MT1-MMP on the cell surface causes focal degradation of gelatin films

Marie-Pia d'Ortho[a], Heather Stanton[b], Michael Butler[b], Susan J. Atkinson[b], Gillian Murphy[b], Rosalind M. Hembry[c,*]

[a] *INSERM U296, Faculté de Médecine, 8 avenue du Général Sarrail, 94010 Créteil, France*
[b] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[c] *Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, UK*

Received 8 December 1997

Abstract The membrane-type matrix metalloproteinases (MT-MMPs) are a subclass of the matrix metalloproteinase (MMP) family which uniquely possess a C-terminal transmembrane domain and are initiators of an activation cascade for progelatinase A (MMP-2). Recent studies have shown that they can also efficiently directly degrade a number of matrix macromolecules. We now show that cells expressing MT1-MMP on their cell surfaces cause subjacent proteolysis of a gelatin film and that this proteolysis is inhibited by TIMP-2 but not by TIMP-1. These data indicate that expression of MT1-MMP on the cell surface may lead to both progelatinase A activation and extracellular matrix degradation.

Key words: Matrix metalloproteinase; Membrane-type matrix metalloproteinase; Gelatinase A; Matrix degradation; Confocal microscopy

1. Introduction

The description of a subclass of the family of matrix metalloproteinases that are membrane associated, the membrane-type matrix metalloproteinases (MT-MMPs), has focussed largely on their ability to act as initiators of the activation of progelatinase A. Most studies have concentrated on MT1-MMP, which has been shown to be displayed on the surface of activated cells [1]. Studies to define its mechanism of action have shown that this is due to the formation of an MT-MMP-TIMP-2 complex as a 'receptor' for progelatinase A which is processed sequentially by active MT1-MMP and active gelatinase A [2,3]. The structural similarity of the catalytic domain of MT1-MMP with that of other MMPs strongly suggests that they also should be able to degrade ECM components, and the ability of MT1-MMP to digest several ECM macromolecules has been confirmed biochemically [4–7]. Using confocal microscopy, we have studied degradation of fluorescently labelled gelatin films by cells expressing MT1-MMP, comparing fibroblast cells (HIFFs) that naturally express MT1-MMP, with CHO cells transfected with MT1-MMP cDNA. We now show that cells expressing MT1-MMP on the cell surface can focally degrade fluorescently labelled gelatin films, and that this degradation may be inhibited by addition of recombinant TIMP-2 but not TIMP-1.

*Corresponding author. Fax: (44) (1223) 411609.
E-mail: rh.srl@mrc-lmb.cam.ac.uk

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; HIFFs, human infant foreskin fibroblasts; NSS, normal sheep serum; PBS, phosphate buffered saline pH 7.4; rt, room temperature; tPA, tissue-type plasminogen activator; TR, Texas red

2. Materials and methods

2.1. Expression and purification of the GST-ΔMT1-MMP fusion protein in order to raise a polyclonal antibody

A C-terminally truncated form of MT1-MMP, lacking the transmembrane domain (residues 511–582), ΔMT1-MMP, was expressed intracellularly in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST) using the pGEX system [8]. To generate MT1-MMP, PCR was carried out with MT1-MMP cDNA [1] using the primers 5′-CGTAGATCTGCGCTCGCCTCCCTCGGC-3′ (R6662) and 5′-AAAAGATCTCATGGGCAGCCCATCCAGTC-3′ (R6310). Amplified DNA was cloned into pIJ2925 [9] at the *Bgl*II site and the nucleotide sequence was determined from the 5′ end to the downstream *Sma*I site and from the 3′ end to the upstream *Bam*HI site using the Sequenase kit version 1.0 (United States Biochemical). The amplified DNA was replaced by the original cDNA between the *Sma*I and the *Bam*HI sites. After digestion with *Bgl*II the DNA was subcloned into the pGEX-2T plasmid at the *Bam*HI site to encode a GST-ΔMT1-MMP fusion protein. The DNA was expressed in *E. coli* DH5α with isopropyl-β-D-galactoside (0.1 mM) induction. Cells were collected by centrifugation and resuspended in PBS (1 ml/l of cell broth). The cells were disrupted by sonication and the inclusion bodies were washed to remove contaminating *E. coli* proteins. Triton X-100 was added to a final concentration of 1%. Reducing sample buffer was added to the inclusion body suspension which was then electrophoresed using a 3.5 mm thick SDS-polyacrylamide gel. After migration, GST-ΔMT1-MMP was detected as a 70 kDa band by staining with Coomassie blue and was electro-eluted with 0.025% SDS, 25 mM Tris, 192 mM glycine at 100 V overnight at 4°C. The preparation was dialysed overnight at 4°C against 2.5% Triton X-100, 50 mM Tris-HC1 pH 7.4, 5 mM $CaCl_2$. Protein concentration was estimated and protein purity was checked by SDS PAGE, followed by silver staining and Western blotting using a goat anti-GST polyclonal antibody (Pharmacia).

2.2. Preparation of E217A mutant MT1-MMP and eukaryotic expression

A cDNA encoding a catalytically inactive form of MT1-MMP was made by site-directed mutagenesis using PCR overlap extension mutagenesis [10] to replace the glutamic acid residue within the zinc binding helix with an alanine residue. Such mutations have been shown to dramatically reduce the catalytic capability of MMPs. The eukaryotic expression construct was produced in essentially three steps. Firstly, the site-directed mutation was made in the existing pGEX plasmid described above. Secondly, the truncated MT1-MMP cDNA was inserted into the eukaryotic expression vector to generate an 'in frame' fusion with the tPA secretion signal. Thirdly, the 3′ end of the original MT1-MMP cDNA was replaced to produce the full length E217A mutant cDNA.

Oligonucleotide R6662 was used with the mutagenic oligonucleotide 5′-CCCCAGGGCATGGCCCAGCGCGTGCACAGCCACCAGG-AG-3′ to generate a 680 nt amplification product. Similarly, the oligonucleotides R6310 and 5′-CTTCCTGGTGGCTGTGCACGC-GCTGGGCCATGCCCTGGGG-3′ produced a 840 nt product. The amplification products were purified, mixed and used as a template for a PCR reaction using oligonucleotides 5′-GAAAGCTTCCATGGC-GACAGGACGG-3′ and 5′-TCACTGGAATTCTCGAGCCCCAG-

PII S0014-5793(97)01555-X

GGCA-3′. The 208 nt product was digested with *Nco*I and *Xho*I and the resulting 187 nt fragment was purified and ligated using the same restriction enzyme sites into the pIJ2925 plasmid carrying the MT1-MMP ΔTM cDNA described above. The ligation mixture was used to transform competent cells of *E. coli* DH5α and colonies were screened by nucleotide sequence analysis for the desired E→A mutation.

The *Bgl*II DNA fragment encoding the E217A mutant ΔTM form was purified and subcloned into the eukaryotic expression vector pEE14.tPA [11,12]. This inserted the cDNA in the appropriate translational reading frame with the tPA secretion signal sequence. Thus, the tPA signal peptide, which has been used to successfully produce many secreted proteins at relatively high levels, replaced the natural signal peptide of MT1-MMP. Full length cDNA was reformed by replacement of the 2.55 kbp *Xho*I-*Eco*RI fragment purified from the parental MT1-MMP cDNA.

2.3. Cell culture

Human infant foreskin fibroblasts (HIFFs) were prepared and subcultured as described [13]. Cells from passages 4–7 were used for the monolayer experiments, plated onto glass coverslips and cultured for 3 days in DMEM supplemented with 10% FBS at 37°C with 5% CO_2. Media were changed to either DMEM alone, DMEM with 2% partially purified cytokines (largely interleukin 1β [14]), DMEM with 2% partially purified cytokines with oncostatin M (Sigma, 50 ng/ml) or DMEM with phorbol myristate acetate (PMA; Sigma, 10 ng/ml), and the cells cultured for 24 h. Monensin (Sigma, 5 μM) was added for the last 19 h of the culture period. Cells were then fixed for immunolocalisation.

To prepare cell lysates, HIFFs were cultured in 75 cm^2 flasks in DMEM supplemented with 10% FBS. At confluence the cell layers were washed with serum free DMEM and then cultured in this medium for a further 24 h with or without PMA at 10 ng/ml. At harvest, cells were washed twice with ice-cold PBS and lysed using a buffer of 50 mM Tris-HCl, pH 8.0, containing 1% Triton X-100 0.15 M NaCl, 0.02% sodium azide, 10 mM EDTA, 1 μg/ml pepstatin A, 100 μM phenylmethylsulphonyl fluoride and 1 μg/ml E-64 (L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane). A volume of 400 μl of ice-cold lysis buffer was used for each 75 cm^2 flask. Cells were scraped into lysis buffer, centrifuged in a microfuge for 10 min at 4°C and the supernatant was stored at −70°C.

CHO-L761H cells [15] were maintained in DMEM supplemented with 10% FBS and passaged with trypsin-EDTA. Cell lysates were prepared from cells that were grown in 55 cm^2 petri dishes and then transfected with either control vector or MT1-MMP cDNAs as described below. CHO-L761H cells are known to synthesise very low levels of progelatinases A and B that are not detectable by immunolocalisation.

2.4. Preparation of sheep antiserum to MT1-MMP

150 μg purified GST-ΔMT1-MMP (1 ml) was emulsified in 1 ml complete Freund's adjuvant and injected into multiple intramuscular sites in a Clun sheep. Booster injections of 100 μg (0.5 ml prepared similarly) were given on days 42 and 307. Blood was removed on days 7, 10 and 14 following each boost injection. Immunoglobulins (IgG) were prepared by triple ammonium sulphate precipitation from the bleed with the highest titre (bleed 6). The fraction of IgGs corresponding to antibodies directed to GST was separated from total IgGs by affinity chromatography using a GST-Sepharose-glutathione column. To prepare this column, soluble GST was obtained by intracellular expression in *E. coli* transformed with pGEX-2T plasmid followed by sonication of cells, and then coupled to CNBr-activated Sepharose B. After washing the GST-Sepharose column with PBS, IgGs were passed through the column, unbound material was collected and the A_{280} was determined. Affinity purified antibody to MT1-MMP was prepared by adsorption of the IgG to the catalytic domain of MT1-MMP [16] immobilised on nitrocellulose. Specific antibodies were eluted from the nitrocellulose using a pH 2.8 glycine buffer, followed by neutralisation using 2 M Tris-HCl pH 8.0.

2.5. Western blotting

Recombinant MMPs and TIMPs [22,23] (0.2–0.5 μg) or 10 μl cell lysate were separated on an SDS-10% polyacrylamide gel under reducing conditions and transferred by electroblotting to nitrocellulose membranes (Hybond ECL, Amersham). The membranes were probed with affinity purified sheep anti-human ΔMT1-MMP (5 μg/ml). Bound antibody was revealed using a peroxidase conjugated second antibody, followed by incubation with a chemiluminescent substrate (SuperSignal Substrate, Pierce, Rockford, IL). Luminescence was detected by exposure to autoradiographic film (Hyperfilm-ECL, Amersham).

2.6. Preparation of gelatin-TR films

10 mg gelatin (Sigma) was dissolved in 1 ml 0.1 M sodium bicarbonate pH 9.0. Texas red sulphonyl chloride (Molecular Probes, Eugene, OR; 1 mg) was dissolved in 100 μl dry dimethyl formamide, added dropwise to the gelatin solution and stirred (30 min, 4°C). Conjugated gelatin-Texas red (gelatin-TR) was separated from unconjugated dye by passing through a Sephadex G25 column equilibrated with PBS, and stored at 4°C until use.

Eight well Labtek slides (ICN Biomedicals) were coated with poly-L-lysine (Sigma; 10 μg in 200 μl H_20 per well, 1 h, rt) followed by gelatin-TR (10 μl in 200 μl H_2O per well, 2 h, rt). This was removed and the coating fixed with 4% formaldehyde (5 min, rt), washed three times with PBS and unreacted aldehyde groups blocked with ammonium chloride (50 μM in PBS, 10 min, rt). After repeated washing in sterile PBS, 400 μl DMEM with 10% FBS was added to each well and incubated overnight (37°C, 5% CO_2/air) prior to plating cells.

CHO-L761H cells were plated onto Labtek slides at 3.2×10^4 cells per well and cultured in DMEM supplemented with 10% FBS at 37°C, with 5% CO_2 for 24 h. Transfection was performed essentially according to Chen and Okayama [17]. Either pEE14 MT1-MMP, pEE14 E217A MT1-MMP, or pEE14 vector only (2.5 μg DNA/cm^2/well) were co-precipitated with calcium phosphate and incubated with the cells for 4 h at 37°C. Media were removed and the cells exposed to 15% glycerol in DMEM for 1 min. After washing in DMEM, the cells were cultured in DMEM with 10% FBS, 37°C, 5% CO_2 for 3 h. Media were then replaced with DMEM only for a further 16 h prior to immunolocalisation. Either recombinant human TIMP-1 (10 μg/well), recombinant human TIMP-2 (10 μg/well), CT1399 (N^4-hydroxy-N^1-(*S*-morpholinosulphonylaminoethylcarbon-

Table 1
MT1-MMP mediated film degradation occurs in the presence of TIMP-1 but not TIMP-2 and is increased by addition of progelatinase A

Transfection	Addition	MT1-MMP cell surface immunofluorescence	Film degradation ($\mu m^2 \pm$ S.E.)
Vector	–	−	0
MT1	–	+	1651 ± 254
E217A-MT1	–	+	0
MT1	rTIMP-1	+	1066 ± 131
MT1	rTIMP-2	+	0
MT1	CT1399	+	0
MT1	Aprotinin	+	1681 ± 790
MT1	pGLA 0.001 μg	+	3803 ± 527
MT1	pGLA 0.01 μg	+	5004 ± 520
MT1	pGLA 0.1 μg	+	8409 ± 1048

CHO cells were plated onto gelatin-TR films and transfected with either vector (V), MT1-MMP (MT1) or E217A MT1-MMP (E217A-MT1) cDNA as described in Section 2. Either rTIMP-1 (10 μg/well), rTIMP-2 (10 μg/well), CT1399 (10 μM), aprotinin (10 μg/ml) or progelatinase A (pGLA, 0.001–1.0 μg/well) were added to some wells. Cells were stained with anti-MT1-MMP IgG, observed by confocal microscopy and scored for cell surface immunofluorescence and focal degradation of the films.

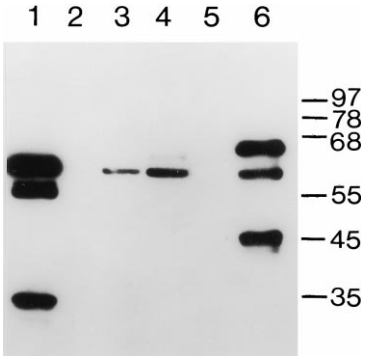

Fig. 1. Western blot of recombinant MMPs and cell lysates using affinity purified antibody to recombinant MT1-MMP. Recombinant MMPs and cell lysates were electrophoresed on an SDS-10% polyacrylamide gel, blotted onto nitrocellulose membrane and probed with affinity purified antibody. Bound antibody was visualised with a peroxidase conjugated second antibody followed by chemiluminescent detection. Lane 1, recombinant MT1-MMP; lane 2, recombinant progelatinase A; lane 3, lysates prepared from untreated HIFFs; lane 4, lysates prepared from HIFFs stimulated with PMA; lane 5, lysate from control vector CHO cells; lane 6, lysate from CHO cells transfected with MT1-MMP. Molecular mass markers are indicated on the right.

yl)-2-cyclohexyl-ethyl)-2-(*R*)-(4 chlorophenyl-propyl) succinamide; Celltech, Slough; 10 μM), aprotinin (Bayer AG; 10 μg/ml) or recombinant progelatinase A (0.001–1.0 μg/well) were added to some wells after the glycerol shock and also included in the serum-free incubations.

2.7. Immunolocalisation

2.7.1. Cell monolayers. Cells were fixed, permeabilised and stained as previously described [18]. They were viewed on a Zeiss photomicroscope III with epifluorescence and standard wide band FITC filters. Photographs were taken on Kodak EPH P1600 film and processed at 1600 ASA.

2.7.2. Gelatin-TR. CHO cells cultured on gelatin-TR films were fixed with 4% formaldehyde in PBS freshly prepared from paraformaldehyde (5 min; rt), and wells washed with PBS. They were then incubated (1 h; rt) with either normal sheep serum IgG (NSS), sheep anti-MT1-MMP IgG (50 μg/ml in PBS) or affinity purified sheep anti-MT1-MMP IgG (38 μg/ml) together with 5% normal pig serum, followed by a pig anti-sheep Fab′-FITC [19] (1:400 in PBS with 5% normal pig serum, 30 min, rt). Slides were mounted in Vectashield (Vector Laboratories Inc, Burlingame, CA) and viewed on a MRC 600 confocal microscope with a krypton/argon laser. Data were collected in two ways: (a) by simultaneously scanning both the 488 nm (FITC, left) and 568 nm (Texas red, right) laser lines with a confocal aperture of 0.5 and Kalman averaging over 10 scans, at slow scan speed; (b) by collecting serial 1 μm sections through cells and films (Z series) and merging images with Bio-Rad Comos software using false colour. Images were taken from the screen onto Agfapan 25 or Kodak E100SW film. To assess the area of film degradation the Biorad Comos software was used to outline on the screen the degradation below at least 10 separate cells in duplicate wells (>20 wells in all) and areas averaged. Each experiment was repeated at least three times.

3. Results and discussion

3.1. Specificity of anti-human ΔMT1-MMP IgG

The specificity and sensitivity of sheep anti-human ΔMT1-MMP IgGs were tested by Western blotting. The affinity purified antibody recognised recombinant MT1-MMP ($\Delta_{502-559}$) [7] detecting pro-enzyme at 60 kDa and processed forms at 55 and 34 kDa (Fig. 1, lane 1) but did not recognise MT2-MMP [20] (data not shown). The antibody recognised wild-type MT1-MMP present in cell lysates from human infant foreskin fibroblasts, detecting a major band at 60 kDa that was upregulated following treatment of the cells with PMA (Fig. 1,

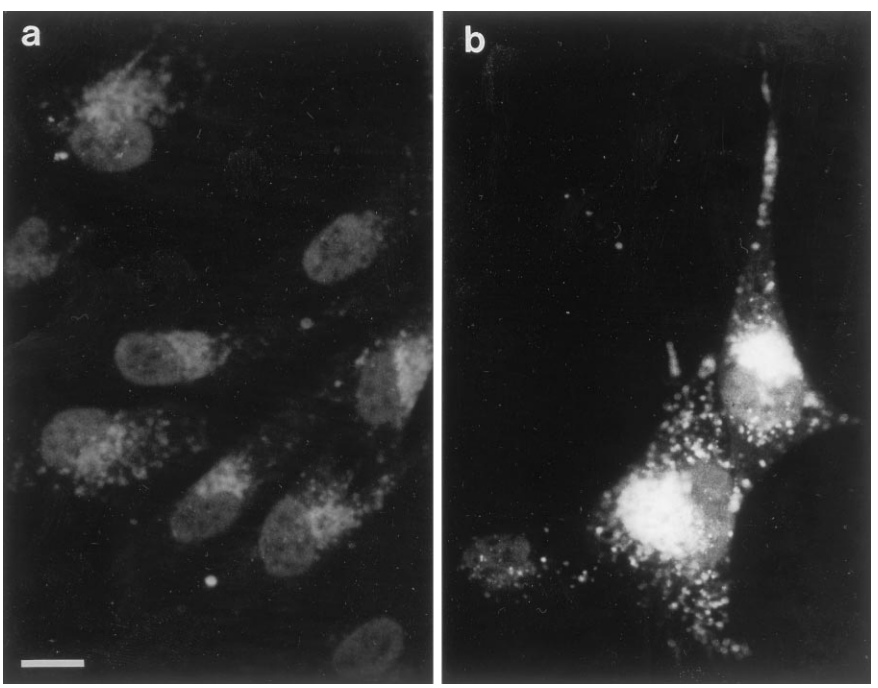

Fig. 2. MT1-MMP synthesis by human fibroblasts is constitutive and upregulated by PMA. Human infant foreskin fibroblasts were grown on glass coverslips, then cultured for 24 h in either (a) DMEM or (b) DMEM with PMA 10 ng/ml. Monensin was added to the culture medium for the last 19 h. The cells were then fixed and stained by indirect immunofluorescence with the affinity purified sheep IgG to MT1-MMP followed by pig anti-sheep Fab-FITC. Bars = 20 μm. a: Cells cultured in DMEM alone have weak perinuclear immunofluorescence indicating constitutive synthesis. b: Cells cultured with DMEM and PMA have enhanced perinuclear staining with many large secretory vesicles demonstrating increased MT1-MMP synthesis.

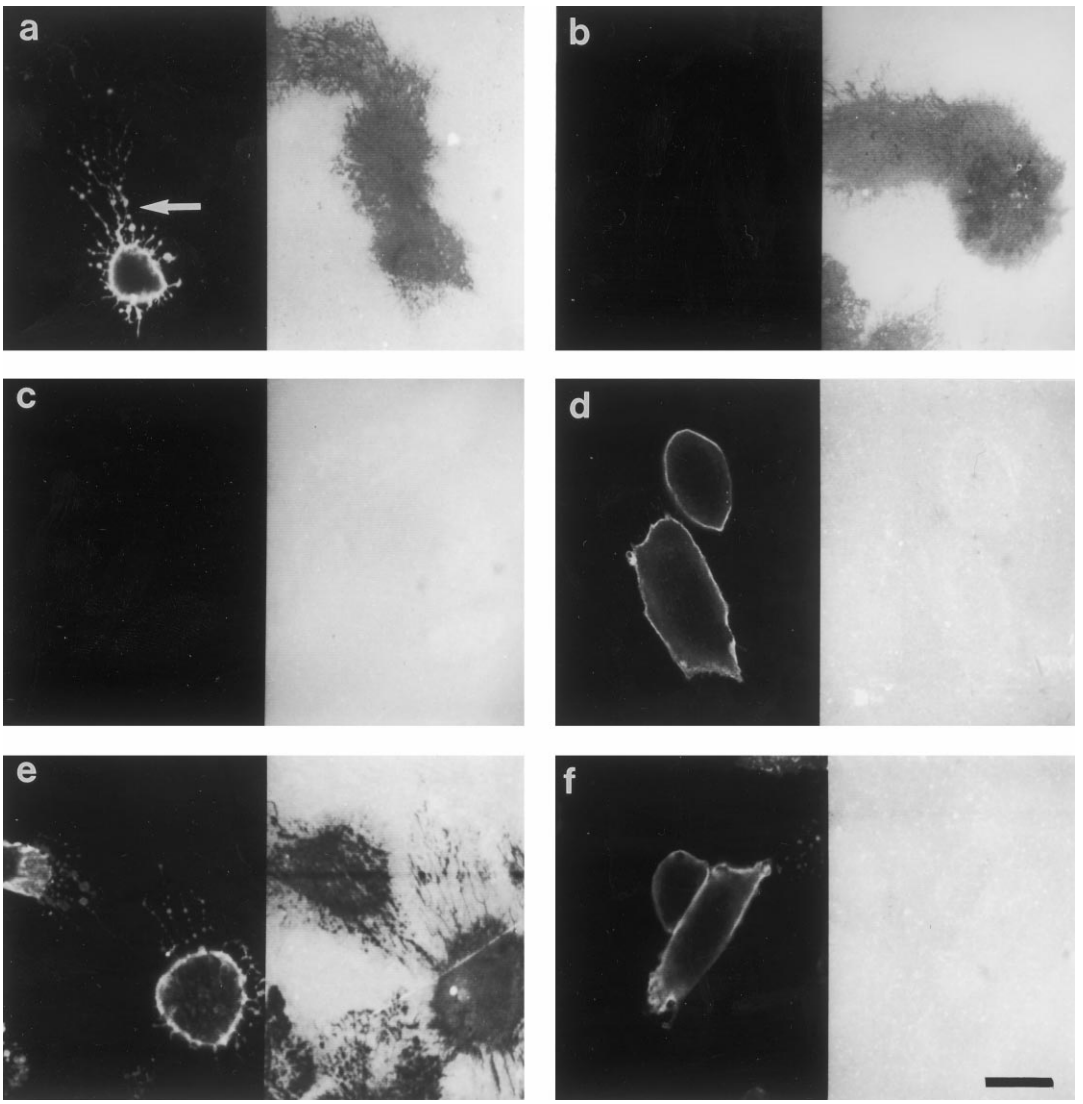

Fig. 3. CHO cells transfected with MT1-MMP express MT1-MMP on the cell surface and degrade gelatin films but cells transfected with E217A MT1-MMP do not degrade the films: film degradation is inhibited by rTIMP-2 but not by rTIMP-1. CHO cells were grown on gelatin-TR films and transiently transfected with equal amounts of either MT1-MMP cDNA (a, b, e, f), control vector cDNA (b) or E217A MT1-MMP cDNA (d) and cultured for 19 h following glycerol shock. Either rTIMP-1 (e) or rTIMP-2 (f) were added to the culture media for 19 h. Films were then fixed and stained by indirect immunofluorescence with sheep anti-MT1-MMP IgG (a, c–f) or normal sheep IgG (b). Simultaneous scans were collected at 488 nm (FITC, left) and 568 nm (Texas red, right). Bar = 25 μm. a: MT1-MMP-transfected cell has MT1-MMP immunofluorescence on cell body and on membrane remaining on coverslip (arrow), and has degraded a pathway across the gelatin film during the culture period. b: MT1-MMP transfected cells stained with NSS show film degradation but no cell immunofluorescence. c: pEE12 vector transfected cells have neither cell immunofluorescence nor film degradation. d: E217A MT1-MMP transfected cells have cell surface immunofluorescence but there is no film degradation. e: MT1 transfected cells cultured with TIMP-1: both cell staining of MT1-MMP and film degradation are evident. f: MT1 transfected cells cultured with TIMP-2: cells have surface staining of MT1-MMP but there is no film degradation.

lanes 3, 4). A minor doublet at 63 kDa was detected in lysates from stimulated cells (Fig. 1, lane 4), but was rarely present in lysates from unstimulated cells. Bands of 60 and 63 kDa have also been reported by Lohi et al. [21] in CCL-137 human fibroblasts, using an antibody raised to the C-terminal intracellular domain of MT1-MMP. Wild-type MT1-MMP was not present in control vector transfected CHO cells (Fig. 1, lane 5); when the pEE14 MT1-MMP construct was overexpressed in these cells, bands of 66, 60 and 45 kDa were detected (Fig. 1, lane 6). We speculate that the 66 kDa form corresponds to the pro-enzyme and that the 45 kDa band is a truncated form of MT1-MMP, resulting from metalloproteinase mediated processing (Stanton and Murphy, unpublished results). This band is of a similar size to that detailed by Lohi et al. [21], who also reported a band of 43 kDa in HT1080 human fibrosarcoma cells stably transfected with MT1-MMP.

When blotted against other MMPs and TIMPs prepared using a mammalian expression system as previously described [22,23], the sheep anti-human ΔMT1-MMP IgGs did not recognise gelatinase A (Fig. 1, lane 2), collagenases 1, 2 and 3, stromelysins 1 and 2, gelatinase B and TIMPs 1, 2 and 3 (data not shown). NSO mouse myeloma cells stably transfected with MT1-MMP cDNA stained brightly with the antibody using immunolocalisation techniques, but NSO cells transfected with TIMP-2 cDNA were negative (data not shown).

3.2. Fibroblast synthesis of MT1-MMP

Atkinson et al. [3] reported that low levels of MT1-MMP

mRNA and protein could be identified in unstimulated HIFFs by Northern and Western blotting respectively. In order to detect MT1-MMP protein by immunofluorescence microscopy in normal (i.e. not transfected) cells, HIFFS were cultured on coverslips and stained with sheep anti-MT1-MMP antibody. Cells showed weak perinuclear immunofluorescence when the secretion inhibiting ionophore monensin was included in the culture medium for 19 h prior to fixation (Fig. 2a) but cells cultured without monensin were negative (data not shown), indicating that MT1-MMP protein synthesis is slow but constitutive in these cells and follows the normal secretory pathway. Cells cultured with monensin but incubated with normal sheep serum (NSS) IgG did not stain (data not shown). However, cells cultured with PMA for 24 h and monensin for the last 19 h showed much increased perinuclear immunofluorescence (Fig. 2b), in agreement with Lohi et al. [21] who demonstrated that human fibroblast expression of MT1-MMP mRNA was constitutive and enhanced 2–3-fold by treatment with PMA. Neither basal nor stimulated cells showed any immunofluorescence of external cell membranes or cell surface associated staining.

3.3. Degradation of gelatin films

Sato et al. [1] showed that cells transfected with MT1-MMP cDNA overexpressed MT1-MMP on the cell surface. MT1-MMP has also been shown to have gelatinolytic activity in vitro [5,7]. The question thus arose whether MT1-MMP could be functionally gelatinolytic when expressed on the cell surface. To address this question, we used a transient transfection model system to overexpress MT1-MMP on the cell surface and cultured these cells on a gelatin substrate. CHO L761H cells were cultured on thin ($\sim$2 μm measured by confocal microscopy) films of gelatin labelled with Texas red, transiently transfected with MT1-MMP cDNA, then stained by indirect immunofluorescence with anti-MT1-MMP antibody to detect cells expressing MT1-MMP on their cell membranes. The results are shown in Fig. 3 and summarised in Table 1. On simultaneously scanning both channels (Fig. 3a) rings of FITC immunofluorescence at the level of the film were seen on 20–30% of cells (Fig. 3a, left) indicating cell surface staining of MT1-MMP on transfected cells and, by collection of 1 μm sections through the cells, staining was seen to cover the entire cell surface (data not shown). In addition, at film level, traces of MT1-MMP immunofluorescence were seen separate from the cells (Fig. 3a, arrow), presumably membrane deposits on the coverslip, detached as a result of cell movement during the culture period. A substantial fraction of the cellular integrins is known to be left behind on the substratum as the cell detaches and locomotes [24], and these data suggest that cell surface bound MT1-MMP may also remain attached to the substratum. The gelatin-TR film had been degraded by these cells, seen as dark feathery patches on an otherwise homogeneous film (Fig. 3a, right). The film degradation caused by MT1-MMP expressing cells was subjacent to the cell and sharply demarcated, similar to the localised proteolysis of opsonised fibronectin films by polymorphonuclear leukocytes in the presence of proteinase inhibitors [25]. Tracks of film clearance were frequently observed, similar to phagokinetic tracks [26], illustrating proteolysis during cell migration. Areas of film degradation were always associated with FITC immunofluorescence of cells and/or membrane deposits. Some cells had no cell surface staining for MT1-MMP and no adjacent film clearance; since degradation of the film was only seen beneath cells with cell membrane staining we concluded that these cells were not transfected and not actively producing cell membrane bound MT1-MMP. MT1-MMP transfected cells stained with NSS showed tracts of film clearance but no staining on the FITC channel (Fig. 3b) while cells transfected with control vector only and stained with anti-MT1-MMP antibody showed neither FITC immunofluorescence nor film degradation (Fig. 3c). These data show that film removal is not due to either cell traction or the transfection process, and the FITC fluorescence is not due to non-specific uptake of sheep IgG by CHO cells. The gelatin film method we developed is similar to those used in other studies [27,28] but, by mild fixation of the gelatin to the glass slide, removal of the film by cell traction is prevented. Scanning on both channels with confocal laser light allows visualisation of small areas of film clearance without interference from flare from surrounding intact film, together with identification of cells expressing enzyme on the cell membrane.

We then transfected CHO cells with an E217A mutant MT1-MMP cDNA that yields inactive MT1-MMP. Fig. 3d shows that cell surface MT1-MMP immunofluorescence was present on these cells but no subjacent film degradation was seen, demonstrating that the film degradation previously observed was due to the catalytic activity of MT1-MMP.

MT1-MMP transfected cells were incubated with recombinant human TIMP-1 (10 μg/well): Fig. 3e shows that cell surface immunofluorescence was present and degradation of the film was observed. However, no film degradation took place when transfected cells were incubated with either recombinant TIMP-2 (10 μg/well; Fig. 3f) or CT1399 (10 μM; data not shown), although FITC immunofluorescence was observed on cell surfaces, confirming the presence of MT1-MMP. CHO cells are known to synthesise very low levels of gelatinases A and B which could have contributed to the film degradation, but TIMP-1 is known to be a good inhibitor of gelatinase A and an extremely poor inhibitor of MT1-MMP [16]. Inclusion of aprotinin in wells containing MT1-MMP transfected cells had no effect on either cell surface staining or film degradation, indicating that plasminogen activators do not play a role in this system. We therefore concluded that degradation of the gelatin-TR film was due to proteolysis by MT1-MMP. Furthermore, for proteolysis to occur, a requirement for MT1-MMP at the cell surface was noted. HIFFs stimulated with PMA and grown on gelatin films expressed MT1-MMP intracellularly, but not at the surface (Fig. 2b) and no film degradation was observed (data not shown). Interestingly, proteolysis of the gelatin film by transfected CHO cells was associated with processing of MT1-MMP to a 45 kDa form as detected by Western blot analysis. In contrast, PMA stimulated HIFFs which did not degrade the films had MT1-MMP bands at 63 and 60 kDa but none of the 45 kDa form was detected in the lysate.

Purified recombinant progelatinase A (0.001–1.0 μg/well) was then added to MT1-MMP transfected cells. MT1-MMP cell surface immunofluorescence was observed in all wells as before, indicating that even at the highest concentration progelatinase did not interfere with antibody-MT1-MMP binding. However, film degradation, again seen as tracts only below cells with FITC staining, increased in area with increasing amounts of progelatinase. At 0.1 μg the margins of the area degraded by a single cell greatly exceeded the cell diameter,

whereas without progelatinase the cell tract or hole approximated the cell diameter (see Fig. 3a,e). This is demonstrated by the area measurements (Table 1): variations in individual cell locomotion and local cell densities contribute to large standard errors. These results suggest that, whereas film degradation by MT1-MMP is immediately subjacent to the cell membrane, degradation by gelatinase may not be. Whether enzyme/cell membrane detached from the cell body during cell locomotion is also able to continue ECM proteolysis has yet to be determined.

These data demonstrate that cells expressing MT1-MMP on their cell membranes exhibit subjacent proteolysis of a gelatin film and that this proteolysis is inhibited by addition of TIMP-2 or a hydroxamate inhibitor, but not by TIMP-1. Thus, expression of MT1-MMP on the cell surface may lead to both progelatinase A activation and extracellular matrix degradation. We are currently investigating the use of other substrates and the gelatin film method developed for this work should be useful for studying the properties of the other MT-MMPs.

Acknowledgements: We thank Mr Chris Green for preparing the photographs and Ms Jennifer Davies for DNA preparations. M.-P. d'Ortho was the recipient of a fellowship from the Institut National de la Santé et de la Recherche Médicale (INSERM, France), and this work was supported by the Medical Research Council (UK), the Arthritis and Rheumatism Council and the Wellcome Trust.

References

[1] Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. (1994) Nature 370, 61–65.
[2] Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A. and Goldberg, G.I. (1995) J. Biol. Chem. 270, 5331–5338.
[3] Atkinson, S.J., Crabbe, T., Cowell, S., Ward, R.V., Butler, M.J., Sato, H., Seiki, M., Reynolds, J.J. and Murphy, G. (1995) J. Biol. Chem. 270, 30479–30485.
[4] Imai, K., Ohuchi, E., Aoki, T., Nomura, H., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1996) Cancer Res. 56, 2707–2710.
[5] Pei, D.Q. and Weiss, S.J. (1996) J. Biol. Chem. 271, 9135–9140.
[6] Ohuchi, E., Imai, K., Fuji, Y., Sato, H., Seiki, M. and Okada, Y. (1997) J. Biol. Chem. 272, 2446–2451.
[7] d'Ortho, M.-P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith B., Timpl, R., Zardi, L. and Murphy, G. (1998) Eur. J. Biochem. (in press).
[8] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[9] Janssen, G.R. and Bibb, M.J. (1993) Gene 124, 133–134.
[10] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[11] Bebbington, C.R. and Hentschel, C.G. (1987) in: DNA Cloning (Glover, D.M. and Hames, B.D., Eds.), pp. 163–188, IRL Press, Oxford.
[12] Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.T., Heyneker, H.L., Goeddel, D.V. and Collen, D. (1983) Nature 301, 214–221.
[13] Heath, J.K., Gowen, M., Meikle, M.C. and Reynolds, J.J. (1982) J. Period. Res. 17, 183–190.
[14] Saklatvala, J., Pilsworth, L.M.C., Sarsfield, S.J., Gavrilovic, J. and Heath, J.K. (1984) Biochem. J. 224, 461–466.
[15] Cockett, M.I., Bebbington, C.R. and Yarranton, G.T. (1991) Nucleic Acids Res. 19, 319–325.
[16] Will, H., Atkinson, S.J., Butler, G.S., Smith, B. and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123.
[17] Chen, D. and Okayama, K. (1987) Mol. Cell Biol. 7, 2745–2752.
[18] Reynolds, J.J. and Hembry, R.M. (1992) Matrix Suppl. 1, 375–382.
[19] Hembry, R.M., Murphy, G. and Reynolds, J.J. (1985) J. Cell Sci. 73, 105–119.
[20] Butler, G.S., Will, H., Atkinson, S.J. and Murphy, G. (1997) Eur. J. Biochem. 244, 653–657.
[21] Lohi, J., Lehti, K., Westermarck, J., Kähäri, V.M. and Keski-Oja, J. (1996) Eur. J. Biochem. 239, 239–247.
[22] Hipps, D.S., Hembry, R.M., Docherty, A.J.P., Reynolds, J.J. and Murphy, G. (1991) Biol. Chem. Hoppe-Seyler 372, 287–296.
[23] Ward, R.V., Hembry, R.M., Reynolds, J.J. and Murphy, G. (1991) Biochem. J. 278, 179–187.
[24] Palecek, S.P., Schmidt, C.E., Lauffenburger, D.A. and Horwitz, A.F. (1996) J. Cell Sci. 109, 941–952.
[25] Owen, C.A. and Campbell, E. (1995) J. Cell Biol. 6, 367–376.
[26] Albrecht-Buehler, G. (1977) Cell 11, 395–404.
[27] Pilcher, B.K., Dumin, J.A., Sudbeck, B.D., Krane, S.M., Welgus, H.G. and Parks, W.C. (1997) J. Cell Biol. 137, 1445–1457.
[28] Nakahara, H., Howard, L., Thompson, E.W., Sato, H., Seiki, M., Yeh, Y.Y. and Chen, W.T. (1997) Proc. Natl. Acad. Sci. USA 94, 7959–7964.