Membrane Type-1 Matrix Metalloproteinase Confers Aneuploidy and Tumorigenicity on Mammary Epithelial Cells

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Abstract

An elevated expression of membrane type-1 matrix metalloproteinase (MT1-MMP) is closely associated with multiple malignancies. Recently, we discovered that recycled MT1-MMP was trafficked along the tubulin cytoskeleton into the centrosomal compartment and cleaved the integral centrosomal protein pericentrin-2. These events correlated with the induction of chromosomal instability and aneuploidy in nonmalignant Madine-Darby canine kidney cells. Accordingly, we hypothesized that MT1-MMP is an oncogene that promotes malignant transformation of normal cells rather than just an enzyme that supports growth of preexisting tumors. To prove our hypothesis, we transfected normal 184B5 human mammary epithelial cells with MT1-MMP (184B5-MT1 cells). MT1-MMP was colocalized with pericentrin in the centrosomal compartment and especially in the midbody of dividing cells. 184B5-MT1 cells acquired the ability to activate MMP-2, to cleave pericentrin, and to invade the Matrigel matrix. 184B5-MT1 cells exhibited aneuploidy, and they were efficient in generating tumors in the orthotopic xenograft model in immunodeficient mice. Because of the absence of tumor angiogenesis and the resulting insufficient blood supply, the tumors then regressed with significant accompanying necrosis. Gene array studies confirmed a significant up-regulation of oncogenes and tumorigenic genes but not the angiogenesis-promoting genes in 184B5-MT1 cells. We believe that our data point to a novel function of MT1-MMP in the initial stages of malignant transformation and to new and hitherto unknown transition mechanism from normalcy to malignancy. (Cancer Res 2006; 66(21): 10460-5)

Introduction

It is generally accepted that oncogenesis arises from an accumulation of genetic mutations in normal cells. Genetic instability occurs at both the gene level (nucleotide instability) and the chromosomal level (microsatellite instability and chromosomal instability) and promotes the accumulation of mutations (1). Chromosomal instability generates most of the chromosomal defects; therefore, tumor cells frequently exhibit chromosomal instability, including either a partial or complete loss/gain of chromosomes (aneuploidy; ref. 2). Commonly observed aneuploidy of malignant cells, especially in solid tumors, suggests a direct link between chromosome segregation errors and tumorigenesis (3).

Centrosomes, the microtubule organizing centers, are the major regulators of chromosome segregation (4). Centrosomal aberrations ultimately result in chromosome instability (5, 6). Centrosomes are essential for microtubule nucleation and mitotic spindle formation (7). The centrosome contains a pair of centrioles, surrounded by a lattice-like pericentriolar material (PCM). Pericentrin is one of the most important scaffolding proteins of the PCM (8). There are several splice variants of pericentrin of which pericentrin A (220 kDa) and pericentrin B (kendrin, 350 kDa) are the most common (9). Microtubule nucleation is mediated by the ring-shaped γ-tubulin ring complex (γ-TuRC) that is responsible for mitotic spindle organization, checkpoint regulation, and chromosomal segregation during mitosis (7). Pericentrin anchors the γ-TuRC to the centrosome (10). Either pericentrin silencing by small interfering RNA or an overexpression of pericentrin fragments encoding the γ-TuRC binding domain induces aberrations in the mitotic spindle and a reduction in the centrosomal γ-tubulin (8).

Cell-surface–associated membrane type-1 matrix metalloprotease (MT1-MMP) is a multifunctional, invasion-promoting enzyme involved in the pericellular proteolysis of the extracellular matrix proteins, the activation of soluble MMPs, and the cleavage of adhesion and signaling cell receptors (11–15). Although MT1-MMP is present in normal tissues, its enhanced expression is directly linked to tumor progression and metastasis. The expression of functionally active MT1-MMP is associated with many aggressive malignancies (16–20). In malignant cells, MT1-MMP acts as a growth factor and usurps tumor growth control (21).

Recently, we discovered that MT1-MMP, after its presentation at the cell surface, is endocytosed, and the internalized protease then cleaves the ALRLLG1156L1157FG motif of pericentrin B and induces chromosomal instability and aneuploidy (22, 23). The transmembrane domain is critical for the trafficking of MT1-MMP to the PCM, suggesting that internalized MT1-MMP is integrated in the vesicle membrane (24).

Here, we have shown that MT1-MMP expression promoted the activation of MMP-2, cell invasion, and the cleavage of pericentrin, and that these events correlated with aberrations in chromosome segregation, up-regulation of gene expression of multiple oncogenic and carcinogenic genes, aneuploidy, and tumorigenicity of 184B5 human mammary epithelial cells. We determined that after reaching a 0.5-cm size in immunodeficient mice, MT1-MMP–bearing tumor xenografts receded because there was insufficient host angiogenic response and inadequate neo-vascularization of the tumors. These observations suggest the existence of a novel, previously uncharacterized mechanism leading, potentially, to the malignant transformation of normal cells into cancerous cells.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Antibodies and cells. Both a mouse monoclonal antibody 5D1 and a rabbit polyclonal antibody against the recombinant catalytic domain of MT1-MMP were generated in our laboratory. A rabbit polyclonal antibody AB215 against the hinge region of MT1-MMP was from Chemicon (Temecula, CA). A monoclonal antibody against the V5 epitope conjugated with FITC was from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies 4B and M8 to the COOH-terminal and NH2-terminal regions of pericentrin, respectively, were kindly provided by Stephen J. Dowsey (University of Massachusetts Medical Center, Worcester, MA). A murine monoclonal antibody against γ-tubulin was from Sigma (St. Louis, MO). Rat anti-mouse CD105 antibody was from BD Biosciences (San Diego, CA). The 184B5 human mammary epithelial cell line was from the American Type Culture Collection (Manassas, VA). Cells were grown in MEGM complete medium (Invitrogen). The human embryonic kidney 293FT cells were from Invitrogen. 293FT cells were maintained in DMEM supplemented with 10% fetal bovine serum.

Lentiviral expression of MT1-MMP gene. The cDNA fragment encoding the full-length human MT1-MMP was cloned into the pLenti6/V5-D-TOPO vector (Invitrogen) by PCR using standard cloning procedures. The authenticity of the resulting MT1-MMP-V5 lentiviral construct was confirmed by DNA sequencing. For production of lentivirus, 293FT cells were used according to the manufacturer's instructions (Invitrogen). 184B5 cells were infected with the pLenti6/V5-GW/lacZ construct, were used as a control. The stable transfectants were selected in DMEM with 10 μg/mL blasticidin. MT1-MMP—expressing clones were identified among the blastidicin-resistant clones by Western blotting and also by immunocytochemistry with the V5 antibody. To avoid clonal effects, blastidicin-resistant clones were combined (184B5-MT cells) and used in our MMP-2 activation, invasion, immunostaining, and tumorigenicity studies, whereas the individual, randomly selected MT1-MMP—positive clones were used in our fluorescence-activated cell sorting (FACS) analysis.

Cell proliferation assay. Cells (1,000 per well) were seeded in the DMEM/10% FCS in the wells of a 24-well plate and cultured for 7 days. The cells were then stained with 0.2% crystal violet in a 20% methanol/water solution and washed thrice in water. The dye was extracted using 1% SDS, and A570 was measured with a plate reader.

Matrigel invasion assay. These assays were done in Boyden chamber-type, 6.5-mm, 24-well Transwell plates (Costar/Corning, Inc., Corning, MA) with the 8-μm pore size membranes (25–27). DMEM (100 μl) containing 2.4 μg of Matrigel (BD Biosciences, Bedford, MA) were dried on the upper surface of the membrane at room temperature overnight. Matrigel was rehydrated in DMEM (100 μl) for 2 hours. Cells (1 × 10^5 per well) were seeded in DMEM (100 μl) into the upper chamber of the Transwell. The bottom well of the chamber received 500 μl of DMEM supplemented with 10% FCS. Cells were allowed to invade the Matrigel for 12 hours at 37°C in a CO2 incubator. The medium was then aspirated, and the cells that migrated onto the membrane’s lower surface were stained for 10 minutes with 0.2% crystal violet in 20% methanol/water solution (300 μl) and washed thrice with water. The dye was extracted with 1% SDS (250 μl), and A570 was measured with a plate reader.

Isolation of centrosomes. Centrosomes were isolated from the cells that had been synchronized with nocodazole (10 μg/ml) and cytochalasin D (1 μg/ml) for 90 minutes (28). Cells were lysed in 1 ml/mM Tris-HCl (pH 8) containing 0.5% Igepal. Cell lysates were spun at 1,500 × g to separate the nuclei and cell fragments. The supernatant fraction was filtered through nylon mesh (70-μm pore size) and centrifuged at a 200 w/v Ficoll-400 cushion at 12,000 rpm for 30 minutes. The crude centrosomal fraction localized at the Ficoll/water interface was collected and further purified by a 40% to 70% sucrose gradient centrifugation at 30,000 rpm for 2 hours. The purified centrosomes (~60% sucrose) were collected and then diluted in 1 ml/mM Tris-HCl (pH 8) containing 0.5% Igepal. The centrosomes were collected by centrifugation at 14,000 rpm in an Eppendorf centrifuge and immediately used for further analysis including Western blotting.

Gelatin zymography. Gelatin zymography of conditioned medium aliquots was used to assess the ability of cellular MT1-MMP to activate MMP-2. Cells were plated in the wells of a 48-well plate (Costar/Corning) in serum-containing DMEM and grown 90% confluent. The medium was changed for serum-free DMEM supplemented with purified MMP-2 proenzyme (10 ng/ml). After a 12-hour incubation, aliquots of the medium were analyzed in gelatin-gels (Novex, San Diego, CA) and processed as described earlier (29).

Flow cytometry. Cells were detached in trypsin-EDTA, fixed in 70% ethanol at –20°C, washed in PBS, and resuspended in 1% bovine serum albumin (BSA)/PBS supplemented with 50 μg/ml propidium iodide. The DNA content of the cells was analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

In vivo tumorigenicity assay and immunostaining. Athymic female BALB/c nu/nu 4-week-old mice were purchased from Charles River (Chicago, IL) and kept under pathogen-free conditions. Mice were anesthetized with Avertin (15 ml/kg body weight). Cell suspension (1 × 10^7/ml) was prepared by trypsinization of exponentially growing cells. 184B5 and 184B5-MT cells (1 × 10^5/0.1 ml of MEGM) were injected in the mammary fat pads (10 mice per group). Tumor growth was monitored weekly. The tumor measurements were made by caliper measurements at two perpendicular diameters. The volume of the tumors was calculated by the formula: \( V = \frac{4}{3} \pi D_1 D_2 \). Twenty-one days after the cell injection, xenograft tumors were excised and fixed overnight in 4% paraformaldehyde in PBS. The resected tumors were embedded in paraffin and sectioned. Sections were heated to and maintained at 100°C for 20 minutes in 50 mmol/L Tris (pH 10) for antigen retrieval (30) and then stained for 4 hours with H&E and with the antibodies to CD105 and MT1-MMP (the V5 epitope) followed by the species-specific secondary antibody conjugated with green Alexa Fluor Flou 488 and red Alexa Fluor 594 (Molecular Probes, Eugene, OR). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI).

For immunostaining of cultured cells, cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, blocked with 1% BSA, and stained with DAPI and with the MT1-MMP 5D1 monoclonal antibody followed by the goat anti-mouse antibody conjugated with green Alexa Fluor 488 (Molecular Probes). Images were acquired at a ×600 original magnification on an Olympus BX51 fluorescence microscope equipped with a cooled MagnaFire camera (Olympus, San Diego, CA).

Isolation of total RNA and DNA-chip RNA expression profiling. 184B5 and 184B5-MT cells (1 × 10^9 per 100-mm dish) were grown until subconfluent in DMEM supplemented with 10% fetal bovine serum. Total cellular RNA was extracted with an RNAeasy kit following the manufacturer’s recommendations (Qiagen, Valencia, CA). RNA was treated with RNase-free DNase to remove contaminating genomic DNA. RNA integrity and purity was assessed by formaldehyde gel electrophoresis and by an A260/280 reading. RNA was aliquoted and stored at –80°C in RNase-free water until fluorescence labeling. Total RNA (500 ng) was reverse transcribed and labeled with biotin using Illumina RNA Amplification kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The cRNA samples were mixed with the Hyb E1 hybridization buffer containing 37.5% (w/v) formamide, and the hybridization mix was dispensed on the center of the Sentrix HumanRef-6 BeadChip (Illumina, San Diego, CA) containing 46,000 genes represented in the consensus RefSeq database. Hybridization was done for 18 hours at 55°C. Array chips were then washed with E1B solution followed by 100% ethanol and E1BC solution again. Next, the chips were blocked with an E1 blocking buffer followed by staining with streptavidin-Cy3 and washing with E1BC solution. We analyzed Illumina HumanRef-6 Expression BeadChips using the manufacturer’s BeadArray Reader and collected primary data using the supplied Scanner software. Data analysis was done in three stages. First, expression intensities were calculated for each gene probed on the array for all six hybridization experiments using Illumina’s Beadstudio #1 software. Second, intensity values were quality controlled and normalized: quality control was carried out by using the Illumina Beadstudio detection P set to <0.1 as a cutoff. At this step, ~24,000 genes, which were not detected in the array, were eliminated from the total of 46,000 genes (~50% reduction). The residual 21,892 genes were normalized using the normalize.quantiles routine of
Bioconductor from the Affymetrix software package. The *normalize.quantiles* procedure accounted for any variation in hybridization intensity between the individual arrays. An assessment of several different normalization techniques using the Bioconductor *maCorrPlot* routine suggested that *normalize.quantiles* was the most appropriate for the data. The normalized data were imported into GeneSpring to identify the differentially expressed genes based on the Welch $t$ test and fold difference of the expression level ($P_{\text{adj}} = 0.05$). Upon publication, complete microarray data for each sample will be made publicly available through National Center for Biotechnology Information’s Gene Expression Omnibus (accession no. GSE5095).

**Results**

**MT1-MMP induces chromosomal instability in 184B5 human mammary epithelial cells.** We specifically selected immortalized 184B5 human normal mammary epithelial cells to show the oncogenic properties of MT1-MMP in our studies. The 184B5 cell line was obtained from normal augmentation mammoplasty material and immortalized by exposure to benzo(a)pyrene (31). 184B5 cells exhibit a stable chromosome number of 47. These normal cells are not malignant or tumorigenic in vivo, and resulted in carcinogenesis (31–33).

We transfected 184B5 cells with the lentiviral construct expressing the full-length MT1-MMP proenzyme gene and selected a pool of MT1-MMP–expressing clones (184B5-MT cells). MT1-MMP expression was not detected in the control 184B5 cells, whereas 184B5-MT cells expressed significant levels of the MT1-MMP proenzyme and the enzyme as well as the 40- to 45-kDa degraded forms of the protease (Fig. 1A). These data are consistent with the pattern of MT1-MMP expression we observed in many tumor cell types (34). Following transfection with MT1-MMP, the cells acquired the ability to activate MMP-2, a well-known target of MT1-MMP, and also to efficiently invade the Matrigel (Fig. 1). The proliferation rate of 184B5 and 184B4-MT cells, however, was similar.

Immunostaining of migrating cells showed the localization of MT1-MMP immunoreactivity, primarily, at the trailing and at the leading edges of 184B5-MT cells, as well as the presence of MT1-MMP in the intracellular compartments (Fig. 1).

In agreement with our earlier observations, MT1-MMP partially colocalized with pericentrin in the centrosomes and extensively colocalized with pericentrin in the midbody of dividing cells (Fig. 2). These data correlate well with the presence of pericentrin in the midbody (35, 36), and they also suggest that MT1-MMP, in addition to its other functions, is involved in the resolution of the membrane connection between two prospective daughter cells during the terminal steps of cytokinesis. The midbody localization of MT1-MMP was also confirmed in HeLa cells (data not shown).

Consistent with our earlier findings, DAPI staining revealed irregular nuclei and chromatid abnormalities in the interphase and metaphase 185B5-MT cells, respectively, suggesting the prerequisite for multiple genetic aberrations in these cells whereas both the
nuclei and the chromatin seemed normal in the 184B5 cell control (Fig. 2). FACS analysis of the individual 184B5-MT cell clones showed the presence of two cell populations: one with decreased and another with increased chromosomal DNA levels, an indication of aneuploidy. In contrast, control 184B5 cells were homogenous in their DNA content, the levels of which were in between those observed in 184B5-MT cells (Fig. 2).

Because our previous works linked MT1-MMP to the proteolysis of pericentrin, we isolated the centrosomes from both the 184B5 control and 184B5-MT cells, and we then analyzed the samples by Western blotting with the antibodies to MT1-MMP, pericentrin, and γ-tubulin. Consistent with our previous data from tumor cells and Madin-Darby canine kidney (MDCK) cells (22), the expression of MT1-MMP correlated with the proteolysis of pericentrin B ( kendrin) in 184B5-MT cells, whereas γ-tubulin remained intact (Fig. 3). In the ex vivo experiments, in which the centrosome samples, isolated from 184B5 control cells, were cocultivated with the soluble catalytic domain of MT1-MMP, we also determined that MT1-MMP cleaved pericentrin, whereas γ-tubulin was resistant to the proteolysis (data not shown).

Microarray analyses of 184B5 and 184B5-MT cells. To characterize changes in gene expression in response to MT1-MMP, total RNA was extracted from subconfluent cultures of 184B5 and 184B5-MT cells and reverse transcribed to cDNA. Differentially expressed genes were determined by looking for genes with statistically significant differences between 184B5 and 184B5-MT samples based on the results of the Welch t test (P < 0.05). The analysis identified 4,511 genes with P < 0.05. From these 4,511 genes, 207 genes exhibited at least a 2-fold difference in the expression levels in 184B5-MT cells when compared with those in 184B5 cells. The mRNA expression of 180 genes increased by a factor of ≥2 in 184B5-MT cells, and the expression of 27 genes decreased in 184B5-MT cells when compared with the 184B5 cell control. The gene array data are shown in Supplementary Fig. S1. The complete information on signal intensity, significance of expression changes, fold changes, and P values is set forth in Supplementary Table S1.

The expression of genes related to the extracellular matrix (especially, fibronectin), to the control of mitosis (CDC2 and cyclins B1 and B2), and to oncogenesis (melanoma-associated gene, B aggressive lymphoma gene, aurora kinase, lipocalin 2, stathmin 1, β-catenin, RAB20, epithelial cell transforming sequence 2 oncogene, and breast carcinoma amplified sequence 4) was significantly up-regulated in MT1-MMP–expressing cells. No up-regulation of the genes involved in angiogenesis, including VEGF, however, was observed in 184B5-MT cells when compared with 184B5 cells. Taken together, our results suggest that the expression of MT1-MMP induces mitotic aberrations and aneuploidy in normal epithelial cells, and that MT1-MMP affects the mRNA expression of the genes known to be directly implicated in oncogenesis. We then addressed the question of whether the expression of MT1-MMP was sufficient to cause the malignant transformation of normal epithelial cells and oncogenesis.

Normal epithelial cells transfected with MT1-MMP induce tumors in mice. To evaluate the tumorigenic potential of 184B5 cells (control) and 184B5-MT cells, we inoculated each type of cell in the breast fat pads of nude mice (107 per animal; 10 mice per group). There was neither noticeable inflammation nor evidence of tumors 1 week after cell injections. In 2 weeks, however, tumors were evident in all of the mice that received 184B5-MT cells (Fig. 4). Surprisingly, 184B5-MT tumors began to regress after 3 weeks. As expected, there were no tumors in mice that received the control cells. The 3-week 184B5-MT tumors were excised, sectioned, and analyzed by immunohistochemistry. The microscopic analysis of the H&E-stained tumor sections showed the presence of a large size lumen and necrotic cells in the tumor central part. MT1-MMP immunoreactivity was localized at the inward side of the tumor xenografts. A similar immunoreactivity pattern was observed when either the antibodies 5D1 or AB815 against MT1-MMP were used to stain the 185B5-MT tumor sections (data not shown). In agreement with the gene array data, the staining of the 185B5-MT tumors with the antibody to the CD105 endothelial marker showed minor immunoreactivity of the capillary network inside the tumor. In contrast, a dense capillary network was observed in the normal mouse mammary fat pad (Fig. 4). These data suggest that MT1-MMP, acting as a potent oncogene, induced the malignant transformation of 184B5-MT cells and supported the growth of tumor xenografts in our orthotopic model system. The expression of MT1-MMP and the subsequent malignant transformation event were, however, insufficient for the induction of host angiogenesis. Because of the absence of tumor angiogenesis and the resulting insufficient blood supply, the tumors regressed with significant accompanying necrosis.

Discussion

Cancer cells regularly exhibit chromosomal instability (2), a hallmark of malignancy and an early event in the transformation
centrosomal scaffolding proteins. Pericentrin B seems to directly interact with multiple microtubule nucleating and regulatory proteins, including CG-NAP (also known as AKAP 350 and AKAP 450); γ-TuRC proteins GCP2, GCP3, and γ-tubulin; calmodulin; PKA; PKC βII; and PCM-1 (7). Thus, the normal functioning of pericentrin is required for centrosome performance and chromosome segregation. Conversely, alterations in pericentrin functionality cause mitotic spindle aberrations (8). Thus, either the expression of the GCP2/3 binding pericentrin fragments or the PKC βII binding fragments induce mitotic spindle aberrations and γ-tubulin uncoupling from the centrosomes (8, 37).

Earlier, we showed that MT1-MMP confers tumorigenicity on nonmalignant MDCK epithelial cells (38) and that MT1-MMP, in addition to its pericellular proteolytic function, causes limited proteolysis of pericentrin B in tumor cells. This intracellular proteolytic event, the topology of which is still incompletely understood, is associated with mitotic spindle aberrations, chromosome instability, and aneuploidy (22). Our current study goals were to determine if MT1-MMP performs as an oncogene and is capable of transferring malignant phenotype to normal cells and causing a transition from normalcy to malignancy. To address this question, we transfected nonmalignant immortalized 184B5 human mammary epithelial cells with the lentiviral construct expressing MT1-MMP. After transfection with MT1-MMP, 184B5 cells acquired pericellular proteolysis and invasive characteristics, including the ability to activate MMP-2 and to invade the Matrigel, abilities that are specific to cancer cells. The expression of MT1-MMP also correlated with the proteolysis of pericentrin, mitotic spindle aberrations, aneuploidy, and up-regulation of the gene expression of multiple cell cycle control and oncogenic genes. These features are also specific to neoplastic cells. Most importantly, MT1-MMP–transfected 184B5 cells generated, with 100% efficiency, tumor xenografts in the orthotopic model in immunodeficient mice. We believe that the cell growth in vivo we observed represented the tumor-like growth of 184B5-MT cells because no neoplastic cell growth of the 184B5 control was observed in mice. The 184B5-MT tumors, after reaching 0.5 cm in size, however, regressed because of insufficient host angiogenic response and the inadequate neovascularization of the xenografts.

Overall, our results suggest that MT1-MMP plays also a role in the initial stages of malignant transformation, whereas the pericellular activity of MT1-MMP promotes invasion and migration of malignant cells and contributes to tumor growth and metastasis. We believe our results shed significant additional light on the functional role of cellular MT1-MMP and explain the direct association of MT1-MMP with multiple cancer types.

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