

Targeting a single function of the multifunctional matrix metalloprotease MT1-MMP.  
Impact on lymphangiogenesis\*

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**Keywords:** MMP-14, membrane type matrix metalloprotease, gelatinase, lymphangiogenesis, proenzyme activation, inhibitory monoclonal antibody, monoclonal antibody therapy

**Background:** Therapeutic strategies for MMP targeting have limited selectivity.

**Results:** A novel, specific mAb against MT1-MMP selectively blocks proMMP-2 activation. This antibody inhibited lymphatic vessel sprouting.

**Conclusions:** A single function of a multifunctional MMP was blocked selectively and completely. MT1-MMP mediated proMMP-2 activation is involved in lymphangiogenesis.

**Significance:** This supports development of therapeutic MMP targeting and the understanding of lymphangiogenesis.

#### SUMMARY

The group of matrix metalloproteases (MMPs) is responsible for multiple processes of extracellular matrix remodeling in the healthy body but also for matrix and tissue destruction during cancer invasion and metastasis. The understanding of the contributions from each individual MMP, both in healthy and pathological events, has been complicated by the lack of specific inhibitors and the fact that some of the potent

MMPs are multifunctional enzymes. These factors have also hampered the setup of therapeutic strategies targeting MMP activity. A tempting target is the membrane-associated MT1-MMP which has well-documented importance in matrix degradation but which takes part in more than one pathway in this regard. In this report, we describe the selective targeting of a single function of this enzyme by means of a specific monoclonal antibody against MT1-MMP, raised in an MT1-MMP knock-out mouse. The antibody blocks the enzyme's ability to activate proMMP-2 without interfering with the collagenolytic function or the general proteolytic activity of MT1-MMP. Using this antibody, we have shown that the MT1-MMP-catalyzed activation of proMMP-2 is involved in the outgrowth of cultured lymphatic endothelial cells in a collagen matrix *in vitro*, as well as in lymphatic vessel sprouting assayed *ex vivo*. This is the first example of the complete inactivation of a single function of a multifunctional MMP and the use of this strategy to pursue its role.

## INTRODUCTION

Matrix metalloproteases (MMPs) are centrally engaged in the processes of extracellular matrix (ECM) turnover that occur both during normal development and in connection with various pathological conditions, including cancer growth, invasion and metastasis (1). MT1-MMP (MMP-14), a transmembrane type MMP, plays a critical role in pericellular collagen remodeling (2,3) and MT1-MMP deficient mice have a profound phenotype including defective angiogenesis and severe bone defects, associated with inadequate collagen turnover (2,4). The human correlate of these bone defects is known as the Winchester syndrome (5) and is associated with mutations in MT1-MMP (6). The collagen degrading properties of MT1-MMP include both a direct collagenolytic activity and the ability to activate proMMP-2, the proform of the secreted gelatinase MMP-2 (4,7-9), but the relative importance of these activities remains elusive. Mutations in the human MMP-2 gene give rise to severe bone disorders (10) and although ablation of the gene in mice was initially reported only to result in minor growth impairments (11), detailed analyses have revealed that MMP-2 deficient mice share many of the characteristics of the human disease in an attenuated form (12,13). In mice expressing a mutated collagen, modified in the classical collagenase cleavage site, concomitant MMP-2 deficiency was shown to severely impact skeletal development (14). This implicates the enzyme in bulk turnover of matrix components in the skeleton. Furthermore, recent studies have pointed to an important function of MMP-2 in lymphangiogenesis, the sprouting of lymphatic vessels (15,16).

Both MT1-MMP and MMP-2 have been tightly linked to tumorigenesis. Both enzymes are highly expressed in human cancers where the levels of MT1-MMP expression and active MMP-2 are positively correlated (17,18) and in mouse cancer models, both proteins contribute to several stages of disease progression (19-24). Thus, it is likely that the role of MT1-MMP in cancer progression includes both direct collagen cleavage and activation of proMMP-2.

Monoclonal antibodies (mAbs) are valuable reagents for the specific functional targeting of extracellular and membrane-bound proteins and have been used successfully in studies of protein

function *in vitro* (25-27), as well as for therapeutic targeting *in vivo* (28). Importantly, a function blocking anti-MT1-MMP mAb has been developed by phage display technology. This antibody is capable of decreasing tumor growth, angiogenesis and invasion *in vivo* (19). However, since it acts as a general inhibitor of MT1-MMP proteolytic activity, it does not allow a distinction between the individual roles of MT1-MMP. To this end, another highly interesting mAb was developed recently and shown to interfere specifically with the collagen binding activity of MT1-MMP's hemopexin domain. This latter antibody was found to counteract MT1-MMP-dependent collagen degradation and, even though the anti-collagenolytic effect was incomplete, it also strongly attenuated cellular invasion *in vitro* (29). In the present work, we have succeeded in developing an MT1-MMP mAb that selectively inhibits the other major function, i.e. proMMP-2 activation, with no effect on the general proteolytic or the collagenolytic activity of MT1-MMP. This blocking effect is complete, thus enabling the selective targeting of a single function of the enzyme. Furthermore, using this antibody, we have shown the importance of MT1-MMP mediated pro-MMP-2 activation in the sprouting of lymphatic microvessels in a collagen matrix.

## EXPERIMENTAL PROCEDURES

*Cells, reagents and antibodies* - The following cells, mAbs and reagents have been described previously or were purchased from commercial sources: Primary murine skin fibroblasts (25), human HT1080 cells and Chinese Hamster Ovary (CHO) cells (ATCC), mAb against trinitrophenyl functional group (a-TNP) (30), murine mAb-2 against MT1-MMP (25), Galardin/GM6001 (31), rat-tail collagen type I (trypsin-resistant collagen I (BD Biosciences) for fibroblast mediated degradation studies (25) and pepsin-extracted collagen (collagen R; Serva electrophoresis) for lymphatic endothelial cell sprouting analyses (16)), recombinant human MT1-MMP for BIAcore analyses (complete extracellular part of the enzyme; Calbiochem), interleukin 1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Peprotech) and recombinant human TIMP-2 (Fuji Chemical Industries). Recombinant human TIMP-1 was a kind gift from Professor Gillian Murphy,

Cambridge Research Institute, UK. BIAcore chips, reagents and rabbit-anti-mouse IgG capture antibody for surface plasmon resonance studies were from GE Healthcare. Novel mAbs against MT1-MMP were generated as described below.

*Recombinant MT1-MMP construct for immunization of mice* - A recombinant, truncated MT1-MMP protein, comprising the propeptide and the catalytic domain of murine MT1-MMP (Gln29-Ser289) and a C-terminal 6-histidine tag, was produced in *Pichia pastoris*. Construction of the expression vector (including a yeast alpha-factor signal sequence and a Kex2 signal cleavage site), transfection of *P. pastoris* strain X-33 cells and protein production were performed as described (32,33), except for the following modifications: The murine MT1-MMP sequence was amplified by PCR using the pMTC/MT1-MMP/suPAR-DIII expression vector (25) as template and the following synthetic oligonucleotide primers (restriction sites underscored): 5'-TCTCTCG AGAAAAGACAAGGCAGCAACTTCAGCCC -3' (forward primer including a XhoI restriction site) and 5'-GCTCTAGATCAATGATGATGATGATGATGCC

CGAAGGGCAGCCCATC-3' (reverse primer including sequence encoding the histidine tag, a stop codon and a XbaI restriction site). The PCR product was then digested with the indicated restriction enzymes and inserted into the *Pichia* expression vector pPICZαA. After transfection and isolation of a strongly producing clone, large-scale culture of cells was performed at pH 6.0, followed by harvest of the supernatant after 24 hours. The protein product, designated MT1-MMP29-289-His, was purified from the filtered supernatant using a 1 ml HisTrap HP column (GE Healthcare) and elution with a linear imidazol gradient ranging from 0.01-1 M imidazol in 20 mM sodium phosphate, 300 mM NaCl, pH 7.0.

*CHO cells transfected with MT-MMPs* - For the construction of expression vectors encoding murine MT1-MMP, MT2-MMP or MT3-MMP, the respective sequences (34) were cloned into the pWPI GWB vector. This vector was modified from the pWPI GW vector (35) by the inclusion of a separate sequence encoding the GFP homolog dsRED (36) to allow an evaluation of transfection efficiency. CHO cells

were transiently transfected with these vectors using Lipofectamine 2000. Uniform transfection levels were confirmed by Western blotting of cell lysates (see below) with a goat dsRED antibody (Santa Cruz Biotechnology).

*Generation of monoclonal antibodies against MT1-MMP*

Immunization of young MT1-MMP deficient mice (2) in a mixed genetic background was performed according to the previously published schedule (25), except that the mice received the first immunization injection at an age between 21 and 28 days and that each injection included 20 µg of recombinant MT1-MMP29-289-His protein mixed with either adjuvant (1 mg Al(OH)<sub>3</sub>, Statens Serum Institut, Copenhagen, Denmark; two initial immunization injections) or PBS (subsequent three booster injections). Immunization was performed with the approval of the National Institute of Dental and Craniofacial Research (NIDCR) Animal Use and Care Committee, NIH. Sera from the immunized mice were tested for anti-MT1-MMP antibodies by ELISA (see below). Spleen cells from an antibody-positive mouse were fused with X63.Ag8.653 myeloma cells (ATCC #CRL-1580) by electrofusion, using equipment and reagents from Cyto Pulse Sciences Inc. and following the procedures recommended by this manufacturer. Fused cells were plated in 96 well cell culture plates at a cell density of 1.5x10<sup>4</sup> cells/well and selection of successfully fused hybridoma cells was performed by cell culture in the presence of 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine (HAT, Sigma-Aldrich cat no H0262). After 10 days of cell culture, culture supernatants were screened by ELISA, performed as described (25), except that the ELISA wells were coated with 15 ng of recombinant murine MT1-MMP29-289-His protein. Clones producing antibody reactive with MT1-MMP were subcloned by limiting dilution and the reactivity of monoclonal antibodies against MT1-MMP was confirmed by Western blot analysis. IgG was purified from the culture supernatants by affinity chromatography on a Protein G column (GE Healthcare). All mAbs were of the IgG1κ subtype, as determined using a mouse monoclonal isotyping kit (Serotec).

*Assays for antibody binding and specificity* - Detergent cell lysates for Western blotting were prepared using a lysis buffer including 1 % Triton X-100, 10 mM Tris/HCl, 140 mM NaCl,

pH 7.4, supplemented with 0.5 % (v/v) protease inhibitor cocktail III (Calbiochem). Cells were incubated at 0°C in this buffer for 20 min, followed by clarification by centrifugation. For transfected cells, this was done 24 h post transfection. Western blotting was performed using the NuPAGE system from Life Technologies with electrophoresis on 4-12 % gradient gels under reducing conditions. After electroblotting, blocking of PVDF membranes with 5 % skim milk powder and incubation with murine primary antibodies in the presence of the same blocking solution, the rabbit anti-mouse IgG based chemiluminescence (ECL) system (GE Healthcare) was used for detection.

Surface plasmon resonance analysis of the binding of MT1-MMP to mAb 9E8 was done using a BIAcore 3000 instrument (GE Healthcare) and software from the same supplier. The BIAcore rabbit anti-mouse catching antibody, coupled on a CM5 sensor chip, was used for capture of injected mAb in a uniform orientation (37). Following the binding of mAb 9E8 on this support, dilution series of purified, recombinant human MT1-MMP in 10 mM HEPES, 150 mM NaCl pH 7.4 with 0.005 % Surfactant P20 (HBS-EP buffer; GE Healthcare) were injected at a flow rate of 10  $\mu$ l/min, after which the binding and dissociation phases were recorded at 20°C. A reference flow channel without coupled antibody allowed for buffer bulk subtraction throughout each sensorgram. For each dilution of MT1-MMP, a parallel run was performed without pre-injection of mAb 9E8. The resulting sensorgram, representing unspecific binding to the capture support, was subtracted from the specific binding curve. Kinetic analysis was performed using the BIAeval software, version 4.1. Data was analyzed according to a Langmuir 1:1 binding model which was consistent with the curves obtained.

*Assays for MT1-MMP function and inhibition*  
-Fluorogenic assays for MT1-MMP endopeptidase (general proteolytic) activity in a purified system were performed with the recombinant murine MT1-MMP catalytic domain (see above), using the quenched fluorescent peptide substrate Mca-K-P-L-G-L-Dpa-A-R-NH<sub>2</sub> (R&D systems). The assay was performed in 100 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl<sub>2</sub>, 1  $\mu$ M ZnCl<sub>2</sub>, 0.05% Brij35, pH 7.5, including 10  $\mu$ M substrate. Approx. 5 nM

MT1-MMP29-289-His was pre-incubated in the presence or absence of potential inhibitors (galardin, mAb 9E8 or TIMP-2). Assay was performed at 25°C and fluorescence recorded continuously during a 50 min period with excitation at 355 nm and emission at 460 nm.

Studies of cell mediated proMMP-2 activation were performed with HT1080 cells cultured in serum free DMEM including 100 nM phorbol-12-myristate-13-acetate (PMA). Cells were cultured for 48 h in this medium alone or in the presence of potential inhibitors (galardin, TIMP-1, TIMP-2, antibodies against MT1-MMP or a-TNP (isotype matched control antibody)). Conditioned media were then analyzed by gelatin zymography (25), using gels and reagents from Invitrogen and following the procedures recommended by the manufacturer.

Assays for fibroblast-mediated degradation of a reconstituted collagen I matrix, with cell culture in serum free medium including 1 nM IL-1 $\beta$  and 10 nM TNF- $\alpha$ , were performed essentially as described (2). In some experiments, <sup>125</sup>I-labeled collagen I was incorporated into the collagen matrix (26), after which the release of radiolabeled collagen degradation products was monitored daily by measuring 10  $\mu$ l of culture supernatant in a gamma counter.

*Outgrowth of lymphatic endothelial cells and microvessels in collagen matrices* – Studies on the outgrowth of lymphatic endothelial-like HTERT-HDLEC cells (38) from collagen-embedded spheroid cultures were performed essentially as described (16). Briefly, cells were seeded and pre-cultured in endothelial basal medium (EBM)-2 containing 0.24% high viscosity methyl cellulose (Sigma Aldrich) in cell culture plates with 2 x 10<sup>3</sup> cells per well for 24 h to form micro-spheres. Subsequently, spheroids were collected, embedded in collagen gels and maintained at 37°C for another 24 hours in medium including 5% FCS, supplemented with 650 nM anti-MT1-MMP mAb 9E8 or irrelevant control antibody (anti-TNP), using duplicate samples for each experimental condition. After this, unstained cell samples were examined by phase-contrast microscopy, using an Axiovert 25 microscope equipped with a 20X NA 0.3 LD A-Plan lens and an AxioCam color digital camera (Carl Zeiss, Jena, Belgium). Images were captured at room temperature, using acquisition software KS400

3.0. Cell migration was then quantified by a computerized method determining the sprouting envelope area, defined as the area of the minimal convex polygon containing the whole spheroid and all sprouting cells. Two independent experiments were performed with identical results.

Lymphatic “ring” assays for *ex vivo* studies on lymphatic sprouting in a 3D collagen matrix were performed as described (15). The explants of murine thoracic ducts (duplicate samples for each experimental condition) were cultured for 11 days in the presence of 650 nM mAb 9E8 or the above-mentioned irrelevant control antibody. Cultures were examined by phase contrast microscopy as above, using a 5X NA 0.12 A-Plan lens (Carl Zeiss) for image capture. Computerized quantification was based on binary images as described (15), using a grid comprised of concentric rings which was generated by successive increments at fixed intervals from the duct boundary. The number of microvessel–grid intersections was then counted and plotted versus the distance from the ring to determine microvessel distribution. At least 5 images per experimental condition were used. The lymphatic sprouting assay was performed twice, using independent duct isolates, yielding identical results.

## RESULTS

*Generation of murine monoclonal antibodies against murine and human MT1-MMP* - In order to obtain reagents that interfere with the individual molecular functions of MT1-MMP, a panel of mAbs against it was generated. This was achieved by immunization of MT1-MMP deficient mice with a recombinant murine MT1-MMP protein comprising the propeptide and the catalytic domain (see Experimental Procedures). A panel of eight anti-MT1-MMP mAbs was obtained.

One of the antibodies directed against the catalytic domain, mAb 9E8 (IgG1 $\kappa$  subtype), was further characterized. This antibody specifically recognized MT1-MMP (Fig. 1). Thus, when lysates of wildtype and MT1-MMP-deficient primary fibroblasts were analyzed by Western blotting using this antibody, the deficient cells showed no reaction (lane 1), whereas a 55 kDa band, corresponding to the molecular mass of active MT1-MMP, was observed in the wildtype cells (lane 2). In

addition, the wildtype cell lysates displayed a very weak band at approx. 60 kDa, corresponding to pro-MT1-MMP that has not yet passed exportation and furin-mediated activation. The antibody also specifically recognized the human form of MT1-MMP as shown with lysates of human HT1080 fibrosarcoma cells, cultured either under standard conditions (lane 3) or in the presence of phorbol-12-myristate-13-acetate (PMA) (lane 4). The Western blot of these lysates displayed the same band pattern as that obtained with wildtype murine fibroblasts, above. To specifically address the question whether the antibody might cross-react with the closely related MT2- and MT3-MMP, we also analyzed lysates of CHO cells transfected with DNAs encoding MT1-MMP, MT2-MMP and MT3-MMP, respectively. Whereas a low level of endogenous MT1-MMP is expressed in CHO cells (39) and could be detected in the untransfected cells with mAb 9E8 (lane 8), a strongly increased signal was noted in cells transfected with MT1-MMP cDNA (lane 5). However, no increase in signal and no additional bands were noted in cells transfected with MT2- or MT3-MMP (lanes 6 and 7).

*mAb 9E8 inhibits the MT1-MMP-catalyzed activation of proMMP-2* To study any effect of the antibody on the function of MT1-MMP, we first examined the effect of mAb 9E8 on proMMP-2 activation, using a cellular proMMP-2 activation assay with HT1080 cells. These cells express both MT1-MMP and proMMP-2 (40). When cultured in serum free medium, HT1080 cells secrete proMMP-2 which can be visualized by gelatin zymography as a ~70 kDa gelatinolytic component (Fig. 2A, lane 1). Upon stimulation of cells with PMA, proMMP-2 is processed into the active enzyme through an intermediate form (41,42) (lane 2). This activation process is dependent on MT1-MMP which could also be specifically detected in the PMA stimulated cells by mAb 9E8, as noted above. Accordingly, proMMP-2 activation could be fully inhibited by galardin, a broad spectrum MMP inhibitor, as well as the physiological MT1-MMP inhibitor TIMP-2 (lane 3 and 5). The first cleavage step was insensitive to TIMP-1, an inhibitor which does not bind to MT1-MMP but efficiently blocks MMP-2 activity (lane 4). However, addition of TIMP-1 did lead to inhibition of the second cleavage event. These

observations are in accordance with previous studies demonstrating that the cleavage generating the intermediate form is catalyzed by MT1-MMP, whereas the second cleavage reaction results from MMP-2 autocatalytic cleavage (41,42).

Very strikingly, addition of mAb 9E8 completely inhibited the proMMP-2 activation process, resulting in a situation identical to that observed with galardin and TIMP-2 (lane 6). The addition of an isotype matched control antibody (a-TNP) or another mAb against murine MT1-MMP (mAb-2) had no effect on the activation process, demonstrating that the effect observed with mAb 9E8 was neither an artifact resulting from the addition of exogenous IgG or a result of generally targeting MT1-MMP on the cell surface (lane 7 and 8).

An analysis of the concentration dependence revealed detectable inhibition of the activation reaction with 10-20 nM mAb 9E8 and almost complete inhibition at 60-80 nM (Fig. 2B). For comparison, the kinetics of the interaction of the mAb with purified MT1-MMP were determined by surface plasmon resonance analysis (Fig. 2C). The  $K_d$  derived from the kinetic data obtained was between 0.5 and 1 nM (Fig. 2C). Thus, mAb 9E8 inhibited the proMMP-2 activation reaction in concentrations 10- to 50-fold higher than the actual  $K_d$  of the interaction with MT1-MMP. Considering the fact that the conditions of the proMMP-2 activation assay included long-term incubation of the antibody with live PMA-stimulated cells, we considered this to be a highly efficient functional effect.

*mAb 9E8 has no effect on the general proteolytic or the collagenolytic activity of MT1-MMP and shows weak interference with TIMP-2 binding* - Next, we investigated whether the inhibitory effect of mAb 9E8 was restricted to the proMMP-2 activation process or also included inhibition of other MT1-MMP functions.

First, the antibody's ability to inhibit the general endopeptidase activity of MT1-MMP was investigated. To this end, the activity of recombinant MT1-MMP was measured using a quenched fluorescent peptide substrate (Fig. 3A). The cleavage of this substrate was fully inhibited by galardin. In contrast, no inhibitory effect of mAb 9E8 was observed, demonstrating that the antibody is not a general inhibitor of MT1-MMP activity.

Second, because an important role of MT1-MMP is the ability to cleave fibrillar collagens directly, we wanted to test whether mAb 9E8 had any effect on this activity. For that purpose, a cell based collagen degradation assay was utilized (2). In this assay, MT1-MMP expressing, primary mouse fibroblasts are cultured upon a reconstituted collagen type I matrix for five days. During this period the fibroblasts degrade the underlying matrix and, as previously demonstrated (2) (25), this degradation process is completely dependent on MT1-MMP since fibroblasts isolated from MT1-MMP deficient littermate mice lack the ability to degrade the matrix (Fig. 3B). As expected, galardin fully inhibited the collagenolytic activity of the wild-type fibroblasts. In contrast, mAb 9E8 had no effect on the degradation process (Fig. 3B). Furthermore, a quantitative variant of the assay was developed by incorporation of radiolabeled collagen into the matrix. In this way, the degradation process could be followed over time by measuring the release of radioactivity into the surrounding medium. In accordance with the results obtained in the original assay, the accumulation of radioactivity in the conditioned medium displayed a clear MT1-MMP dependency and was fully inhibited by galardin but was not affected by mAb 9E8 (Fig. 3C).

A third functional interaction of MT1-MMP is the binding to TIMP-2. In terms of extracellular proteolysis, the consequences of this interaction are quite complex, depending on the local concentrations of the reactants. Thus, TIMP-2 is capable of inhibiting the proteolytic activity of MT1-MMP but is also an essential component of the MT1-MMP:TIMP-2:proMMP-2 activation complex involved in MT1-MMP dependent proMMP-2 activation. (43). In this trimolecular complex, TIMP-2 acts as a bridging entity between proMMP-2 and MT1-MMP. Because in this complex MT1-MMP acts as a docking device and is inactivated through the binding of TIMP-2, a neighbouring "TIMP-2 free" MT1-MMP molecule serves to mediate the proteolytic cleavage of the bound proMMP-2 (40,44,45). Therefore, the TIMP-2 concentration is a crucial determinant for the level of proMMP-2 activation. At low concentrations, TIMP-2 facilitates the activation reaction, whereas at high TIMP-2 concentrations

no activation is observed due to the lack of inhibitor free MT1-MMP (46).

Consequently, we studied any interference of mAb 9E8 with the TIMP-2 mediated inhibition of MT1-MMP (Fig. 4). For this purpose, we utilized the above mentioned quenched fluorescent peptide substrate to assay MT1-MMP activity in the presence of the inhibitor. In the first experiment, we employed a TIMP-2 concentration (100 nM) resulting in almost complete inhibition of MT1-MMP activity. As seen in Fig. 4A, addition of mAb 9E8 to this system had no impact on the effect of TIMP-2. Thus, at least under these conditions, the antibody is incapable of preventing the access of the inhibitor to the active site of the enzyme.

This, however, did not exclude a weak interference with the formation of the inhibitor complex that might be apparent only at low concentrations of TIMP-2. To study this possibility, we first established a TIMP-2 concentration range resulting in partial inhibition of MT1-MMP activity (data not shown). We then used conditions leading to approx. 60 % inhibition to study any interference of mAb 9E8 with the effect of the inhibitor (Fig. 4B). Interestingly, under these conditions, we could indeed observe a partial rescue effect of the antibody on the inhibition profile. The lack of complete rescue, even at a high antibody concentration, indicates that this is only a weak effect. Nevertheless, a weak interference with TIMP-2 binding may contribute to explain the inhibitory function of mAb 9E8 against proMMP-2 activation because this interference would counteract the build-up of the proMMP-2 activator complex.

*mAb 9E8 delays lymphatic endothelial cell outgrowth in collagen* – Having characterized the ability of mAb 9E8 to block a single function of MT1-MMP, we next wanted to utilize this as a strategy to differentiate between the different functions of the protease in a matrix-dependent cellular system. The migration of lymphatic endothelial cells (LECs) into 3-dimensional collagen matrices is dependent on proteolytic activity (47) and MMP-2 has been shown to play a major role in this process (16). This opens the question whether the proteolytic cascade step of MT1-MMP catalyzed pro-MMP-2 activation is involved. In particular, this question is important because other proteases may also serve to activate pro-MMP-2 (4,48). To study this

question, we first employed a simple system with spheroids of cultured LECs (HTERT-HDLEC) embedded in a collagen gel. The outgrowth of these cells can be followed microscopically and quantified in an established computerized system (16). When cellular outgrowth was followed in the presence of mAb 9E8, a marked reduction in migration was noted, relative to cells cultured in the presence of an irrelevant control antibody (Fig. 5). Thus, the migration of these cells was indeed supported by the MT1-MMP – proMMP-2 cascade activation.

Finally, to study this phenomenon in a more complex biological system, we employed an established *ex vivo* system, the “lymphatic ring assay” using explants from mouse thoracic ducts. When cultured in a collagen gel in the presence of serum, these explants expand into the surroundings in the form of tubular outgrowths that retain the characteristics of lymphatic microvessels (15). When mAb 9E8 was added to these cultures, a marked reduction in lymphatic sprouting was noted (Fig. 6), in accordance with the previously reported importance of MMP-2 activity in this process (16). This result shows that the responsible mechanism is indeed MT1-MMP mediated proMMP-2 activation.

## DISCUSSION

In this study, we have developed and utilized an MT1-MMP monoclonal antibody that selectively inhibits the complicated process of proMMP-2 activation. To our knowledge, this is the first example of an approach enabling the complete and selective blocking of a single function of a single MMP.

The understanding and prevention of dysregulated MMP activity during pathological processes such as cancer invasion is a subject of extraordinary interest. However, it has become evident that the wide spectrum of MMPs, some with overlapping function and others with opposing roles, complicates both understanding and successful intervention (49). Today, it is clear that the apparent failure of premature clinical trials in this regard was due, at least in part, to a lack of selectivity in the intervention strategies utilized (50). Consequently, current efforts are focused on the development of highly specific reagents, directed against single MMPs (51,52). An additional level of complexity arises because some of these enzymes have multiple

substrates and functional roles (53), which may necessitate the development of even more narrow, function-specific strategies of intervention. Our current work demonstrates that this type of strategy can indeed be pursued.

MT1-MMP is a particularly attractive target in the development of anti-invasive therapy because this protease has a well-established function in matrix degradation (2) and a central role in invasive growth, being expressed by both tumor and stromal cells (54,55). Whereas tumor studies in genetically based mouse cancer models are hampered by the severe phenotype of MT1-MMP deficient mice (20), a mAb has recently been developed which efficiently blocks MT1-MMP activity and transplanted tumor growth *in vivo* (19). However, the exact mechanism through which MT1-MMP serves to promote tumor progression in these systems is not clear. Thus, whereas MT1-MMP was first discovered as the result of the search for the endogenous activator of the MMP-2 proenzyme (9), the enzyme was subsequently also demonstrated to display direct activity against various ECM proteins, including native collagens (7,8).

Since then, both the MT1-MMP dependent proMMP-2 activation and the activity of MT1-MMP towards collagens have been studied extensively. However, although both of these functions appear to be important, their individual contributions to physiological and pathological processes are still insufficiently known. Nevertheless, several observations suggest that MT1-MMP mediated proMMP-2 activation plays an important role, both during normal development and in disease progression (2,4,10-13,19-23).

The novel MT1-MMP antibody, mAb 9E8, inhibits MT1-MMP mediated activation of proMMP-2 on the cell surface. Although the mechanism of inhibition is unknown, our studies suggest that a weak interference with the MT1-MMP:TIMP-2 interaction could contribute to this effect, since it may counteract the formation

of the MT1-MMP:TIMP-2:proMMP-2 complex involved in the activation process.

The fact that mAb 9E8 has no effect on the general proteolytic or the collagenolytic activity of MT1-MMP provides a unique opportunity to discriminate between the effects of MT1-MMP exerted through the activation of proMMP-2 and those exerted by direct proteolytic attack on ECM components. We have utilized this property to demonstrate the importance of the current enzyme system in two experimental settings with sprouting of lymphatic endothelial cells in a collagen matrix. Importantly, these findings do not only provide independent proof of the importance of MMP-2 in this process but also directly identifies the involvement of the MT1-MMP – proMMP-2 activation reaction.

The specific inhibitory properties of mAb 9E8 complement those of other interesting targeting reagents. These include the above-mentioned neutralizing mAb against MT1-MMP, developed by Devy et al., which completely blocks all proteolytic activity of the enzyme (19), as well as the more recently described antibody developed by Basu et al., which displays an interference with the collagenolytic function alone (29). Furthermore, mAb 9E8 has a broad potential utility since it reacts with both murine and human MT1-MMP. This is a valuable property since antibodies with cross-species reactivity can be used, e.g. in mouse models with transplanted human tumor cells, to target antigens expressed by both tumor and stromal cells.

Altogether, the specific mechanism of proMMP-2 activation has enabled its selective targeting through the current strategy, leading to a complete blocking of this function of MT1-MMP while leaving all of its other functions intact. Through the complete and selective blocking of a single function of a multifunctional protease, mAb 9E8 may provide the first example of a new generation of MMP inhibitors.

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## FOOTNOTES

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The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloprotease; MT1-MMP, membrane type-1 MMP; mAb, monoclonal antibody; PMA, phorbol-12-myristate-13-acetate.

## FIGURE LEGENDS

### **FIGURE 1. Specific reaction of mAb 9E8 with murine and human MT1-MMP.**

Detergent lysates of the following cell types were analyzed by Western blotting, using mAb 9E8 as the primary antibody: MT1-MMP deficient mouse fibroblasts (MT1-MMP<sup>-/-</sup>) (lane 1), fibroblasts from a littermate wildtype mouse (MT1-MMP<sup>+/+</sup>) (lane 2), unstimulated and PMA-stimulated human HT1080 fibrosarcoma cells (lane 3 and 4, respectively), CHO cells transfected with cDNAs encoding MT1-MMP (lane 5), MT2-MMP (lane 6) and MT3-MMP (lane 7), and untransfected CHO cells (lane 8). Equal numbers of successfully transfected cells were analyzed for lanes 5-7. The electrophoretic migration of molecular mass marker proteins is indicated (kDa), along with the predicted migration of MT1-MMP and proMT1-MMP.

### **FIGURE 2. mAb 9E8 inhibits MT1-MMP dependent proMMP-2 activation.**

A, antibody-mediated inhibition of the activation reaction. HT1080 cells were cultured in serum free medium in the absence (lane 1) or presence of PMA (lane 2-8). Where indicated 20  $\mu$ M galardin, 250 nM TIMP-1, 120 nM TIMP-2, 200 nM mAb 9E8, 200 nM mAb-2 (MT1-MMP control antibody) or 200 nM a-TNP (isotype matched irrelevant control antibody) was added. After 48 hours, the culture supernatants were harvested and analyzed by gelatin zymography. B, antibody concentration dependence. The experiment was performed as in A in the presence of PMA, using the indicated

concentrations of mAb 9E8 (nM). C, Surface plasmon resonance analysis of the binding of MT1-MMP to mAb 9E8. A BIAcore chip, coupled with an anti-murine IgG catching antibody, was used for capture of mAb 9E8, forming a stationary phase with the immobilized mAb. Different concentrations of purified, recombinant MT1-MMP were then injected, after which the binding and dissociation phases were recorded. Data are presented after subtraction of buffer bulk and unspecific binding (see Experimental Procedures). The binding and dissociation curves (black) followed a simple theoretical Langmuir 1:1 binding model (fitted curves superimposed; orange), allowing the calculation of kinetic parameters (indicated below the graph).

**FIGURE 3. mAb 9E8 has no effect on MT1-MMP endopeptidase or collagenase activity.**

A, endopeptidase activity of MT1-MMP. The activity of recombinant MT1-MMP catalytic domain was measured using a peptide substrate. The reaction was performed with 5 nM MT1-MMP catalytic domain and 500 nM mAb 9E8 or 20  $\mu$ M galardin as indicated. Enzyme activity is represented as arbitrary fluorescence units. Curves represent the mean of triplicates with standard deviation indicated as vertical lines. B and C, collagenolytic activity. B, a droplet of  $5 \times 10^4$  wild-type (MT1-MMP+/+) or MT1-MMP-/- fibroblasts was seeded on a collagen I matrix. Cells were then cultured as detailed in Experimental Procedures. Where indicated, 20  $\mu$ M galardin or 500 nM mAb 9E8 was included in the culture medium. After 5 days of culture, cells were detached and the remaining collagen matrix was stained with Coomassie blue to reveal areas of collagen clearance. C, wild-type (MT1-MMP+/+) or MT1-MMP-/- fibroblasts were seeded on a collagen I matrix including a trace amount of  $^{125}$ I-labeled collagen I and cultured under the same conditions as described in B. The release of  $^{125}$ I-labeled collagen was monitored daily, by measuring 10  $\mu$ l culture supernatant in a gamma counter. Each column represents the mean of a duplicate sample with indication of range. cpm: counts per minute.

**FIGURE 4. Effect of mAb 9E8 on TIMP-2 binding.**

A, mAb 9E8 does not prevent the inhibition of MT1-MMP at a high concentration of TIMP-2. The proteolytic activity of 5 nM recombinant MT1-MMP catalytic domain in the presence or absence of 100 nM TIMP-2 was measured using the same peptide substrate as in Fig. 3A. Where indicated, MT1-MMP was preincubated with 500 nM mAb 9E8. Curves represent the mean of triplicates with standard deviation indicated as vertical lines. B, mAb 9E8 shows partial interference with TIMP-2 binding at low inhibitor concentrations. The experiment was performed as described in A, but with 3 nM TIMP-2.

**FIGURE 5. mAb 9E8 delays the MMP-2 dependent outgrowth from lymphatic endothelial cell spheroids.**

A, Spheroids of HTERT-HDLEC cells in a 3D collagen matrix. Cell spheroids were grown for 24 h in the presence of an irrelevant control antibody (mAb against TNP; left) or anti MT1-MMP mAb 9E8 (right) and examined by phase contrast microscopy. Duplicate samples are shown. Bar = 250  $\mu$ m. B, quantification of cellular outgrowth (convex envelope; see Materials and Methods) in the experiment shown in A. Statistical analysis was performed by non-parametric Wilcoxon test. \*:  $p < 0.0001$ .

**FIGURE 6. mAb 9E8 delays the sprouting of lymphatic vessels *ex vivo*.**

The sprouting of murine thoracic duct explants was followed in the presence of mAb 9E8 or control antibody (mAb against TNP), as indicated. Micrographs show the explant outgrowth in a collagen matrix after seven days (A) and 11 days (B). Bar = 500  $\mu$ m. Duplicate samples are shown. Computerized quantification is shown to the right of each panel.  $N_i$  = number of microvessel intersections with the grid at increasing distances; see Experimental procedures.

Figure 1

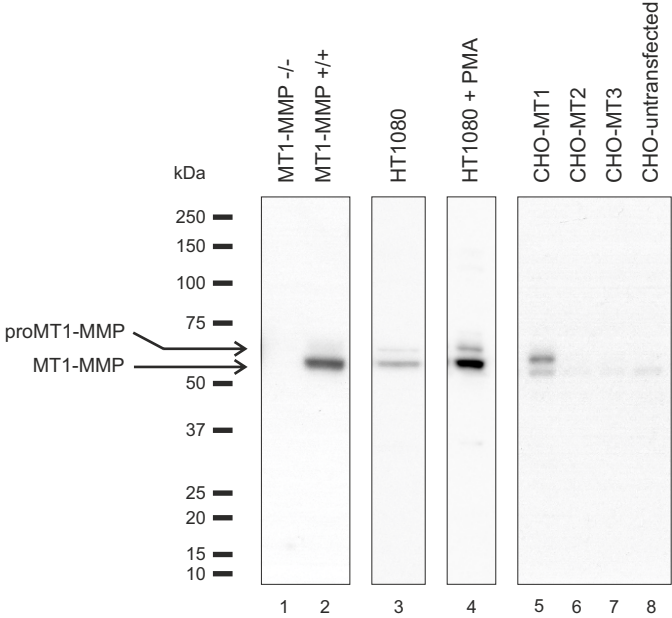
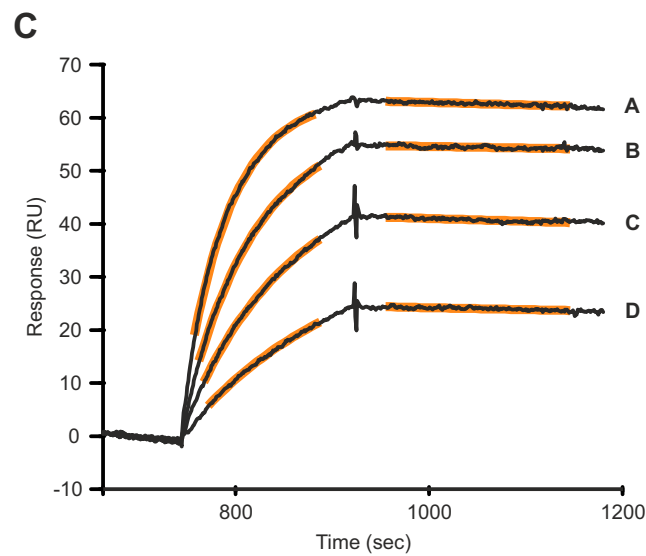
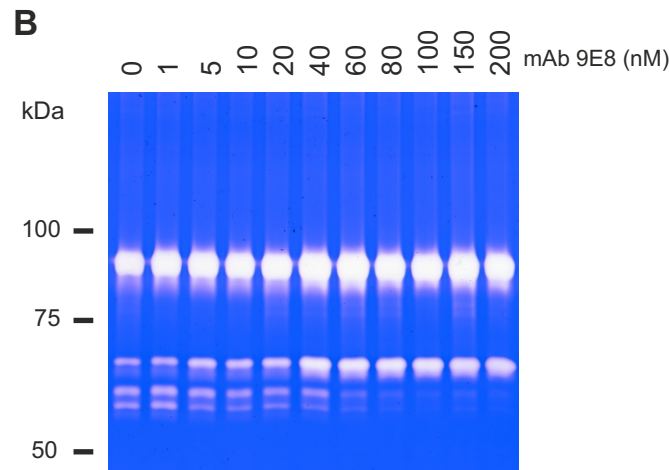
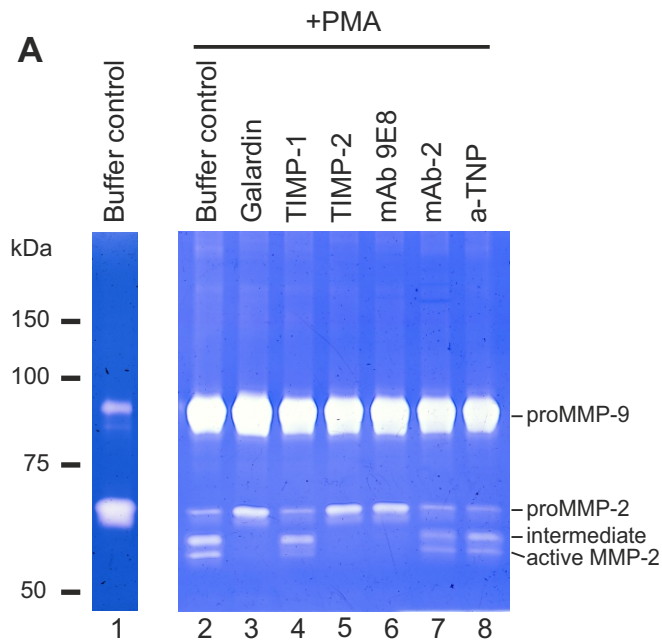


Figure 2



Curve	Concentration MT1-MMP (nM)	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_d$ (nM)
A	200	$1.0 \times 10^5$	$7.6 \times 10^{-5}$	0.7
B	100	$1.3 \times 10^5$	$5.7 \times 10^{-5}$	0.4
C	50	$1.8 \times 10^5$	$1.3 \times 10^{-4}$	0.7
D	25	$2.4 \times 10^5$	$1.5 \times 10^{-4}$	0.6
K <sub>d</sub> (nM) Average ± standard deviation				<b>0.6 ± 0.1</b>

Figure 3

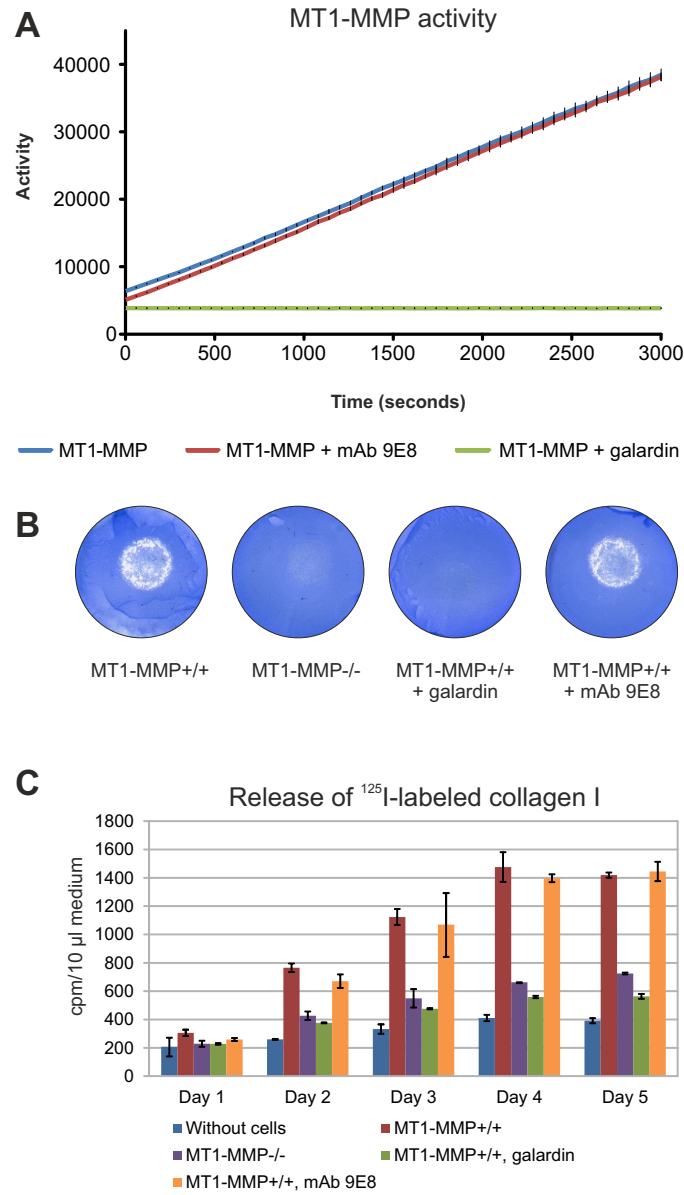


Figure 4

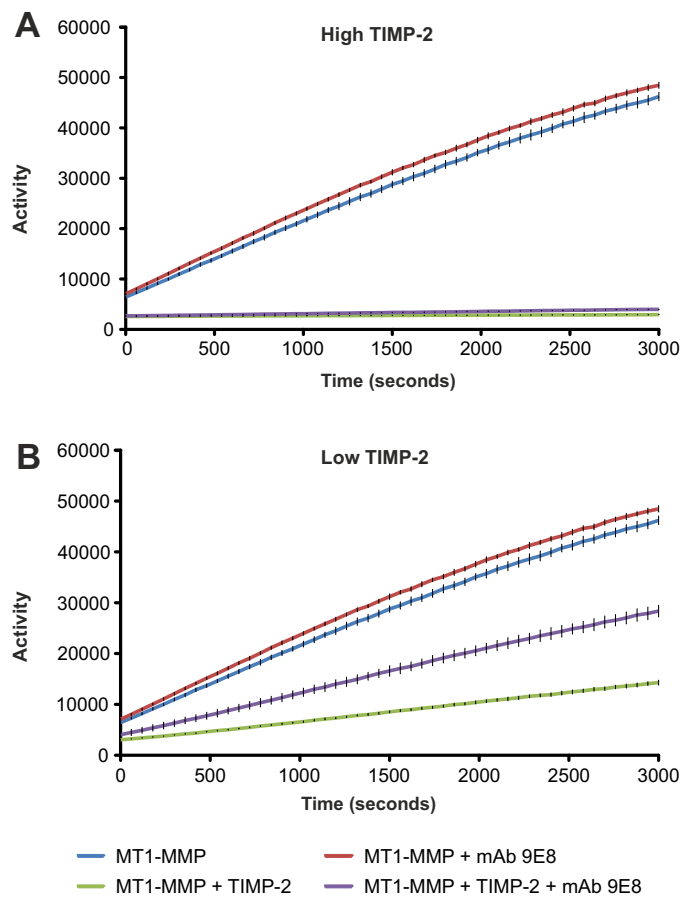


Figure 5

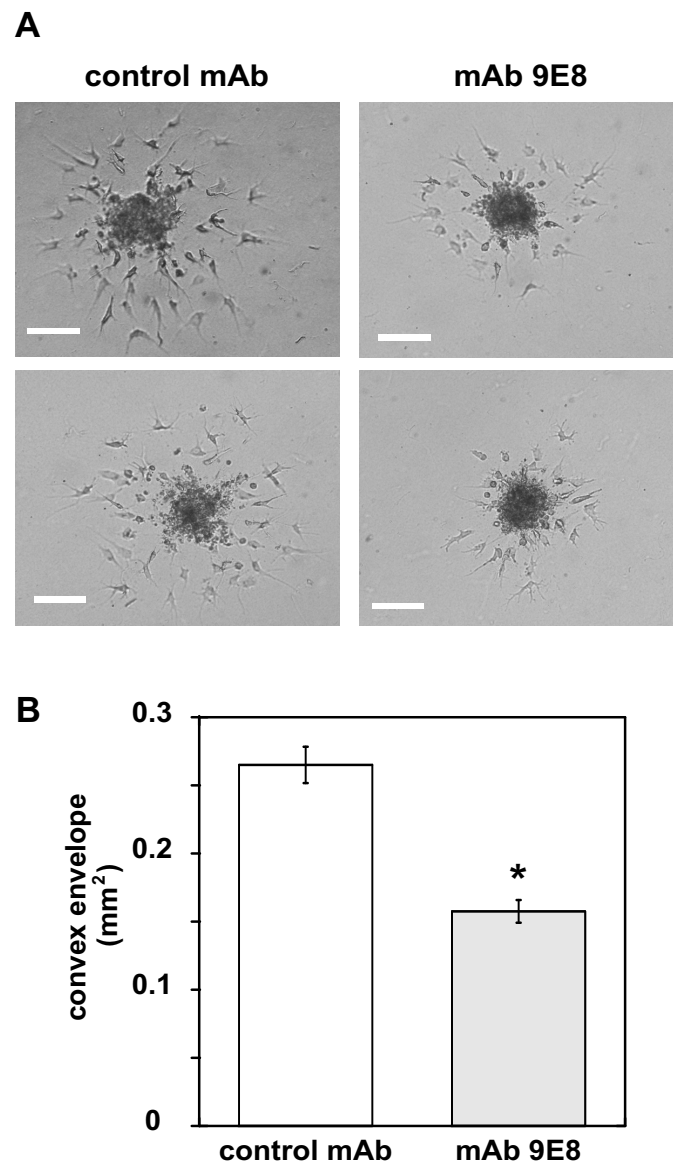


Figure 6

