involved in shedding C3.

To analyze the relationships between MT1-MMP and C3b in more detail, we determined the rate of deposition of C3b on MCF-zeo and -MT cells. For these purposes, antibody-sensitized cells were incubated with human C5-depleted serum for different periods of time and the deposition of C3b was determined by flow cytometry. FACS analysis showed that in 15–60 min the resultant deposition of C3b was significantly lower on MCF-MT cells than on MCF-zeo (Fig. 5). These data agree well with the observations of others (35, 36) who evaluated the effect of the distinct but functionally similar cathepsin L protease on the deposition of C3b on cell surfaces. Our findings show that MT1-MMP inhibits complement activation by clearing cell surface-deposited C3b and C4b from a tumor cell with the resultant release of their respective inactive soluble forms into the extracellular milieu.

MT1-MMP Protects Breast Carcinoma Cells from Complement-Mediated Injury—To investigate further the functional role of MT1-MMP in protecting MCF7 cells from the complement-mediated attack, we performed a kinetic assay. In this assay complement-mediated lysis was assessed by measuring the release of the cytosolic glucose-6-phosphate dehydrogenase from the cells. Our results indicated that the release of glucose-6-phosphate dehydrogenase reached a plateau following a 30-min treatment of MCF-zeo and MCF-MT cells with complement and afterward remained fairly constant for an additional 30 min (Fig. 6A). The lysis by complement was significantly repressed in MCF-MT cells. These data correlate well with the other works where a maximal lytic effect of complement was observed following an ~30-min treatment (37, 38).

To corroborate these findings, we evaluated complement-treated and untreated cells by light microscopy (Fig. 6B). Visual observation showed that MCF-zeo cells did not survive treatment with complement, whereas MCF-MT cells were largely resistant to complement-mediated lysis. We also stained cells with propidium iodide and employed fluorescence
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microscopy to identify the stained, dying cells (Fig. 6B). Following treatment with human complement, MCF-zeo cultures exhibited a large number of dead cells stained with propidium iodide. In contrast, only a few of the propidium iodide-positive cells were observed in MCF-MT cultures.

The release of glucose-6-phosphate dehydrogenase measured in these individual cell samples, directly confirmed the data of Table I. The data from one representative experiment performed in triplicate are shown.

MT1-MMP Does Not Affect the Expression of the Cellular Complement Regulatory Proteins—Tumor cells are known to defend themselves against the complement by expressing the complement regulatory proteins CD35, CD46, CD55, and CD59 (10, 11, 39). CD35, however, is not expressed by MCF7 cells (40). We used flow cytometry to determine the expression levels of CD46, CD55, and CD59 in MCF-zeo and -MT cells. There was no correlation between the expression of MT1-MMP and expression levels of CD46, CD55, or CD59 (Table I). Thus, we exclude the possibility that differential expression of the complement regulatory proteins explains the resistance of MCF-MT cells to complement. Conversely, these data further support the unique role of MT1-MMP in defending malignant cells against complement.

**DISCUSSION**

We believe that MT1-MMP directly contributes to tumor cell immune resistance and is thus associated with the survival of metastatic cells in the bloodstream. The efficient survival of MCF-MT cells in cytotoxicity tests (Fig. 6) strongly supports this hypothesis.

Our *in vitro* and cell-based data show that MT1-MMP inhibits complement by efficiently releasing the C3b and C4b complement components from tumor cell surfaces. Very recently, Tam *et al.* (41) has also demonstrated, by using isotope-coded affinity tag mass spectrometry, that C3 is a target of MT1-MMP proteolysis, thereby directly supporting our data. Conversely, our results identify the temporal and causal link associating MT1-MMP with C3, and demonstrate the biochemical mechanism and the subsequent physiological effect of the MT1-MMP interactions with C3.

Evidently, the complement-suppressing function of MT1-MMP is a novel proteolytic defense mechanism. The multifunctional MT1-MMP enzyme plays a number of important roles in connection with malignant cells. There is a strong correlation of MT1-MMP expression with the tumorigenicity of many cancer cell types (19, 20). In particular, MT1-MMP has been detected in all invasive breast tumor samples characterized by lymph node metastasis and lymph vessel invasion (42–44). In agreement, the recent work of many authors has demonstrated that MT1-MMP is a potent protease involved in the cleavage of the extracellular matrix, in the activation of several soluble MMPs, and in the processing of the cellular adhesion and signaling receptors such as integrins, CD44, tissue transglutaminase, lipoprotein receptor-related protein (LRP), and gC1q-R (45–52). Reciprocally, inhibition of the MMP activity by natural and synthetic inhibitors was shown to reduce tumor growth and metastasis (53).

Our current work supports and extends these earlier studies and provides evidence that the complement proteins C3b and C4b are novel and important targets of MT1-MMP. MT1-MMP decreases the deposition of C3b and C4b at the cell surface, and inhibits amplification of the complement cascade. Notably,
deficient mice will clarify the role of MT1-MMP in the tumor lesions employing the expression of murine MT1-MMP in murine nontumoral cells. Our findings warrant additional studies. The protease plays in connection with invading metastatic malignancies to escape immune attack. These mechanisms are additive to the mechanisms involving the CD46, CD55, and CD59 proteins. We conclude therefore that MT1-MMP expression with the levels of the complement regulatory CD46, CD56, and CD59 proteins. We failed to find any correlation between MT1-MMP expression and the incidence and growth rate of MCF7 tumor xenografts in nude rats (10).

The level of C3b deposition is important in tumor immunity because C3b and its cleavage fragments on the cell surface serve as receptors for immune effector cells. Thus, in a mouse melanoma model, the deposition of C3b enhanced phagocytosis of mastocytoma P815 cells by macrophages (8, 54). Our data agree well with these studies and indicate that the modulation of C3b and C4b deposition by MT1-MMP is likely to help malignant cells to evade host immune surveillance and thus to grow more efficiently as tumors and as metastases. There are extensive data suggesting that antibody-dependent cell-mediated cytotoxicity enhanced by complement is a powerful mechanism to limit tumor growth (55). MT1-MMP-dependent proteolysis of C3b and C4b is thus very likely an important factor in tumor immunity.

It is well established that normal cells synthesize a number of the complement regulatory proteins to control and avoid aberrant activation of the complement system. These soluble and plasma membrane-associated proteins are frequently overexpressed by malignant cells to escape complement attack (11, 12). We failed to find any correlation between MT1-MMP expression with the levels of the complement regulatory CD46, CD56, and CD59 proteins. We conclude therefore that MT1-MMP directly inhibits complement. This hypothesis correlates well with the earlier observations that indirectly indicated the existence of the regulatory mechanisms that are used by tumor cells to escape immune attack. These mechanisms are additive to the mechanisms involving the CD46, CD55, and CD59 complement regulatory proteins (12, 40).

We believe that our studies have identified a biochemical mechanism that involves MT1-MMP in tumor immunity and have shed light on the exceptionally important role that this protease plays in connection with invading metastatic malignant cells. Our findings warrant additional studies. The studies employing the expression of murine MT1-MMP in murine tumor cell lines followed by tumor xenografts in complement-deficient mice will clarify the role of MT1-MMP in the tumor defense mechanisms.

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Cellular Membrane Type-1 Matrix Metalloproteinase (MT1-MMP) Cleaves C3b, an Essential Component of the Complement System
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