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Membrane type 1 matrix metalloproteinase and gelatinase A synergistically degrade type 1 collagen in a cell model

Susan J. Atkinson, Margaret L. Patterson, Michael J. Butler[1], Gillian Murphy*

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

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Abstract A fibrosarcoma cell line transfected with the matrix metalloproteinase MT1 MMP showed an enhanced ability to degrade ^{14}C-labelled collagen films. As previously shown for proMMP 2 activation, TIMP 1 was an ineffective inhibitor of the process of collagenolysis whereas TIMP 2 was efficient and completely prevented collagen degradation. In the presence of the calcium ionophore, ionomycin, proteolytic processing of MT1 MMP was restricted and collagenolysis did not occur indicating that the 63 kDa form of the enzyme is not a functional collagenase. The collagenolytic activity of MT1 MMP was shown to be enhanced by the addition of proMMP 2, but TIMP 1 inhibition remained poor relative to that of TIMP 2. The study demonstrated that synergy between two non-conventional collagenases effectively degrades insoluble pericellular collagen. Due to the membrane localisation of MT1 MMP, this could potentially occur in a highly localised manner.

Key words: MT1 MMP; MMP 2; TIMP; Collagen; Proteolysis

1. Introduction

Membrane type matrix metalloproteinases (MT MMPs) are a subset of the matrix metalloproteinase (MMP) family of enzymes which together are capable of degrading the macromolecular components of the extracellular matrix (ECM). MT1 MMP (MMP 14) is the first member of this subset which are distinguished by the presence of a transmembrane domain and a short cytoplasmic 'tail' [1]. In addition they have the propeptide domain, zinc binding catalytic domain and hemopexin-like C-terminal domain of the soluble MMPs. A further characteristic of this membrane associated subset is the presence of a furin recognition site in the propeptide allowing activation by the furin-like proprotein convertases. Many reports to date have emphasised the ability to activate progelatinase A (MMP 2) as the major function of MT1 MMP in vivo [2]. This is believed to involve the formation of a MT1 MMP-TIMP 2 'receptor' on the surface of activated cells which enables binding of proMMP 2 and initiates a two step activation cascade by adjacent TIMP 2 free MT1 MMP [3–6]. A parallel cell surface activation mechanism is proposed for MMP 13 [7,8] which, unlike MMP 2, may also be activated, in common with other soluble MMPs, by activation cascades involving plasmin.

Biochemical studies using recombinant soluble MT1 MMP have shown that MT1 MMP has powerful proteolytic properties against a range of macromolecular substrates [9–11] including fibronectin, tenascin, nidogen, aggrecan and perlecan. It can also cleave fibrillar collagens at a specific locus resulting in the 3/4, 1/4 fragments typically generated by the activity of the neutral collagenases, MMP 1, MMP 8 and MMP 13. In common with these MMPs the C-terminal hemopexin-like domain of MT1 MMP is essential for this activity. These data on the soluble proteinase suggest that cell associated MT1 MMP may function as an efficient collagenase in vivo unless its membrane localisation precludes access to the substrate. A recent report describes severe skeletal defects exhibited by MT1 MMP deficient mice [12]. These include craniofacial dysmorphism, dwarfism, osteopenia, arthritis and fibrosis of soft tissues. Preliminary studies also demonstrated that skin fibroblasts from new born MT1 MMP deficient mice were unable to degrade reconstituted type 1 collagen films when stimulated by IL1-β or TNF-α. It was concluded that the phenotype was due to inadequate collagen turnover owing to the failure of collagenolytic and gelatinolytic activity by MT1 MMP. Additionally, Hotary et al. [13] demonstrated that MDCK cells cultured on type 1 collagen matrices were induced to invade and form tubular networks in response to scatter factor/HGF. This behaviour was not modified by overexpression of soluble MMPs, 1, 2, 3, 7, 9, 11 or 13 but was accelerated by transfection of MDCK cells with MT1 MMP and accompanied by complete degradation of the collagen in the presence of HGF.

We have previously demonstrated the ability of cell associated MT1 MMP to degrade gelatin both directly and indirectly by the activation of MMP 2 [14]. In this report we have used an established human cell model to further examine and quantify the specific collagenolytic potential of MT1 MMP. Human fibrosarcoma HT1080 cells which can be induced to overexpress MT1 MMP were cultured on ^{14}C-labelled type 1 collagen films in order to determine to what extent the collagenolytic activity of these cells is MT1 MMP dependent. Additionally we asked whether MT1 MMP and

*Corresponding author. Fax: (44)-1603-592250.
E-mail: g.murphy@uea.ac.uk

[1] Present address: Department of Genetics, John Innes Centre, Colney, Norwich NR4 7UH, UK.

Abbreviations: MMP, matrix metalloproteinase; MT MMP, membrane type MMP; TIMP, tissue inhibitors of metalloproteinase; ECM, extracellular matrix; DMEM, Dulbecco's modification of Eagle's medium; FCS, foetal calf serum; ECL, enhanced chemiluminescence

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MMP 2, when presented together at the cell surface, are able to cooperate in focussed degradation of the pericellular ECM, and the extent to which TIMPs may regulate this process.

2. Materials and methods

2.1. Inducible expression of MT1 MMP using the Tet-off system

A HT1080 human fibrosarcoma cell line, HTC 75, into which the pTet-off control element had been stably transfected (kindly provided by Dr B. van Steensel, Rockefeller University, New York, USA) was further transfected with human MT1 MMP cDNA (generous gift from Professor M. Seiki, University of Tokyo, Japan) using the pTRE plasmid and pSV2neo (for selection) as described by Butler et al. [15]. Highly expressing stable clones were selected by screening for MT1 MMP using gelatin zymography (see below) to detect activation of endogenous MMP 2. Clone L163 #6 was chosen for these studies in which gene transcription could be repressed in the presence of 100 ng/ml doxycycline (Sigma, UK). Cells were maintained in DMEM and 10% FCS containing 500 μg/ml geneticin (Gibco Life technologies) and 100 ng/ml doxycycline (Sigma) prior to use.

2.2. Immunolocalisation of MT1 MMP

HTC 75 cells were seeded at 10^4 cells/well in the presence or absence of 100 ng/ml of doxycycline in DMEM with 10% FCS (tet approved for induced cells) in four-well chamber slides (Falcon).

Cells were incubated at 37°C, 5% CO_2 for 24 h and then washed, fixed and stained by indirect immunofluorescence for MT1 MMP expression using a polyclonal antibody raised in sheep against human MT1 MMP as previously described [14].

2.3. Preparation of ^{14}C-labelled type 1 collagen films

Radiolabelled collagen films were prepared in 24-well tissue culture plates essentially as previously described [16], but with minor modifications. Aliquots of ^{14}C acetylated rat skin type 1 collagen (150 μg (10 000 dpm) in 300 μl 10 mM sodium phosphate buffer pH 7.4 containing 300 mM NaCl and 0.02% NaN_3) were dispensed into culture wells and incubated for 2 h at 37°C. They were then left overnight in a laminar flow culture hood so that the collagen dried into films. The films were washed twice with sterile distilled water to remove salt crystals and azide before equilibrating with two changes of DMEM followed by plating the cells.

2.4. Culture of HTC 75 cells on ^{14}C collagen films

The cells were seeded onto the films at 10^5 cells/well in DMEM and 10% FCS and 100 ng/ml doxycycline (for uninduced cells) or DMEM and 10% tet approved FCS (Clontech). Cells were left to attach overnight at 37°C then the medium was removed, the cells washed free of serum and replaced by 300 μl/well DMEM and ITS (insulin, transferrin and selenium liquid supplements (Sigma)), with or without 100 ng/ml doxycycline. TIMP 1, TIMP 2 or ionomycin (Sigma) were included in some experiments. In others exogenous proMMP 2 was also included. The cells were incubated at 37°C for 24 h and 48 h, harvesting and replacing medium at each time point. At the end of the experiments any remaining collagen was digested using 50 μg/ml bacterial collagenase (Roche Diagnostics). ^{14}C released into the supernatants was counted (100 μl sample) in a liquid scintillation counter. The remaining supernatants were analysed for MMP 2 activation by gelatin zymography. In some experiments cell lysates were prepared for Western blotting to detect MT1 MMP or MMP 1.

2.5. Purification and preparation of TIMPs

Recombinant forms of TIMP 1 and TIMP 2 were expressed and purified from transfected NS∅ mouse myeloma cells as previously described [17,18], dialysed into phosphate buffered saline and filter sterilised.

2.6. Gelatin zymography

Cell supernatants were analysed by gelatin zymography in order to monitor MMP 2 processing/activation as an indication of the activity of MT1 MMP by the induced cells. Samples of supernatants were electrophoresed on 7% polyacrylamide SDS gels which had 0.5 mg/ml gelatin incorporated into them and stained with Coomassie Brilliant Blue [19].

2.7. Western blotting

Cell lysates were prepared as described by Stanton et al. [20] and protein content was determined using the BCA assay (Sigma, UK). Aliquots containing equivalent amounts of protein were run on 10% polyacrylamide SDS gels, transferred to nitrocellulose using a semi-dry blotter and probed for MT1 MMP using a sheep polyclonal antibody to human MT1 MMP [14]. Blots were visualised using the ECL detection system (Pierce Warriner, UK). Cell supernatants were concentrated 3–5-fold and blotted or analysed by ELISA for the detection of MMP 1.

3. Results and discussion

3.1. Transfected HTC 75 cells express MT1 MMP at the cell surface when repression of gene transcription is removed

HT1080 cells are known to express endogenous MT1 MMP which, in unstimulated cells, is retained in an as yet unidentified intracellular compartment. Upon stimulation, for example by phorbol esters or by culturing on ECM components such as fibronectin or collagen, MT1 MMP is thought to be recruited to the plasma membrane by trafficking events which

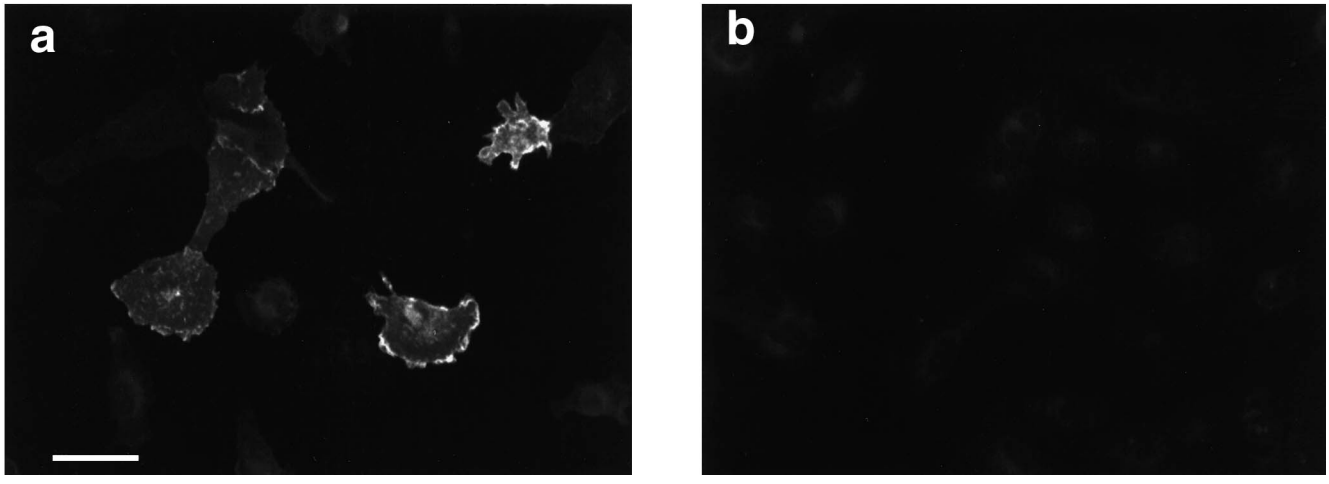

Fig. 1. HTC 75 cells in the absence (a) or presence (b) of doxycycline were stained without permeabilisation for MT1 MMP expression as described in the text. Cell surface staining can be seen in cells induced to express MT1 MMP by removal of doxycycline whereas none is apparent in the uninduced cells. Bar = 50 μm.

remain to be elucidated. At the cell surface it participates in proMMP 2 activation [3,20,21]. The inducible cell line which we have established expressed high levels of MT1 MMP on the cell surface as detected by indirect immunofluorescence when repression of gene transcription was removed by culturing the cells in the absence of doxycycline (Fig. 1). This inducible expression allowed us to examine how cell surface associated MT1 MMP contributes to the proteolysis of the pericellular ECM in the form of a collagen film model.

3.2. MT1 MMP expressing cells show an enhanced ability to degrade collagen

Cells over-expressing MT1 MMP degraded the collagen films up to four times more efficiently than the non-expressing cells after 24 h (Fig. 2a). Analysis of the culture supernatants by gelatin zymography showed the presence of both MMP 9 and MMP 2. MMP 9 was detected only in the inactive proform (92 kDa) in all the samples whether or not the cells were induced. At 24 h, MMP 2 in supernatants from induced cells was all in the fully active (59 kDa) form (Fig. 2b, lanes 3 and 4) compared to that detected in supernatants from repressed cells where approximately 90% was in the proform (66 kDa) (Fig. 2b, lanes 1 and 2). However by 48 h approximately 50% of MMP 2 in the supernatants from non-induced cells could be detected in the fully active form (Fig. 2c, lanes 1 and 2). These results differ from experiments in which the inducible cells are grown on tissue culture plastic. Gelatin zymography of culture supernatants from these experiments demonstrated that MMP 2 is not activated by the repressed cells even after 48 h incubation indicating that MMP 2 activation by these

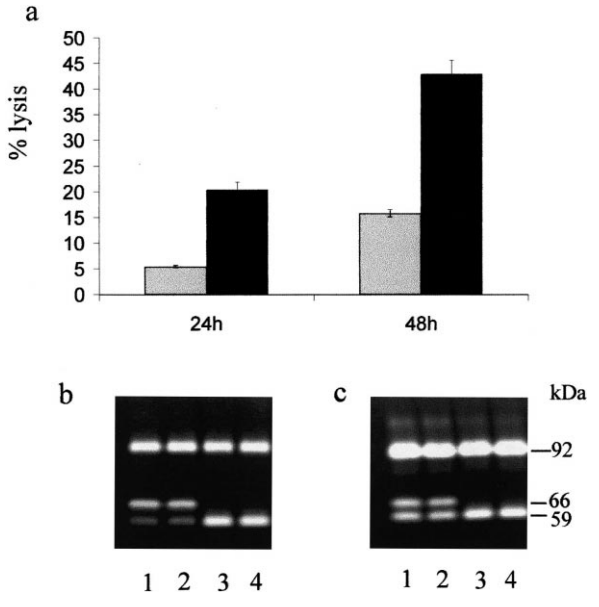

Fig. 2. HTC 75 cells were cultured, as described in the text, on ^{14}C collagen films in the presence (light shading) or absence (dark shading) of 100 ng/ml doxycycline for 24 or 48 h. Radioactive counts released into the cell supernatants after each time point are shown as a percentage of the total counts (a). Each bar represents the mean of four replicate wells. Values from cell free control wells have been subtracted (2–5%). Aliquots of supernatants from the same experiment were analysed on gelatin zymograms as described. Lanes 1 and 2, supernatants from uninduced cells; lanes 3 and 4, supernatants from induced cells. 24 h (b), 48 h (c). Note that approximately 50% MMP 2 is in the active form in 48 h supernatants from uninduced cells as explained in Section 3.

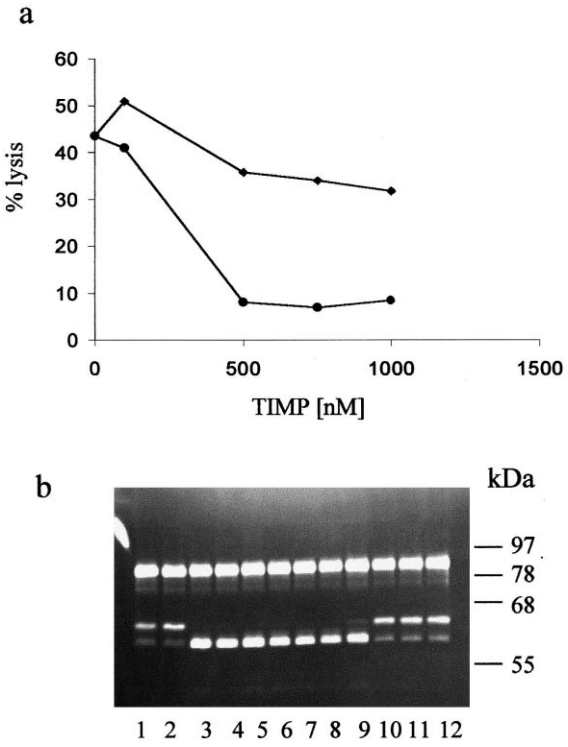

Fig. 3. a: HTC 75 cells were cultured for 24 or 48 h on ^{14}C-labelled collagen films in the presence or absence of doxycycline and varying amounts of TIMP 1 (♦) or TIMP 2 (●). Results are shown as a percentage of the total radioactivity released after 48 h, each point representing the mean of duplicate wells. Data are representatives of three separate experiments. b: Aliquots of 24 h supernatants from the same experiment were analysed by gelatin zymography as described in Section 2. Lanes 1, 2, uninduced cells; lanes 3, 4, induced cells; lanes 5, 6, 7, 8, cells incubated with 100, 500, 750, 1000 nM TIMP 1 respectively; lanes 9, 10, 11, 12, cells incubated with 100, 500, 750 and 1000 nM TIMP 2 respectively.

cells cultured on plastic is MT1 MMP dependent (data not shown). MMP 2 activation by the repressed cells cultured on ^{14}C collagen confirms earlier reports of MMP 2 activation induced by ECM components [21] and is likely to be due to the endogenous levels of MT1 MMP present in these cells. The levels are low however since only a limited increase in ^{14}C collagen degradation was seen between 24 and 48 h and the induced cells still showed enhanced collagenolysis compared to the repressed cells.

3.3. Collagenolysis by MT1 MMP expressing cells is inhibited by TIMP 2

The contribution of MT1 MMP to collagenolysis was further confirmed by the different inhibition profiles seen with TIMP 1 and TIMP 2 (Fig. 3a). MT1 MMP expressing cells incubated in the presence of 1 μM TIMP 1 degraded the collagen by 10% less than those incubated alone. At lower concentrations there was less inhibition of collagenolysis and indeed some apparent protection of the intact enzyme and hence potentiation at 100 nM. TIMP 1 is known to be a poor inhibitor of MT1 MMP [4,22] whereas it is an efficient inhibitor of MMP 2 in vitro. TIMP 2 inhibited collagenolysis by MT1 MMP expressing cells in a dose dependent manner. Only 10% inhibition was observed with a dose of 100 nM TIMP 2 but at 500 nM, TIMP 2 completely inhibited degra-

dation down to levels comparable to cell free controls. At the same concentration TIMP 1 was a very poor inhibitor (Fig. 3a). When TIMPs were removed and the cells cultured for a further 24 h their ability to degrade the collagen was restored. The results from these experiments suggest that in this cell system the majority of the collagenolysis observed is likely to be due to MT1 MMP and that other MMPs have little involvement. No MMP 13 and only negligible levels (<2 ng/ml) of MMP 1 could be detected in association with these cells by ELISA or Western blotting of concentrated 48 h cell supernatants (data not shown) and did not vary between induced and repressed cells.

Gelatin zymography of cell supernatants from these experiments demonstrated that inhibition of MMP 2 activation mirrored inhibition of collagen degradation (Fig. 3b). These data therefore support the previous biochemical data, demonstrating that TIMP 1 does not act as a regulator of MT1 MMP/MMP 2 mediated collagenolysis in a cell model. However it should be noted that TIMP 2 has an apparent inhibition constant of <1 nM when reacted with MT1 MMP in solution [23], indicating that accessibility of the exogenous TIMP to degradative sites is poorer in cell model systems.

3.4. 63 kDa MT1 MMP has no collagenolytic activity

Three forms of MT1 MMP can be identified in lysates from cells which activate MMP 2. These have molecular masses of 63 kDa, 60 kDa and 45 kDa, corresponding to the pro, fully active and truncated forms of MT1 MMP [6,24,25]. Some reports have suggested that the 63 kDa form is not always cleaved to the 60 kDa form as a prerequisite for catalytic activity [26], however work published whilst this manuscript was in preparation demonstrated that processing of MT1 MMP to the 60 kDa form in CHO K1 cells was required for proteolytic activity. When these cells were co-transfected with MT1 MMP and α_1 antitrypsin Portland (α_1PDX), which was shown to inhibit the processing of proMT1 MMP to the active 60 kDa form, no degradation of gelatin was observed compared to cells transfected with MT1 MMP alone [27]. In order to establish which forms of MT1 MMP were catalytically active in our cell model we incubated induced HTC 75 cells with or without ionomycin, a calcium ionophore which has previously been shown to block conversion of MT1 MMP from the 63 kDa proform to the fully active 60 kDa [28,29]. Collagenolysis was inhibited in a dose responsive manner by the presence of ionomycin (Fig. 4a). Analysis of cell lysates by Western blotting revealed that those from cells incubated in the presence of ionomycin had only a major band of MT1 MMP at 63 kDa compared to lysates from cells induced in the absence of ionomycin where the predominant MT1 MMP bands were 60 kDa and 45 kDa (Fig. 4b). These results indicate that ionomycin is able to prevent the generation of fully mature, active MT1 MMP which is believed to result from cleavage in the propeptide domain by proprotein convertases such as furin. The recent study by Yana and Weiss [27] identified a proprotein convertase–MT1 MMP axis which allows both furin dependent and furin independent MT1 MMP processing either intracellularly or at the cell surface. At present it is unclear whether ionomycin, by allowing an intracellular influx of calcium inhibits MT1 MMP processing by preventing trafficking to the sites of proprotein convertase activity. It is however clear that 63 kDa MT1 MMP has no proteolytic activity in this cell model.

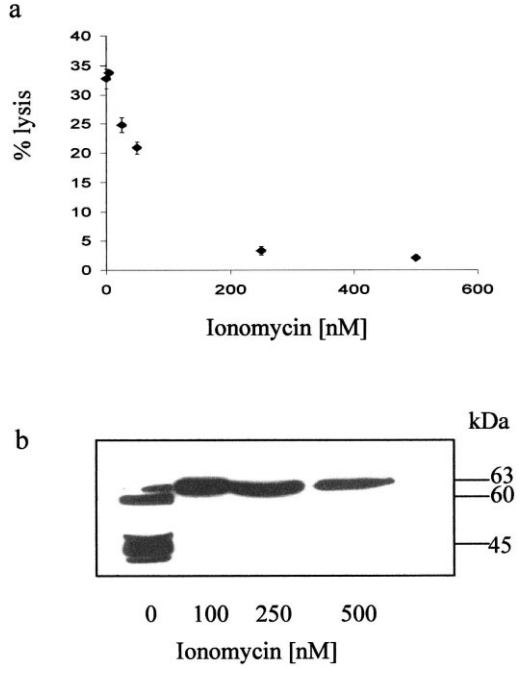

Fig. 4. a: HTC 75 cells were cultured for 48 h on ^{14}C collagen films in the absence of doxycycline and varying amounts of ionomycin. Results are shown as radioactivity released as a percentage of the total. Each point is mean of three replicate wells. Values for cell free controls (5–6%) have been subtracted. b: Western blot analysis of cell lysates from a separate experiment in which cells were cultured on ^{14}C collagen in the absence of doxycycline and in the presence of 0, 100, 250 or 500 nM ionomycin.

3.5. Collagenolysis can be enhanced by the addition of exogenous proMMP 2

The addition of exogenous proMMP 2 resulted in enhanced degradation of collagen by the induced cells (Fig. 5). A proportion of this enhanced degradation was due to activation of MMP 2 by endogenous MT1 MMP since there was also a significant dose responsive increase in degradation of collagen by the non-induced cells (which activate endogenous MMP 2 when cultured on type 1 collagen, Fig. 2). At a dose of 1 μg/ml proMMP 2 collagenolysis was enhanced to almost double that seen in MT1 MMP expressing cells incubated without added proMMP 2. 500 nM TIMP 2 completely abolished degradation of collagen by the induced cells cultured in the presence of 1 μg/ml of exogenous proMMP 2. However TIMP 1 at the same concentration inhibited the enhanced degradation only partially (approximately 30%, data not shown) which suggests either that enhanced collagenolysis was largely effected by MT1 MMP, or that TIMP 1 was unable to efficiently inhibit membrane bound MMP 2. Previous studies have shown that TIMP 1 may be less effective at inhibiting MMP 2 in situations where the C-terminal domain is unavailable (i.e. membrane bound MMP 2), since an MMP 2 mutant lacking the C-terminal domain bound TIMP 1 poorly in vitro [30].

The results from these experiments demonstrate that cell associated MT1 MMP is an efficient interstitial collagenase. Expression of MT1 MMP was accompanied by increased activation of MMP 2, allowing cooperation between these enzymes to facilitate collagenolysis. It is clear that in cell associated activation cascades involving membrane tethered

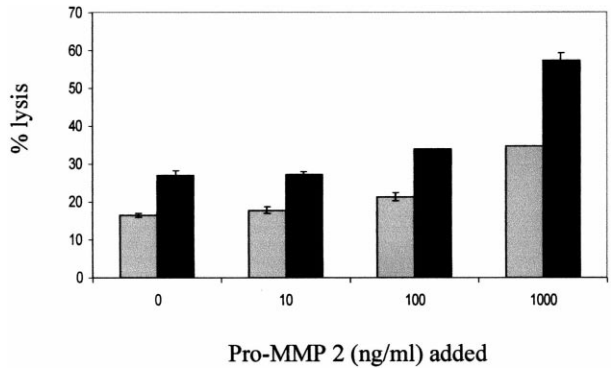

Fig. 5. HTC 75 cells were cultured for 30 h on ^{14}C collagen films in the presence (light shading) or absence (dark shading) of 100 ng/ml doxycycline. Exogenous proMMP 2 was added at the concentrations indicated to three replicate wells. Results are shown as radioactivity released as a percentage of the total with values for cell free controls subtracted.

MMPs relative levels of enzyme and inhibitors are crucial in determining the overall degradation of ECM components. Taken together these results provide evidence that MT1 MMP is able to participate in proteolysis of the pericellular ECM by directly cleaving fibrillar collagens which are then susceptible to further degradation. The cell surface associated nature of this activity provides a focussed and highly regulated mechanism for remodelling the local extracellular environment. The phenotype of the MT1 MMP deficient mouse demonstrates that this is particularly important in bone and cartilage matrix turnover in both development and disease and the ability to cleave fibrillar collagens is of crucial importance in these processes. It is interesting to speculate what the role of the 'traditional' specific interstitial collagenases MMP 1 and MMP 13 might be relative to MT1 MMP. The importance of MMP 1 for cell migration on collagen has been demonstrated for keratinocytes and other epithelial cells [31]. We can further speculate that proteolytic cascades which can be regulated very specifically may be involved in other processes which require focussed degradation of the ECM. In this laboratory co-workers have shown that tubule formation by human umbilical vein endothelial cells grown in fibrin gels involves MT MMP expression and can be inhibited by TIMP 2 but not by TIMP 1. A point mutated form of TIMP 2, which is known to have a greatly increased K_i value for MT1 MMP and is therefore a poor inhibitor of MT1 MMP compared to MMP 2 where inhibition is largely unaffected [23], was however unable to inhibit tubule formation (M. Lafleur and D. Edwards, personal communication). These data invoke a role for cell associated MT1 MMP in angiogenesis. Similar mechanisms are most likely involved in well documented studies correlating MT1 MMP and MMP 2 expression and activation in tumour cell migration.

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