

# Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 *in vivo*

Takahiro Nonaka, Kunika Nishibashi,  
Yoshifumi Itoh, Ikuo Yana, and Motoharu Seiki

Division of Cancer Cell Research, Institute of Medical Science,  
University of Tokyo, Minato, Tokyo, Japan

## Abstract

Membrane type 1 matrix metalloproteinase (MT1-MMP) is a potent modulator of the pericellular environment and promotes tumor cell invasion and proliferation in many types of tumor. The activation of proMMP-2 and processing of collagen I by MT1-MMP have been thought to be important for its tumor-promoting function. These activities can be inhibited by mutant forms of MT1-MMP lacking the catalytic domain. However, the effect of such dominant-negative mutants has never been evaluated *in vivo*. Various mutants lacking the catalytic domain (dCAT) were prepared and confirmed to inhibit MT1-MMP activity in human fibrosarcoma HT1080 cells, and tumor cells expressing these mutants were implanted *s.c.* into nude mice to monitor tumor formation. Only the membrane-anchored form of a dCAT construct through the transmembrane domain [dCAT(1)] showed potent antitumor activity not only in HT1080 cells but also in gastric carcinoma MKN28 and MKN45 cells expressing MT1-MMP. A soluble form of dCAT lacking the transmembrane domain did not show such activity. The expression of dCAT(1) in MKN28 or MKN45 further prevented the metastatic spread of tumor cells into the peritoneal cavity; however, dCAT(1) showed no effect against TMK-1, another gastric carcinoma cell line expressing no MT1-MMP. It is of note that the tumorigenicity of TMK-1 cells enhanced by MT1-MMP overexpression was, in turn, canceled by the additional expression of

dCAT(1). Thus, MT1-MMP expressed in tumor cells seems to play a pivotal role in tumor growth in mice. The results also suggest new possibilities to abrogate the tumor-promoting function of MT1-MMP other than the conventional protease inhibitor-based approach. [Mol Cancer Ther 2005;4(8):1157–66]

## Introduction

Multiple matrix metalloproteinases (MMP) are overexpressed in malignant tumors and believed to contribute to tumor proliferation, invasion, and metastasis (1, 2). Each MMP presumably has a different role in tumor progression depending on the type of enzyme, producer cell, and tumor stage (1), however, our knowledge about the exact roles of these MMPs in tumors remains very limited. Some may contribute to antitumor activity as shown by a recent study in which MMP-8-deficient mice showed an increased incidence of skin tumor development (3). This may also be relevant to the recent disappointing clinical trials of general MMP inhibitors, which did not improve the tumor burden of patients (4–7). Thus, it is of great interest to identify which MMPs should be targeted for effective tumor therapy.

Membrane type 1 (MT1)-MMP, an integral membrane protease responsible for pericellular proteolysis, is one such MMP expressed in tumors (1, 8). MT1-MMP was identified as a tumor-specific activator of proMMP-2, believed to be important for tumor cells to invade basement membranes (9). MT1-MMP also acts as a cell-associated collagenase, particularly cleaving collagen I at a specific site (10, 11). Recent studies have emphasized the importance of cell-mediated collagenolysis during tumor cell invasion and proliferation (12–14). Indeed full-length MT1-MMP promotes tumor cells to invade and proliferate in the collagen matrix (13, 14). On the other hand, a truncated mutant lacking the COOH terminus including the transmembrane domain (soluble MT1-MMP) did not show such activity. Consistently, soluble collagenases such as MMP-1 and MMP-13 could not substitute MT1-MMP for activity to promote cell proliferation and invasion (13, 14).

The catalytic domain of MT1-MMP is primarily responsible for its proteolytic activity (10, 11), however, the collagenase activity of MT1-MMP is further modulated through other parts of the enzyme. For example, the hemopexin-like domain (HPX) at the COOH terminus can bind collagen I and unwind the triple helix for cleavage (11, 15). Because of this activity, recombinant protein composed of HPX with a hinge sequence abrogated MT1-MMP to bind collagen I and inhibited collagenolysis as a result (11, 15).

Received 4/26/05; revised 6/4/05; accepted 6/14/05.

**Grant support:** Special Coordination Fund for promoting Science and Technology from the Ministry of Science and Technology of Japan and a grant-in-aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Y. Itoh is currently at the Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 1 Aspenlea Road, Hammersmith, London W6 8LH, United Kingdom.

**Requests for reprints:** Motoharu Seiki, Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato, Tokyo 108-8639, Japan. Phone: 81-3-5449-5255; Fax: 81-3-5449-5414. E-mail: mseiki@ims.u-tokyo.ac.jp

Copyright © 2005 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0127

HPX also plays a role in the activation of proMMP-2 by MT1-MMP expressed on the cell surface. The catalytic domain of MT1-MMP is sufficient to carry out the activation in solution by introducing a cleavage into the propeptide sequence of proMMP-2, and the reaction is inhibited by tissue inhibitor of metalloproteinase-2 (TIMP-2), a natural inhibitor of MMPs (16, 17). However, additional factors are required for the activation when it occurs on the cell surface mediated by the membrane-anchored form of MT1-MMP. It has been shown that an appropriate amount of TIMP-2 is indispensable in the reaction although it causes inhibition at higher doses (17). The inhibitor domain of TIMP-2 at the NH<sub>2</sub>-terminal binds the catalytic site of MT1-MMP (18), whereas its COOH-terminal domain binds proMMP-2 (19). Thus, TIMP-2 can act as an adaptor to mediate a complex composed of MT1-MMP, TIMP-2, and proMMP-2 (8). Through formation of the complex, proMMP-2 in the media can be recruited to the cell surface where MT1-MMP exists; however, proMMP-2 cannot be activated by MT1-MMP in the complex, because it is inhibited by TIMP-2. Neighboring MT1-MMP has to act as an activator by attacking proMMP-2 in the complex. Thus, at least two MT1-MMP molecules have to be close together for the activation reaction to occur on the cell surface. Multiple portions of MT1-MMP, such as the HPX, transmembrane, and cytoplasmic tail, have been reported to play roles in maintaining an appropriate distance between the two molecules mediating the formation of a homo-oligomer (20–22). Interestingly, MT1-MMP mutants lacking the catalytic domain again inhibited the activation of proMMP-2 acting dominant-negatively against MT1-MMP (20). Presumably, these mutants induced competition with MT1-MMP in the formation of the homo-oligomer. Expression of the mutant in an invasive human fibrosarcoma HT1080 cell line inhibited invasion of the cells into Matrigel (20).

The dominant-negative function of the abovementioned mutants encouraged us to test the efficacy against tumor growth *in vivo*. Here we report the expression of a mutant MT1-MMP lacking the catalytic domain (dCAT) in different types of tumor cells, effectively suppressing their tumor growth in mice. The mutant expression also inhibited the peritoneal spread of gastric carcinomas. However, the mutant was not effective in another gastric tumor cell line, TMK-1, that did not express endogenous MT1-MMP. Additional expression of MT1-MMP in TMK-1 cells enhanced the tumorigenic activity of the cells and this enhancement was effectively canceled by the mutant. Thus, the dominant-negative MT1-MMP mutant abrogates the functions of intrinsic MT1-MMP both *in vitro* and *in vivo*.

## Materials and Methods

### Cells and Cell Culture

All human tumor cell lines were obtained from the Japanese Cancer Resource Bank. HT1080 is a fibrosarcoma cell line, and MKN28, MKN45, and TMK-1 are gastric carcinoma cell lines. HT1080 cells were cultured in DMEM (Sigma, St. Louis, MO) with 10% fetal bovine serum and

gastric carcinoma cells were in RPMI 1640 (Sigma) with 10% fetal bovine serum. Culture media were supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin (Sigma) for HT1080 cells, and 100 µg/mL kanamycin (Invitrogen Corp., Carlsbad, CA) for gastric carcinoma cells. Cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

Stable transfectants were established as follows: genes carried by pCEP4, which replicate autonomously, were transfected using FuGENE6 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions, and transfectants were selected by culturing the cells in the presence of 200 µg/mL hygromycin. The hygromycin-resistant colonies were pooled and used for the experiments. MT1-MMP cDNA carried by an eukaryotic expression vector pcDNA3.1 (Invitrogen) was transfected into TMK-1 cells using FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Zeocin-resistant transfectants were selected and single colonies were isolated. Thus, transfectant clones (MT1-1, MT1-2, and MT1-3) expressing MT1-MMP were established.

### Antibodies and Inhibitors

An anti-MT1-MMP mouse monoclonal antibody (mAb; 222-1D8) against the HPX domain was generated as described previously (Daiichi Fine Chemical, Ltd., Takaoka, Japan; ref. 23). The anti-FLAG epitope M2 mAb was purchased from Sigma. The anti-actin mouse antibody was purchased from Chemicon International (Temecula, CA). The peptidyl hydroxamate MMP inhibitor BB94 was a gift from Dr. Peter D. Brown (British Biotech Pharmaceuticals, Ltd., Oxford, United Kingdom).

### Detection of mRNA

The expression of mRNA was monitored by reverse transcription-PCR. Cells were lysed in TRIzol reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 µg of total RNA using 0.3 µg of each random primer (Invitrogen) and 200 units of Superscript II RNase H reverse transcriptase (Invitrogen). For PCR, 1 µL of the reverse transcriptase product was used as a template. The following primer pairs were used to amplify the reverse transcriptase products: MT1-MMP, 5'-AGGCAAAGCTGATGCAGAC-3' (forward) and 5'-CCATTCAGATCCTCATTCCCT-3' (reverse) generate a 459 bp product after 30 cycles of amplification (96°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds). MMP-2: 5'-TCCCCTTCTTGTTCAATGGCAA-3' (forward) and 5'-ACACCA-CATCTTTCCGTCCTACT-3' (reverse) generate a 402 bp product after 30 cycles of amplification. Glyceraldehyde-3-phosphate dehydrogenase: 5'-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3' (forward) and 5'-TTCCCGTC-TAGCTCAGGGATGACCTTGCCC-3' (reverse) generate a 500 bp product after 30 cycles of amplification.

### Construction and Transfection of Plasmids

A mammalian expression vector pCEP4 (Invitrogen), which replicates autonomously depending on the encoded EBNA-1, was used for the stable expression of the transfected genes. All mutants were generated by a PCR-based

method and had the following structures: dCAT(1) mutant is a catalytic domain-deleted mutant ( $\Delta\text{Tyr}^{112}\text{-Gly}^{288}$ ) of MT1-MMP; sdCAT(1) additionally lacks the transmembrane/cytoplasmic domain ( $\Delta\text{Ala}^{536}\text{-Stop}$ ) of dCAT(1); dCAT(1)gpi is a chimera of the ectodomain of dCAT(1) ( $\text{Phe}^{289}\text{-Cys}^{508}$ ) and the glycosylphosphatidylinositol-anchoring signal derived from MT4-MMP ( $\text{Gly}^{526}\text{-Leu}^{607}$ ); dCAT(4) is a chimera in which  $\text{Cys}^{319}\text{-Cys}^{508}$  in dCAT(1) is replaced with  $\text{Cys}^{336}$  to  $\text{Cys}^{527}$  of mouse MT4-MMP. A FLAG epitope (DYKDDDDK) was inserted downstream of the propeptide sequence of MT1-MMP and the mutants, and the mutant genes were subcloned into the pCEP4 and FLAG-tagged MT1-MMP into pcDNA3.1. All cDNA constructs were confirmed by DNA sequencing.

#### Western Blot Analysis

Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Following a blockage with 5% fat-free dried milk in PBS containing 0.05% Tween 20, the membrane was probed with primary antibodies specific to each protein. The membrane was further probed with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) or horseradish peroxidase-conjugated anti-mouse IgG (Amersham Bioscience, Piscataway, NJ) for visualization.

#### Immunostaining

Cells cultured on glass coverslips were fixed with 3% paraformaldehyde in PBS for 15 minutes at room temperature, washed thrice with PBS, and then blocked with 5% goat serum and 3% bovine serum albumin in PBS for 1 hour at room temperature. Cells were then treated with mouse anti-FLAG M2 monoclonal antibody (5  $\mu\text{g}/\text{mL}$ ) in the blocking solution at room temperature for 2 hours. After washing thrice with PBS, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) in 1:1,000 dilution in a blocking solution for 2 hours at room temperature. The cells were then washed thrice with PBS. Fluorescence signals were analyzed using a Bio-Rad (Hercules, CA) MRC-1024 confocal laser microscope.

#### Gelatin Zymography

Gelatin zymography was conducted with a SDS-polyacrylamide gel containing gelatin (1.0 mg/mL) as described previously (20). Samples were mixed with a nonreducing SDS sample buffer, and separated in 8.5% acrylamide gel. After electrophoresis, the gels were washed in a sample buffer containing 2.5% Triton X-100 for 1 hour to remove SDS and then incubated for 8 hours at 37°C in a reaction buffer [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L  $\text{CaCl}_2$ , 5  $\mu\text{mol}/\text{L}$   $\text{ZnCl}_2$ , 0.02%  $\text{NaN}_3$ ]. The gels were stained with Coomassie brilliant blue R250, and gelatinolytic activities were detected as clear bands against a blue background.

#### Matrigel Invasion Assay

The Matrigel invasion assay was carried out according to the manufacturer's instructions (Becton Dickinson, San Jose, CA). Cells ( $1 \times 10^5$ ) suspended in a serum-free medium were seeded on the upper chamber of a Matrigel-coated transwell filter (20  $\mu\text{g}$ , 8  $\mu\text{m}$  pore). Fetal bovine serum (10%) was added to the medium in the lower chamber and incubated for 24 or

48 hours. Noninvading cells remaining on the upper surface were removed by scraping, and the cells on the lower surface were fixed with 100% methanol, stained by crystal violet, and counted under a microscope.

#### Cell Growth Assay

Cells ( $1 \times 10^4$ ) were seeded on a type 1 collagen-coated culture dish and cultured at 37°C in a humidified  $\text{CO}_2$  incubator. The cell number was counted every 24 hours for 6 days using hemacytometry. Cell viability was assessed with trypan blue staining. For the cell growth assay in the collagen matrix, cells were suspended in 3 mg/mL of type 1 collagen (Nitta gelatin) and seeded onto a culture dish. After collagen gel was formed, cells were cultured at 37°C in a humidified  $\text{CO}_2$  incubator. After 7 days, the cell number was counted using hemacytometry after dissolving the collagen gel with 100  $\mu\text{L}$  of 1% collagenase (Invitrogen).

#### Animals

Animal experiments were carried out according to the guidelines issued by the Committee of Animal Experiments, Institute of Medical Science, at The University of Tokyo. Male athymic BALB/c nude mice (*nu/nu*) were purchased from CLEA Japan, Inc., Tokyo, Japan. The mice were bred and maintained under specific pathogen-free conditions, provided with sterilized food and water ad libitum, and housed in a barrier facility with 12-hour light/dark cycles. Experimental procedures were done on 6-week-old mice. The animals were euthanized when they appeared moribund.

#### Tumor Growth Assay

Cell tumorigenicity was examined in 6-week-old male BALB/c nude mice. Briefly,  $1 \times 10^6$  (HT1080) or  $2 \times 10^6$  (MKN28, MKN45, and TMK-1) of cells in an exponential growth phase were suspended in 1 mL culture medium with 10% fetal bovine serum. An aliquot (200  $\mu\text{L}$ ) of the suspension was injected s.c. into the dorsal side of the mice. The volume of the implanted tumor was measured twice a week with a caliper, using the formula  $V = (LW^2) \pi / 6$ : where  $V$ , volume ( $\text{mm}^3$ );  $L$ , biggest diameter (mm);  $W$ , smallest diameter (mm). The mice were euthanized 4 weeks after tumor inoculation.

#### Histopathology

Animals were sacrificed 14 days after inoculation and tumors were embedded in an ornithine carbamyl transferase compound and stored at  $-80^\circ\text{C}$ . Thin tumor sections (5  $\mu\text{m}$ ) were prepared, fixed with cold acetone, blocked with 3% hydrogen peroxidase, and stained with Ki-67 (NovoCastra, New Castle, United Kingdom) to measure the proliferation index. The apoptotic index was analyzed by terminal nucleotidyl transferase-mediated nick end labeling assay using an *in situ* Apoptosis Detection kit according to the manufacturer's instructions (TaKaRa, Otsu-city, Shiga, Japan).

#### Intraperitoneal Dissemination Assay

Exponentially growing cells (MKN28, MKN45, and TMK-1) were collected and suspended in culture medium with 10% fetal bovine serum. The cells ( $2 \times 10^6/500 \mu\text{L}$ ) were injected directly into the peritoneal cavity of 6-week-old male BALB/c nude mice. The mice were sacrificed at 4 weeks after injection and i.p. nodules were counted macroscopically.

### Statistical Analysis

All results are presented as the mean  $\pm$  SE. For statistical analysis, the nonparametric Mann-Whitney test was used. For all the survival experiments, the Kaplan-Meier log rank test was applied. *P* values  $<0.05$  were considered statistically significant.

## Results

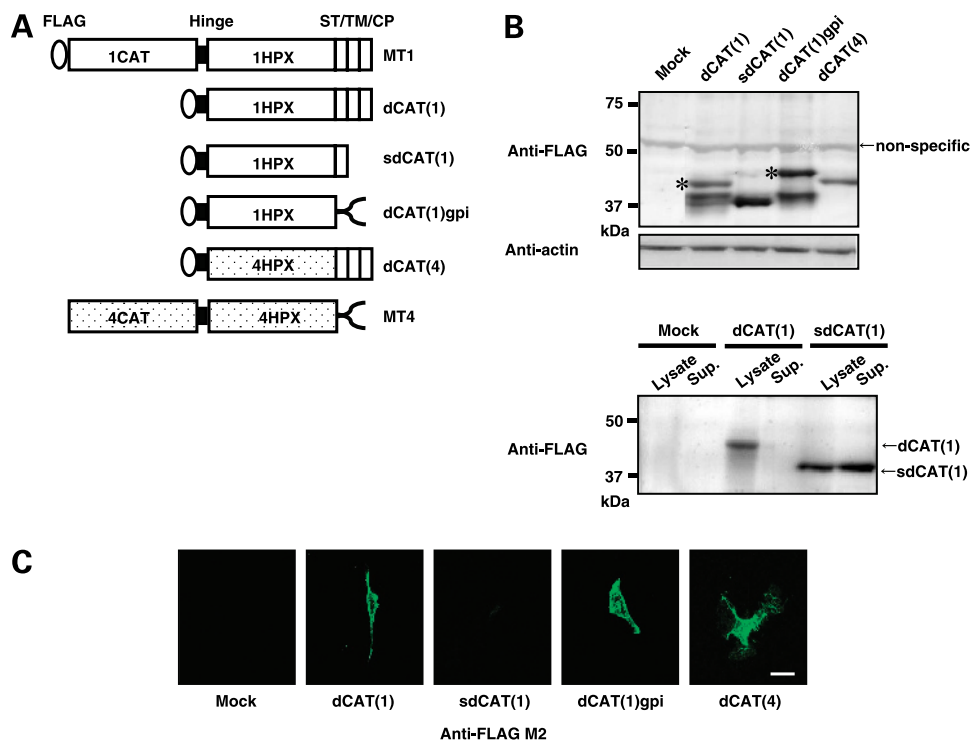
### Expression of MT1-MMP Mutants in Human Fibrosarcoma HT1080 Cells

Various MT1-MMP mutants lacking dCAT were prepared with a FLAG-tag downstream of the propeptide sequence (Fig. 1A) and stably introduced into human fibrosarcoma HT1080 cells. The cells express MT1-MMP constitutively and its down-regulation using the small interfering RNA inhibited activation of proMMP-2 and also inhibited invasion of the cells into Matrigel (24). Simple deletion of the catalytic domain of MT1-MMP generated dCAT(1) with the entire COOH terminus of MT1-MMP including the hinge, HPX, transmembrane, and cytoplasmic tail domains. The transmembrane and cytoplasmic tail of dCAT(1) was either truncated to generate a soluble form,

sdCAT(1), or substituted with the glycosylphosphatidylinositol-anchoring signal derived from MT4-MMP to generate dCAT(1)gpi. We also prepared dCAT(4) with HPX derived from MT4-MMP, which is distantly related to MT1-MMP and has no collagenase activity. HT1080 cells were stably transfected with each construct carried by pCEP4 which can replicate in the cells. Mutant expression was confirmed by Western blotting as shown in Fig. 1B, and sdCAT(1) secretion into the media was confirmed. The localization of dCAT(1), dCAT(1)gpi, and dCAT(4) on the cell surface was also confirmed by immunostaining under nonpermeabilized conditions (Fig. 1C).

### Effect of the Mutants on the Functions of Endogenous MT1-MMP

ProMMP-2 activation is the best characterized function of MT1-MMP (8, 22). First, we confirmed the suppression of proMMP-2 activation in HT1080 cells by the mutants. The activation of proMMP-2 mediated by MT1-MMP can be enhanced by treating the cells with concanavalin A (25), and MMP-2 in the medium was detected by gelatin zymography (Fig. 2A). Cells expressing either dCAT(1) or dCAT(1)gpi caused significant reduction in the amount of the active form of MMP-2, whereas those expressing sdCAT(1) or dCAT(4)



**Figure 1.** Expression of mutant forms of MT1-MMP lacking the catalytic domain. **A**, schematic presentation of FLAG-tagged mutant forms of MT1-MMP lacking the catalytic domain. *MT1*, MT1-MMP; *MT4*, MT4-MMP; *FLAG*, FLAG epitope; *1CAT*, catalytic domain of MT1-MMP; *1HPX*, hemopexin-like domain of MT1-MMP; *4CAT*, catalytic domain of MT4-MMP; *4HPX*, hemopexin-like domain of MT4-MMP; *gpi*, glycosylphosphatidylinositol anchor; *ST*, stalk region; *TM*, transmembrane domain; *CP*, cytoplasmic tail. **B**, HT1080 cells were transfected with plasmids encoding the indicated mutants and the expression of the mutant proteins was examined by Western blotting using anti-FLAG M2 mAb (top). Asterisk, the bands corresponding to the ones having propeptide sequence. Actin was also similarly detected using the anti-actin antibody. To detect sdCAT(1) secreted in the culture supernatant, proteins in the medium were precipitated with 10% trichloroacetic acid and analyzed similarly (bottom). **C**, mutant proteins expressed on the cell surface were detected by immunostaining. Transfected cells were reacted with anti-FLAG M2 mAb without permeabilization and the bound antibody was visualized with Alexa 488-conjugated anti-mouse IgG. Bar, 25  $\mu$ m.

did not (Fig. 2A). Thus, the membrane-anchored forms of the mutants effectively inhibited proMMP-2 activation. Because the ability of HT1080 cells to activate proMMP-2 has been shown mostly to be mediated by MT1-MMP in a small interfering RNA study (24), we believe that the function of endogenous MT1-MMP was inhibited dominant-negatively by the mutants.

Endogenous MT1-MMP also plays a major role in HT1080 cells to invade the reconstituted basement membrane (Matrigel; ref. 24). The expression of the membrane-anchored forms of the mutants [dCAT(1) and dCAT(1)gpi], but not sdCAT(1) and dCAT(4), significantly suppressed invasion (Fig. 2B). Thus, membrane anchoring of the ectodomain of the mutants seems essential for effective inhibition. In our assessment, dCAT(1)gpi showed a weaker effect than dCAT(1). This difference may be due to the lack of the transmembrane domain and cytoplasmic tail (21, 26) or to fragile membrane anchoring through glycosylphosphatidylinositol as reported previously (27).

None of these mutants affected the growth of HT1080 cells on the collagen-coated matrix (Fig. 2C), however, MT1-MMP is reported to regulate cell growth in a three dimensional type 1 collagen matrix (13). Indeed, cell growth was partly dependent on MMP activities in the three-dimensional collagen matrix, as it was inhibited >50% by BB94. The expression of dCAT(1) in the cells inhibited growth to the same level as BB94 (Fig. 2D). Weak inhibition was observed in cells expressing dCAT(1)gpi.

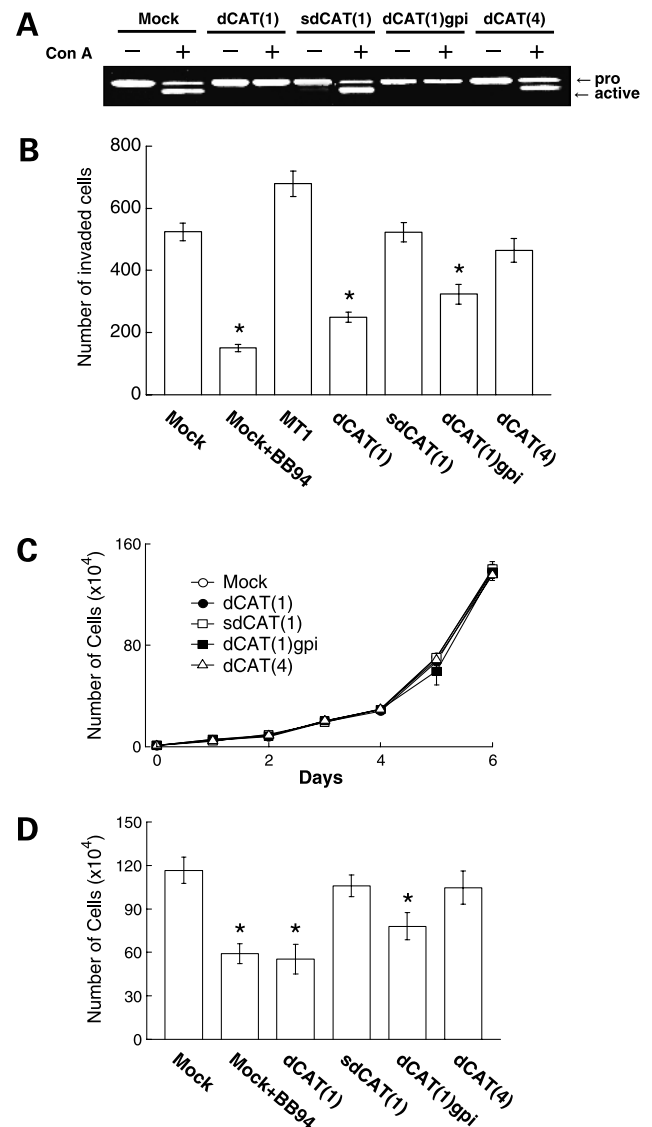
#### Effect of the Mutants on Tumor Formation by HT1080 Cells

To evaluate the effect of the mutants on tumor formation in mice, HT1080 cells expressing the mutants were implanted s.c. in nude mice and the tumor size was monitored. Mock-transfected cells (Mock) formed tumors, as indicated in Fig. 3A, which neither invaded the surrounding tissue nor metastasized spontaneously (data not shown). The strong inhibition of tumor growth (87–94% inhibition in volume) was observed with cells expressing dCAT(1). The expression of dCAT(1)gpi also reduced the tumor size, but the effect was limited compared with that of dCAT(1). Other mutants, sdCAT(1) and dCAT(4), showed no significant effect on the tumor. Suppression observed with cells expressing dCAT(1) was not the result of increased apoptosis *in vivo*, because the ratio of apoptotic cells in the tumor did not differ from that of control cells [Mock and dCAT(1); Fig. 3B]. On the other hand, the ratio of proliferating cells evaluated with Ki-67 antigen was decreased markedly with dCAT(1) in the tumor parenchyma (Fig. 3C). Thus, the expression of dCAT in HT1080 cells inhibited the proliferation of tumor cells *in vivo*.

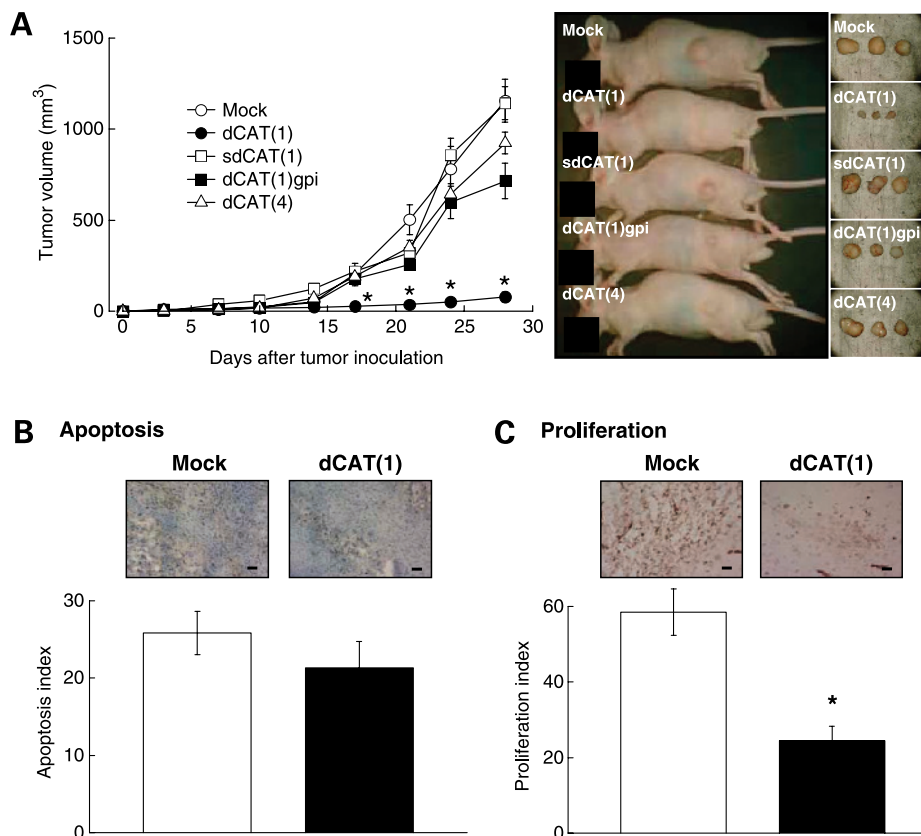
#### Antitumor Activity of dCAT(1) against Gastric Tumor Cells

Next, we used human gastric carcinoma cell lines to test the effect of dCAT(1) on tumor growth and specificity to MT1-MMP. MKN28 and MKN45 cells express endogenous MT1-MMP (Fig. 4A and B) representing reported clinical gastric carcinoma cases (28). However, other gastric

carcinoma TMK-1 cells do not express MT1-MMP and were used as a control for MT1-MMP-negative cells. Either dCAT(1) or wild-type MT1-MMP (MT1) was stably expressed in the cells (Fig. 4C) and was assessed using a



**Figure 2.** Effect of dCAT mutants on MT1-MMP-mediated functions. **A**, effect of the mutants on the activation of proMMP-2. HT1080 cells expressing the indicated mutants were cultured in serum-free medium in the absence or presence of concanavalin A (50  $\mu$ g/mL) for 24 h. The conditioned media were collected and subjected to gelatin zymography. Arrows, proMMP-2 (pro) and the activated form of MMP-2 (active). **B**, effect of the mutants on invasion into Matrigel. Transfected cells ( $1 \times 10^5$  cells) were seeded onto a transwell chamber using a membrane with 8  $\mu$ m pores and coated with Matrigel. Cells were cultured for 24 h and the number of cells invading the lower chamber was counted (mean  $\pm$  SE). **C**, effect of mutants on cell growth in a collagen-coated dish. Cells expressing the indicated mutants were cultured on a type 1 collagen-coated dish and the number of cells was counted on the indicated day. **D**, effect of mutants on cell growth in collagen matrix. Transfected cells were suspended in type 1 collagen solution and seeded onto a culture dish. After collagen gel was formed, cells were cultured for 7 d at 37°C, harvested after collagenolysis and counted.



**Figure 3.** Effect of dCAT mutants on tumor formation by HT1080 cells. **A**, HT1080 cells ( $1 \times 10^5$  cells) transfected with the indicated plasmids were inoculated s.c. into athymic nude mice. The size of the tumors was monitored for 28 d and expressed as the mean  $\pm$  SE ( $n = 14$ ; left). Middle, tumors at the inoculated sites; right, dissected tumors. **B**, sections of mock and dCAT(1)-transfected tumors were examined for apoptosis with terminal nucleotidyl transferase-mediated nick end labeling ( $\times 200$ ) staining (top). Bar, 100  $\mu$ m. Positive cells were counted and expressed as the mean  $\pm$  SE (bottom). **C**, the same sections were immunostained for proliferation marker Ki-67 ( $\times 200$ ; top). Bar, 100  $\mu$ m. Positive cells were counted and presented as the mean  $\pm$  SE (bottom).

Matrigel invasion assay (Fig. 4D). All three parental cells invaded Matrigel to varying extents. MKN28 and MKN45 cells seemed to use MMP activity in the invasion to some extent, as BB94 inhibited it significantly. BB94 did not affect the invasion by TMK-1 cells. The expression of dCAT(1) in MKN28 and MKN45 cells significantly inhibited the invasion, but not in TMK-1 cells. In contrast, the expression of MT1-MMP enhanced invasion in all three cell lines. Both MKN28 and MKN45 cells activated proMMP-2 and it was inhibited by dCAT(1) which was similar to HT1080 cells, although TMK-1 cells showed no ability to activate proMMP-2 (data not shown).

Transfectants were implanted s.c. into mice and tumor formation was monitored. The tumor growth of MKN28 and MKN45 cells was dramatically inhibited by dCAT(1) compared with the control cells as representative results show for MKN28 in Fig. 5A. On the other hand, dCAT(1) expression had no significant effect on the tumor growth of TMK-1 cells (Fig. 5B). In contrast, the additional expression of MT1-MMP in these cells enhanced tumor growth as reported for many other tumor cells (Fig. 5B).

Dissemination into the i.p. cavity is the central route for the spread of gastric carcinomas and a prime cause of recurrence (29, 30). To examine the effect of dCAT(1) in the dissemination model, the gastric carcinoma cells were inoculated into the abdominal cavity and mice were sacrificed on the 28th day for analysis. All three cell lines formed metastatic nodules at the intra-abdominal caul,

mesenterium, parietal peritoneal, and diaphragm. The expression of dCAT(1) in MKN28 and MKN45 cells resulted in a decrease in the number of nodules by 77% compared with mock-transfected cells as representative results for MT1-MMP-expressing cells (MKN28) show in Fig. 5C, but this was not the case for TMK-1 cells (Fig. 5D). In contrast, the exogenous expression of MT1-MMP enhanced peritoneal spread in all cells. The survival of mice bearing MKN28 expressing dCAT(1) was significantly extended compared with the controls, but cells expressing MT1-MMP (MT1) shortened survival (Fig. 5E). Mice inoculated with TMK-1 cells expressing dCAT(1) showed no difference in survival compared with the control, although MT1-MMP-expressing cells shortened it (Fig. 5F).

#### Specificity of the Antitumor Effect of dCAT(1)

The expression of dCAT(1) in three cell lines (HT1080, MKN28, and MKN45) that express endogenous MT1-MMP suppressed tumor growth in mice. However, dCAT(1) had almost no effect on TMK-1 cells that express an undetectable level of endogenous MT1-MMP. Thus, the effect of dCAT(1) seems to be specific to cells employing endogenous MT1-MMP as a driving force for their growth *in vivo*. It is apparent that TMK-1 cells are not using MT1-MMP for their tumor growth, but the enforced expression of MT1-MMP enhanced growth, suggesting that the cell can exploit the enzyme for growth (Fig. 5B and D). To confirm whether the antitumor activity of dCAT(1) is the result of its antagonistic action against MT1-MMP, we

established three independent stable transfectants expressing FLAG-tagged MT1-MMP by integrating the gene into the chromosome (MT1-1 to MT1-3) and used them to ask whether dCAT(1) could abolish the tumor-promoting effect by MT1-MMP.

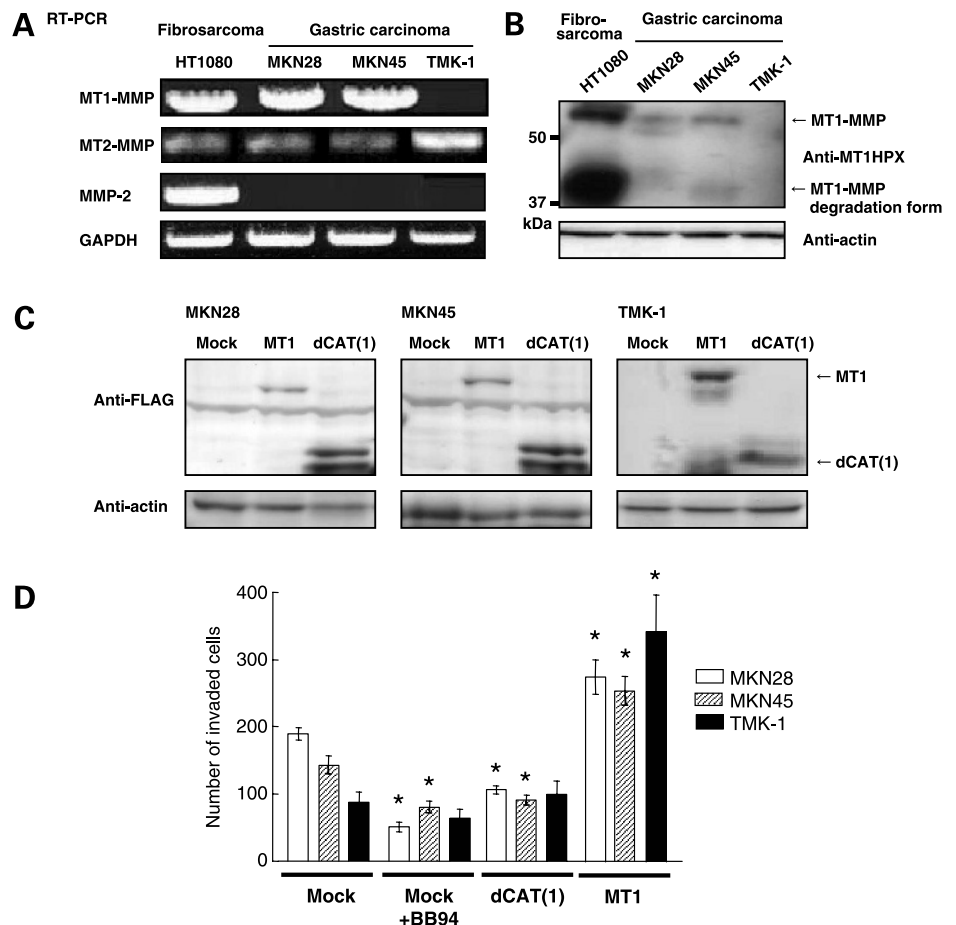
Mock-transfected TMK-1 cells had no ability to activate proMMP-2 as parental cells (Mock, Fig. 6A and B), whereas transfected cells expressing MT1-MMP did (MT1-1, -2, and -3, Fig. 6A and B). As expected, activation was inhibited by an excessive amount of TIMP-2. The additional expression of dCAT(1) in TMK-1 cells transfected with MT1-MMP (Fig. 6A) abolished the activation (Fig. 6B). An inhibitory effect of dCAT(1) was observed with the Matrigel invasion assay (Fig. 6C). Enhanced invasion by MT1-MMP was canceled by dCAT(1) to the level of mock-transfected cells (Mock).

Transfected TMK-1 cells were then inoculated into mice. s.c. tumor growth and peritoneal spread were enhanced by the expression of MT1-MMP (MT1-1/Mock) reproducing the results in Fig. 5 (Fig. 6D and E). Further expression of dCAT(1) in the cells [MT1-1/dCAT(1)] reversed the tumor-promoting effect of MT1-MMP to the level of mock-transfected cells (Mock/Mock) in mice. According to the inhibition of peritoneal spread of dCAT(1)-expressing cells [MT1/dCAT(1)], the survival of the mice was also

significantly extended. Thus, the effect of MT1-MMP expression on tumor growth *in vivo* can be antagonized by dCAT(1) even in TMK-1 cells and the MT1-MMP-independent ability of the cells was not affected.

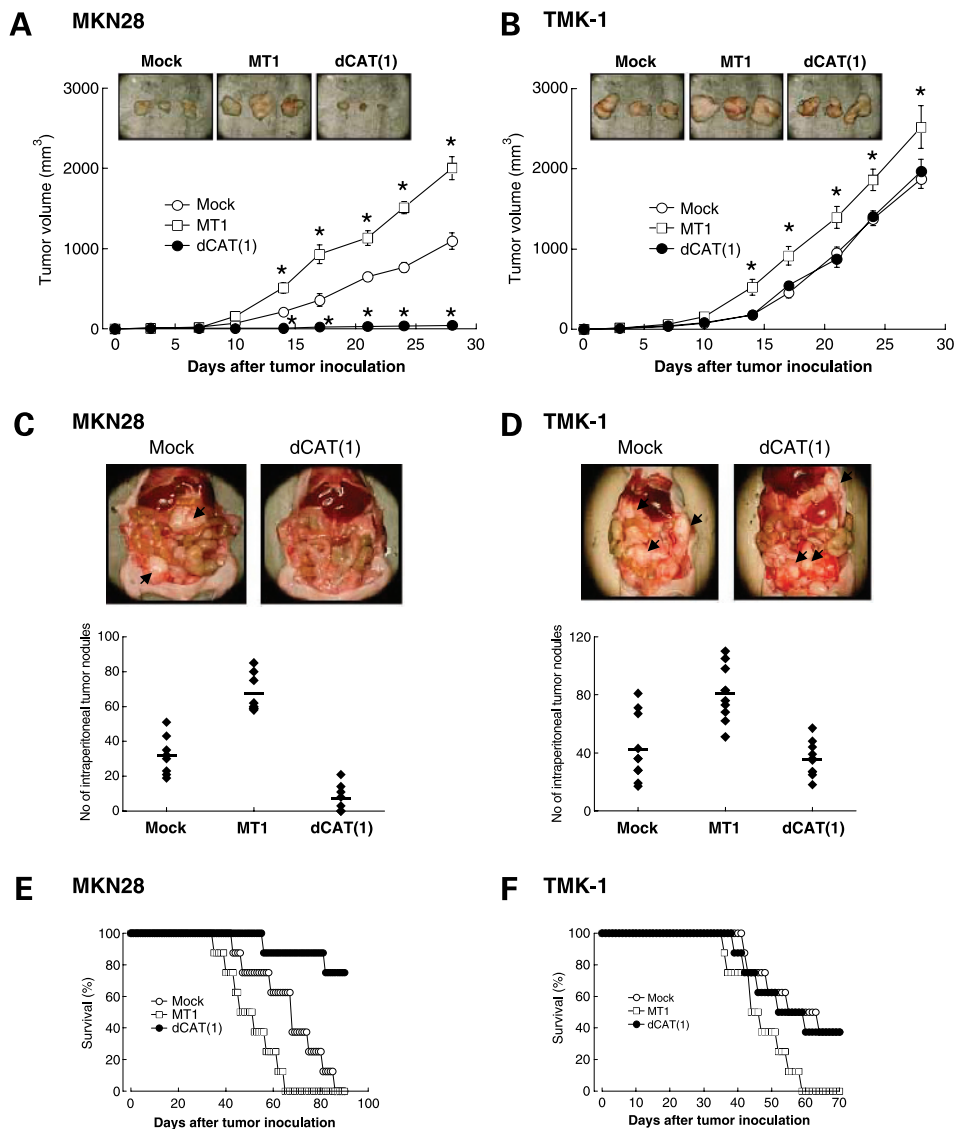
## Discussion

MT1-MMP is a potent modulator of the pericellular environment that regulates tumor cell proliferation and invasion (1, 2, 8, 31). MT1-MMP mutants lacking the catalytic domain have been reported to abrogate the functions of MT1-MMP such as the activation of proMMP-2 and degradation of collagen I at least *in vitro* (11, 20, 32). In this study, we applied the mutants to mouse tumor models to clarify whether MT1-MMP dysfunctions in tumor cells by mutants contribute to tumor suppression *in vivo*. Among the tested constructs, the membrane-anchored forms of dCAT(1) and dCAT(1)gpi showed antitumor activity against HT1080 cells (Fig. 3), although the effect of dCAT(1)gpi was low. The mutant effect in mice showed a good correlation with the activity to counteract MT1-MMP functions *in vitro*, such as the inhibition of proMMP-2 activation, invasion into Matrigel, and proliferation into collagen gel (Fig. 2). Thus, it is likely that the antitumor activity of dCAT(1) reflects its dominant-negative effect against endogenous MT1-MMP as



**Figure 4.** Effect of dCAT mutants on gastric carcinoma cells. **A**, the expression of MT1-MMP was examined in three human gastric carcinoma cells by reverse transcription-PCR. Total RNA was extracted from the cells and mRNAs for MT1-MMP and MMP-2 were examined by reverse transcription-PCR using specific primers. Amplified fragments were analyzed by agarose gel electrophoresis. **B**, the cells were lysed and analyzed by Western blotting using antibodies against the HPX domain of MT1-MMP (Anti-MT1HPX) and actin (Anti-actin). Arrows, specific bands detected by anti-MT1HPX. **C**, the expression plasmid of either FLAG-tagged MT1-MMP (MT1) or dCAT(1) was transfected into the indicated gastric carcinoma cells and stable transfectants were selected. The protein expression was analyzed by Western blotting using anti-FLAG M2 mAb (Anti-FLAG). **D**, transfected cells ( $1 \times 10^5$  cells) were seeded onto a Matrigel-coated membrane with 8  $\mu\text{m}$  pores in a transwell chamber and cultured for 24 or 48 h in the absence or presence of 10  $\mu\text{mol/L}$  BB94. The number of cells invading the lower chamber was counted and presented as the mean  $\pm$  SE.





**Figure 5.** Effect of dCAT mutants on tumor formation by gastric carcinoma cells. **A**, MKN28 cells ( $2 \times 10^6$  cells) transfected with the indicated expression plasmids were inoculated s.c. into athymic nude mice. The tumor size was monitored and presented as the mean volume  $\pm$  SE ( $n = 8$ ). *Insets*, dissected tumors from mice on the 28th d. **B**, TMK-1 cells transfected with the indicated plasmids were analyzed similarly. **C** and **D**, peritoneal dissemination of MKN28 (**C**) and TMK-1 (**D**) cells was examined. Cells ( $2 \times 10^6$  cells) transfected with the indicated plasmid were inoculated i.p. into athymic nude mice. After the 28th d, the mice were sacrificed and the number of tumor nodules in the cavity was counted. The pictures are representative cases showing metastatic nodules of either mock- or dCAT(1)-transfected cells in the peritoneum. *Arrows*, tumor nodules. (**E** and **F**): the survival of mice implanted with either MKN28 (**E**) or TMK-1 (**F**) cells i.p. was monitored and presented by a Kaplan-Meier plot.

observed *in vitro* (11, 20, 32). Membrane anchoring is important, presumably because mutants can compete with endogenous MT1-MMP more effectively when they are on the cell surface. Although dCAT(1)gpi showed weaker activity than dCAT(1), it is not clear whether the transmembrane and cytoplasmic portions play a role in this competition or merely indicate weaker membrane anchoring. However, it is of note that MT1-MMP lacking the cytoplasmic tail could activate proMMP-2 but failed to promote the invasion into Matrigel, whereas it had no effect on invasion into collagen I (26).

The specificity of the antitumor effect of dCAT(1) was also observed with gastric carcinoma cells. The antitumor activity of dCAT(1) was observed against cells expressing endogenous MT1-MMP (MKN-28, and MKN-45), whereas it showed no effect against TMK-1 cells expressing no endogenous MT1-MMP. Although TMK-1 cells are one example of an MT1-MMP-negative tumor cell line, other

cell lines that can form tumors in mice without expressing MT1-MMP are rare; thus, it was advantageous that MT1-MMP-transfected TMK-1 cells (MT1) showed enhanced tumorigenicity compared with mock-transfected cells. The expression of dCAT(1) in TMK-1(MT1-1) cells abrogated the tumorigenicity enhanced by MT1-MMP. Thus, it is clear that dCAT(1) antagonizes the tumor-promoting function of MT1-MMP even *in vivo*. At the same time, endogenous MT1-MMP of the tumor cells (MKN-28, MKN-45, and HT1080) seemed to contribute to their tumorigenicity, at least in those cells tested. This result is consistent with and complementary to previous observations that the enforced expression of MT1-MMP in tumor cells enhances their tumorigenicity (13, 33, 34).

As additional information, we found that TMK-1 cells expressed MT2-MMP about 5-fold higher than the other three cell lines (data not shown). MT2-MMP, like MT1-MMP, is reported to activate proMMP-2 and contributes to



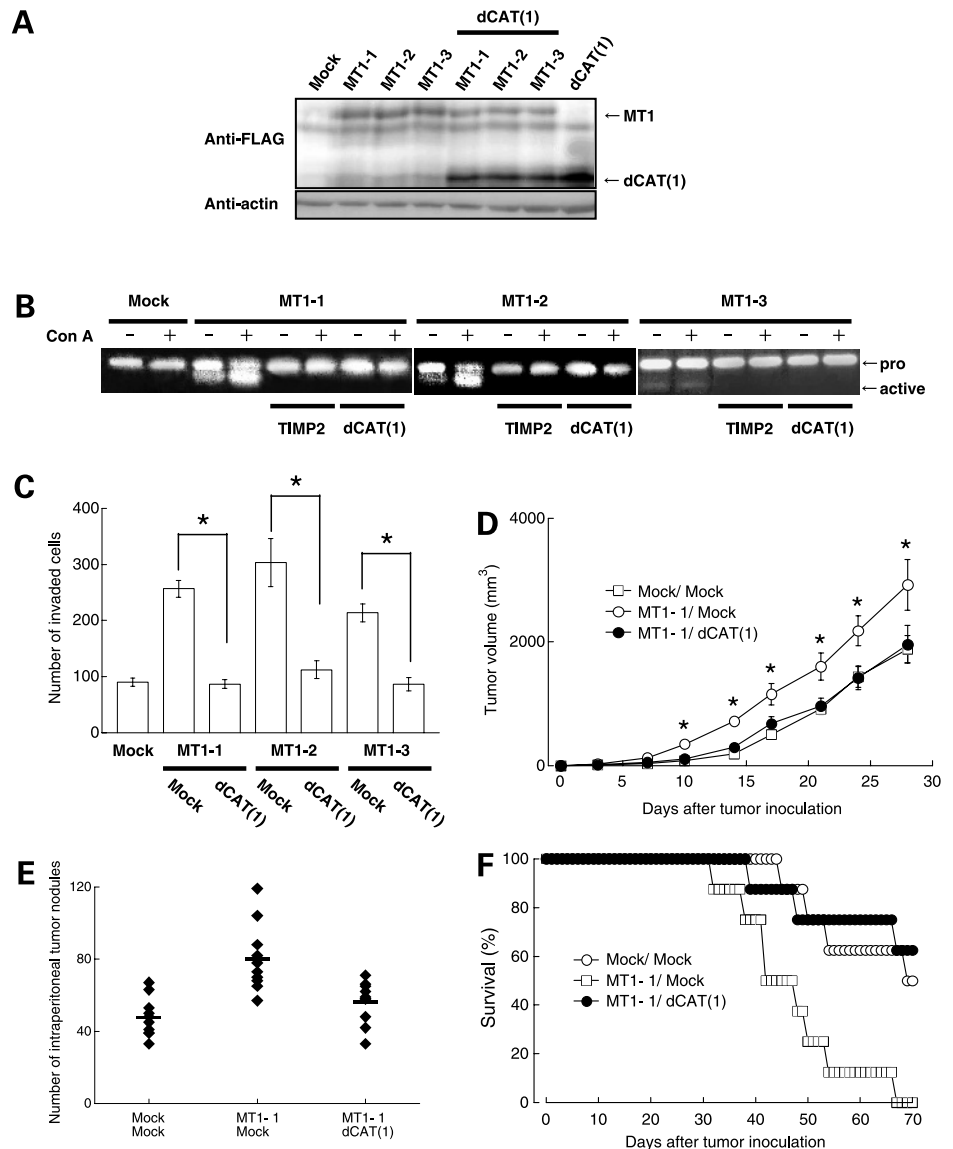
the cell's ability to invade the collagen matrix (12). Because CD44 shedding by MT2-MMP can be inhibited by dCAT(1) (data not shown) or by a similar deletion construct (35), it is possible that dCAT(1) can also inhibit some functions of MT2-MMP in TMK-1 cells. However, MT2-MMP expressed in TMK-1 cells may not be used actively, because the cells could not activate proMMP-2 even after treatment with concanavalin A. Although the effect of dCAT(1) seems to be specific to MT1-MMP expressed in tumor cells, it is possible that the expression of dCAT(1) affects the functions of other proteins that share interacting partners with MT1-MMP. Despite this possibility, dCAT(1) showed no effect on TMK-1 cells in culture and *in vivo*.

Many potential substrates of MT1-MMP have been reported (1, 8, 36), although it is difficult to test them all for dCAT(1). dCAT(1) inhibited at least proMMP-2 activation, cell growth in collagen matrix, and invasion

against Matrigel as shown in Fig. 2. However, it is possible that the expression of dCAT(1) affects a wider range of proteolytic events by MT1-MMP on the cell surface and contributes to antitumor activity as a whole. Among the inhibitory activities of dCAT(1), the inhibition of collagenase activity of MT1-MMP may directly reflect the effect *in vivo* as proposed by Hotary et al. (13). However, this does not necessarily mean that the effect of dCAT(1) on other substrates has negligible antitumor activity. In particular, it should be noted that the antitumor activity of dCAT(1) in mice is greater than in the proliferation assay with collagen gel.

Many synthetic MMP inhibitors have been developed, although a series of clinical trials recently ended in disappointment (5, 7, 37). This is not surprising because MMPs have diverse functions depending on the enzymes, cells, and tissues (1). In tumor-bearing patients, certain MMPs may be important in tissue repair and host

**Figure 6.** Antitumor activity of dCAT(1) was selective in cells expressing MT1-MMP *in vitro* and *in vivo*. **A**, TMK-1 cells were transfected with pcDNA3.1 carrying FLAG-tagged MT1-MMP gene and three zeocin-resistant clones were isolated (MT1-1, MT1-2, and MT1-3). These cells were further transfected with the indicated mutant genes carried by pCEP4 and hygromycin-resistant transfectants were selected. The expression of transfected genes was examined by Western blotting using anti-FLAG M2 mAb. Actin was similarly detected using an anti-actin antibody. **B**, transfected TMK-1 cells were cultured in serum-free medium in the absence or presence of concanavalin A (50  $\mu$ g/mL) for 24 h. The conditioned media were collected and subjected to gelatin zymography. Arrows, proMMP-2 (*pro*) and the activated form of MMP-2 (*active*). **C**, transfected clones ( $1 \times 10^5$  cells) were applied to a transwell chamber with an 8  $\mu$ m pore membrane and coated with Matrigel. After cells were cultured for 48 h, the number of cells invading the lower chamber was counted and expressed as the mean  $\pm$  SE. **D**, the transfected MT1-1 cells ( $2 \times 10^6$  cells) expressing the indicated gene products were inoculated s.c. into athymic nude mice. The tumor size was monitored and presented as the mean volume  $\pm$  SE ( $n = 8$ ). **E**, the MT1-1 cells ( $2 \times 10^6$  cells) transfected with the indicated plasmid were inoculated i.p. into athymic nude mice. After the 28th d, the mice were sacrificed and the number of tumor nodules in the cavity was counted. **F**, the survival of mice implanted with TMK-1 cells expressing the indicated gene products was monitored and presented by a Kaplan-Meier plot.



defense systems rather than promoting malignant tumors (3, 38, 39). Thus, knowing which MMP should be targeted for cancer therapy is important for us to overcome a deadlock situation. This study showed that MT1-MMP expressed in tumor cells is an appropriate target for therapy and functional inhibition of the enzyme can be attained by using a dominant-negative type of mutant. The results also suggest an alternative approach to inhibit MT1-MMP without using synthetic MMP inhibitors, such as gene therapy delivering the dCAT(1) gene to tumors and antibodies to prevent the enzyme from interacting with substrates and modulator proteins.

## References

- Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161–74.
- Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–4.
- Balbin M, Fueyo A, Tester AM, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 2003;35:252–7.
- Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387–92.
- Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2002;2:657–72.
- Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* 2002;115:3719–27.
- Pavlaki M, Zucker S. Matrix metalloproteinase inhibitors (MMPi): the beginning of phase I or the termination of phase III clinical trials. *Cancer Metastasis Rev* 2003;22:177–203.
- Seiki M. Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Lett* 2003;194:1–11.
- Sato H, Takino T, Okada Y, et al. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 1994;370:61–5.
- Ohuchi E, Imai K, Fujii Y, et al. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J Biol Chem* 1997;272:2446–51.
- Tam EM, Wu YI, Butler GS, Stack MS, Overall CM. Collagen binding properties of the membrane type-1 matrix metalloproteinase (MT1-MMP) hemopexin C domain. The ectodomain of the 44 kDa autocatalytic product of MT1-MMP inhibits cell invasion by disrupting native type I collagen cleavage. *J Biol Chem* 2002;277:39005–14.
- Hotary K, Allen E, Punturieri A, Yana I, Weiss SJ. Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. *J Cell Biol* 2000;149:1309–23.
- Hotary KB, Allen ED, Brooks PC, et al. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* 2003;114:33–45.
- Sabeh F, Ota I, Holmbeck K, et al. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J Cell Biol* 2004;167:769–81.
- Tam EM, Moore TR, Butler GS, Overall CM. Characterization of the distinct collagen binding, helicase and cleavage mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): the differential roles of the MMP hemopexin c domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities. *J Biol Chem* 2004;279:43336–44.
- D'ortho MP, Will H, Atkinson S, et al. Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. *Eur J Biochem* 1997;250:751–7.
- Kinoshita T, Sato H, Okada A, et al. TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J Biol Chem* 1998;273:16098–103.
- Higashi S, Miyazaki K. Reactive site-modified tissue inhibitor of metalloproteinases-2 inhibits the cell-mediated activation of progelatinase A. *J Biol Chem* 1999;274:10497–504.
- Overall CM, King AE, Sam DK, et al. Identification of the tissue inhibitor of metalloproteinases-2 (TIMP-2) binding site on the hemopexin carboxyl domain of human gelatinase A by site-directed mutagenesis. The hierarchical role in binding TIMP-2 of the unique cationic clusters of hemopexin modules III and IV. *J Biol Chem* 1999;274:4421–9.
- Itoh Y, Takamura A, Ito N, et al. Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. *EMBO J* 2001;20:4782–93.
- Lehti K, Lohi J, Juntunen MM, Pei D, Keski-Oja J. Oligomerization through hemopexin and cytoplasmic domains regulates the activity and turnover of membrane-type 1 matrix metalloproteinase. *J Biol Chem* 2002;277:8440–8.
- Itoh Y, Seiki M. MT1-MMP: an enzyme with multidimensional regulation. *Trends Biochem Sci* 2004;29:285–9.
- Mori H, Tomari T, Koshikawa N, et al. CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *EMBO J* 2002;21:3949–59.
- Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. *Oncogene* 2003;22:8716–22.
- Ward RV, Atkinson SJ, Slocombe PM, et al. Tissue inhibitor of metalloproteinases-2 inhibits the activation of 72 kDa progelatinase by fibroblast membranes. *Biochim Biophys Acta* 1991;1079:242–6.
- Uekita T, Itoh Y, Yana I, Ohno H, Seiki M. Cytoplasmic tail-dependent internalization of membrane-type 1 matrix metalloproteinase is important for its invasion-promoting activity. *J Cell Biol* 2001;155:1345–56.
- Movahedi S, Hooper NM. Insulin stimulates the release of the glycosyl phosphatidylinositol-anchored membrane dipeptidase from 3T3-L1 adipocytes through the action of a phospholipase C. *Biochem J* 1997;326:531–7.
- Nomura H, Sato H, Seiki M, Mai M, Okada Y. Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res* 1995;55:3263–6.
- Nakajima T, Harashima S, Hirata M, Kajitani T. Prognostic and therapeutic values of peritoneal cytology in gastric cancer. *Acta Cytol* 1978;22:225–9.
- Yonemura Y, Sawa T, Kinoshita K, et al. Neoadjuvant chemotherapy for high-grade advanced gastric cancer [discussion 61–2]. *World J Surg* 1993;17:256–61.
- Seiki M. The cell surface: the stage for matrix metalloproteinase regulation of migration. *Curr Opin Cell Biol* 2002;14:624–32.
- Takino T, Miyamori H, Watanabe Y, et al. Membrane type 1 matrix metalloproteinase regulates collagen-dependent mitogen-activated protein/extracellular signal-related kinase activation and cell migration. *Cancer Res* 2004;64:1044–9.
- Tsunezuka Y, Kinoh H, Takino T, et al. Expression of membrane-type matrix metalloproteinase 1 (MT1-MMP) in tumor cells enhances pulmonary metastasis in an experimental metastasis assay. *Cancer Res* 1996;56:5678–83.
- Deryugina EI, Soroceanu L, Strongin AY. Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis. *Cancer Res* 2002;62:580–8.
- Suenaga N, Mori H, Itoh Y, Seiki M. CD44 binding through the hemopexin-like domain is critical for its shedding by membrane-type 1 matrix metalloproteinase. *Oncogene* 2005;24:859–68.
- Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM. Membrane protease proteomics: isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 2004;101:6917–22.
- Hidalgo M, Eckhardt SG. Development of matrix metalloproteinase inhibitors in cancer therapy. *J Natl Cancer Inst* 2001;93:178–93.
- Dong Z, Kumar R, Yang X, Fidler IJ. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 1997;88:801–10.
- Silzle T, Randolph GJ, Kreutz M, Kunz-Schughart LA. The fibroblast: sentinel cell and local immune modulator in tumor tissue. *Int J Cancer* 2004;108:173–80.