Cellular cholesterol regulates MT1 MMP dependent activation of MMP 2 via MEK-1 in HT1080 fibrosarcoma cells

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Abstract Unstimulated human fibrosarcoma cells (HT1080) constitutively secrete matrix metalloproteinase 2 (MMP 2) as a proenzyme requiring proteolytic cleavage by membrane type-1 MMP (MT1 MMP) for activation. Physiological and pharmacological stimuli induce clustering of MT1 MMP/tissue inhibitor of MMP 2 "receptors", promoting binding and activation of MMP 2. We now report that cholesterol depleted HT1080 cells accumulated MT1 MMP on the cell surface and activated MMP 2. A specific inhibitor of mitogen activated protein kinase kinase 1/2 inhibited both MMP 2 activation and extracellular signal-related kinase phosphorylation induced by cholesterol depletion. Our data indicate that the cholesterol content of unstimulated cells is critical for secretion of MMP 2 as an inactive zymogen and control of pericellular proteolysis.

Keywords: Cholesterol depletion; MT1 MMP; MMP 2 activation; ERK phosphorylation

1. Introduction

Membrane type-1 matrix metalloproteinase (MT1 MMP), the first of the cell surface-associated subset of the MMP family, undergoes constitutive endocytosis and recycling to the plasma membrane [1]. Invading tumour cells localise MT1 MMP in specialised structures termed invadopodia [2], where rapid renewal is required as it is subject to constant autocatalytic degradation [3,4]. The trimolecular activation complex between MT1 MMP, tissue inhibitor of metalloproteinases 2 (TIMP 2) and pro-MMP 2, is well-documented [5–7]. In addition, MT1 MMP efficiently degrades extracellular matrix (ECM) components, processes bioactive cell surface molecules and regulates migration, invasion and proliferation of cancer cells [8–10]. These observations have significant implications for tumour cell biology, as increased levels of MT1 MMP and active MMP 2 correlate well with a poor prognosis for cancer patients [11]. Understanding the trafficking pathways involved in the secretion of MT1 MMP to the plasma membrane and its fate thereafter will be important in the development of potential cancer therapies.

Both clathrin coated pit and caveolae mediated pathways have been implicated in the endocytosis and recycling of MT1 MMP. The enzyme, together with MMP 2, co-localises with caveolin-1 [12,13] and is present in isolated low density, caveolin-1 positive membrane fractions [1,12–14]. Cellular cholesterol is a fundamental requirement for the formation of the characteristic flask shaped plasma membrane invaginations, known as caveolae, whose major structural proteins are the caveolins. Cholesterol depleting drugs, e.g., methyl-β-cyclodextrin (MβCD), cause loss of caveolae structure so that they become flattened and more accessible to the ECM. Under these conditions they can no longer function as intracellular trafficking vesicles and participate in endocytosis. Caveolae are a morphologically distinct subset of a much larger group of cholesterol enriched membrane lipid domains, collectively known as lipid rafts (reviewed by Lai [15]). All of these domains are participants in signal transduction and membrane and protein sorting, thus multiple additional effects of cellular cholesterol depletion are documented including activation and delocalisation of signalling molecules and growth factor receptors [16–18] and alterations to the actin cytoskeleton.

Inhibition of endocytosis with vacuolar H + ATP-ase inhibitors, folimycin or bafilomycin A, allows an accumulation of MT1 MMP at the cell surface and concomitant activation of MMP 2 [19,20]. We now demonstrate that perturbing endocytosis by means of cholesterol depletion results in MMP 2 activation. We show that this is MT1 MMP dependent and examine the changes in the cellular localisation and status of MT1 MMP protein. We also investigate intracellular signal transduction pathways involved in this induced activation of MMP 2 and provide evidence for a requirement for extracellular signal-related kinase (ERK) activation.

2. Materials and methods

2.1. Antibodies

Anti-human MT1 MMP sheep polyclonal antibody was as described [21]. Secondary antibodies conjugated to horseradish peroxidase and anti-biotin monoclonal antibody were all from Jackson Immunoresearch, Stratech Scientific Ltd, UK. Monoclonal antibodies to phospho-ERK and ERK 2 were from Santa Cruz, Insight biotechnology, UK.

2.2. Cell culture

Human fibrosarcoma cell line, HT1080 cells were seeded at 10^5 cells/well in DMEM with 10% FCS and incubated at 37 °C, 5% CO_2 for 24

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Abbreviations: MMP, matrix metalloproteinase; MT1 MMP, membrane type-1 MMP; MβCD, methyl-β-cyclodextrin; TIMP, tissue inhibitor of MMP; ERK, extracellular signal-related kinase; MEK, mitogen activated protein kinase kinase

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h. Media were replaced by DMEM with 0.1% FCS and the cells incubated at 37 °C overnight before cholesterol depletion experiments.

2.3. Cholesterol depletion

Washed cells were incubated at 37 °C, 5% CO_2 in DMEM, 50 mM HEPES, pH 7.2, and 0.1% BSA, with varying concentrations of MβCD (Sigma, UK) for 30 min, then washed and incubated for 1 or 2 h in DMEM. Cell supernatants were concentrated (1 h samples) before analysis by gelatin zymography. Cell lysates were prepared as described [3] and Western blotted for MT1 MMP.

2.4. Gelatin zymography

Samples were electrophoresed on SDS–7% PAGE containing 1 mg/ml gelatin, washed in Triton X-100, incubated overnight at 37 °C and stained with Coomassie brilliant blue.

2.5. Cholesterol repletion

For cholesterol repletion experiments, cholesterol/(MβCD) complexes were prepared as described [22]. For use the complexes were diluted to 0.2 mM cholesterol using DMEM/HEPES pH 7.2, and 0.1% BSA. After an initial incubation with MβCD as before, the cells were incubated for 10, 20, 30 or 40 min in DMEM/HEPES, pH 7.2, 0.1% BSA with or without cholesterol/MβCD complexes. After each time point, the cells were washed and incubated for a further 2 h in DMEM. Cell supernatants were harvested for gelatin zymography as before.

2.6. TIMP inhibition

TIMP 1 [23] or TIMP 2 [24] (100 nM as determined by active-site titration [24]) was added during the final 1-h incubation as described above.

2.7. Inhibition of signalling pathways

SB20358 (25 μM), LY294002 (25 μM), UO126 (10 μM), all from Calbiochem, Merck Biosciences, UK, PD 98059 (20 μM, Qbiogene-Alexis Ltd, UK) or Staurosporine (100 nM, Sigma, UK) were added to cultures during 30 min pre-incubation, 30 min incubation with or without MβCD and during 2 h incubation in DMEM.

2.8. Biotinylation of cell surface proteins

HT1080 cells were seeded into 6-well plates (1/20 80% confluent T75 flask/well) and incubated overnight at 37 °C in DMEM with 10% FCS. Cells were serum depleted for 18 h and then washed and incubated for 30 min with or without MβCD. Biotinylation of cell surface proteins was performed, either immediately afterwards or after a further 60 min incubation in DMEM, essentially as described [1] using 0.5 mg/ml EZ-link non-permeable sulfo-NHS-LC-biotin (Perbio Science, UK) for 20 min at room temperature.

2.9. Immunoprecipitation

Lysates were pre-cleared with protein G–Sepharose overnight at 4 °C and biotinylated proteins were immunoprecipitated with anti-biotin as described [1]. Bound antibody/antigen complexes were washed and eluted from the beads with reducing Laemmli sample buffer.

2.10. Western blot analysis

Immunoprecipitates were separated on SDS–10% PAGE and proteins transferred overnight onto nitrocellulose membranes and probed for biotinylated and unbound MT1 MMP with the polyclonal antibody (1 μg/ml). Lysates were Western blotted as above and probed for MT1 MMP or for phosphorylated ERK or total ERK 2.

3. Results and discussion

Despite intense investigation into the role of MT1 MMP as an activator of MMP 2 and latterly as a regulator of the pericellular environment and tumour cell proliferation, little is understood about the mechanisms underlying its transport to the plasma membrane and sites of function. In this report we describe for the first time how, in unstimulated HT1080 cells, its MMP 2 activating capacity is controlled by the cholesterol content of the cell.

3.1. MβCD dose dependently induces activation of MMP 2

Depletion of cholesterol with MβCD for 30 min induced dose dependent MMP 2 activation. Pro, intermediate and active MMP 2 were detected in media from treated cells compared to only the pro form in untreated cells (Fig. 1, upper panel). Cholesterol depletion was more effective than phorbol ester stimulation or folimycin at this short time point and an accumulation of the intermediate form, generated by MT1 MMP cleavage [6] was apparent with 10 mg/ml MβCD, suggesting increased availability of MT1 MMP. Increased inactive (43–45 kDa) MT1 MMP was detected in lysates from cells treated with 2.5 or 5 mg/ml MβCD, compared to untreated cells (Fig. 1, lower panel). These products of MT1 MMP autocatalytic degradation are often closely correlated with levels of MMP 2 activation [3,4,25]. Higher concentrations of MβCD (10 mg/ml) caused some loss of cells and we therefore chose a concentration of 5 mg/ml for subsequent experiments. Incubation with MβCD, PMA or folimycin for 30 min at the concentrations shown did not affect cell viability as assessed by trypan blue exclusion (data not shown). In addition, two other drugs, filipin and nystatin, induced similar MMP 2 activation (data not shown), indicating that the effect was due to the depletion of cholesterol and not specific to MβCD.

3.2. MβCD induced MMP 2 activation is MT1 MMP mediated

In order to confirm that MβCD induced MMP 2 activation was MT1 MMP mediated, we investigated its inhibition by TIMPs 1 and 2. TIMP 1 inhibited only the final autocatalytic step in accordance with its negligible inhibitory capacity versus MT1 MMP [26]. In contrast, TIMP 2 completely inhibited any processing of MMP 2 (Fig. 2, upper panel). Additionally TIMP 2, but not TIMP 1, abolished proteolytic cleavage of active MT1 MMP resulting in increased 60 kDa MT1 MMP (Fig. 2, lower panel) and confirming that the 43–45 kDa forms are products of MT1 MMP autocatalysis. Thus, the TIMP inhibition profile identified MMP 2 activation induced by cholesterol depletion as MT1 MMP dependent.

3.3. Cholesterol replenishment reverses MMP 2 activation

We next investigated whether replenishing cholesterol in cholesterol-depleted cells could reverse MMP 2 activation.

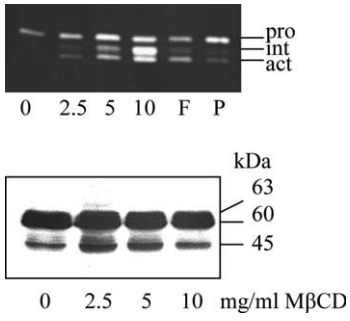

Fig. 1. MβCD induces MMP 2 activation in HT1080 cells. Serum starved HT1080 cells were incubated for 30 min at 37 °C with or without MβCD (0, 2.5, 5, 10 mg/ml). Cells were incubated further for 1 h in DMEM only and concentrated cell supernatants were analysed by gelatin zymography for MMP 2 activation. For comparison cells were incubated with 0.5 μg/ml folimycin (F) or 20 ng/ml PMA (P) (left panel). MβCD (0, 2.5, 5, 10 mg/ml) treated cell lysates from a separate experiment were analysed by Western blot for MT1 MMP (right panel).

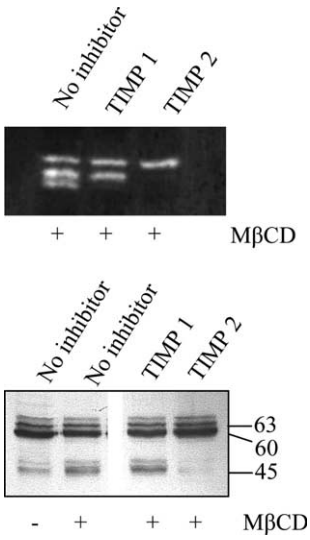

Fig. 2. TIMP 2 inhibits MMP 2 activation induced by MβCD. HT1080 cells were incubated for 30 min at 37 °C with 5 mg/ml MβCD. DMEM with or without TIMPs (100 nM) was added and the cells incubated further for 1 h after removal of MβCD. Concentrated cell supernatants were analysed by gelatin zymography (left panel). Cell lysates were analysed by Western blotting for MT1 MMP (right panel).

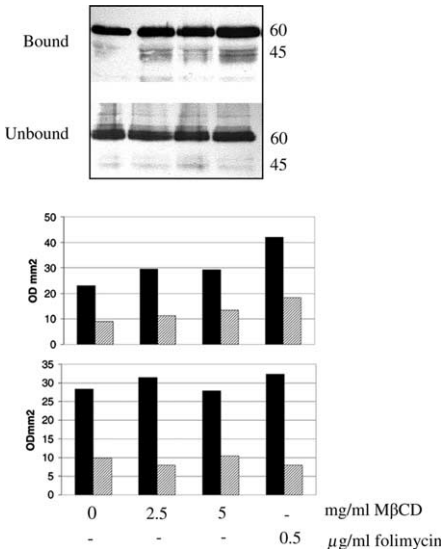

Fig. 4. Cholesterol depletion increases the amount of MT1 MMP on the cell surface. HT1080 cells were incubated for 30 min at 37 °C with or without MβCD or with folimycin, then further incubated for 60 min in DMEM. Surface proteins were biotinylated as described and immunoprecipitated with an anti-biotin antibody. The immunoprecipitates and unbound fractions were analysed by Western blotting for MT1 MMP. Upper panel, biotinylated MT1 MMP (cell surface) in the bound fraction; lower panel, non-biotinylated MT1 MMP (intracellular) in the unbound fraction. Bar charts represent densitometric analysis of the blots; solid bars, 60 kDa bands, hatched bars, 43–45 kDa bands.

Cells were incubated for various times after cholesterol depletion with cholesterol/MβCD complexes that have the ability to restore the cholesterol content of the plasma membrane [16,27]. At least 30 min incubation with the complexes was required to reverse MMP 2 activation (Fig. 3). Neither MβCD nor cholesterol/MβCD complexes had any effect on MMP 9, which is constitutively secreted in the pro-form (92 kDa) by these cells (Fig. 3).

3.4. MβCD increases the amount of MT1 MMP on the cell surface

A major effect of cholesterol depletion in cells is disruption of the structural integrity of caveolae resulting in inhibition of endocytosis. We examined cell surface expression of MT1 MMP after incubation with MβCD or folimycin by biotinylation of cell surface proteins. Both treatments resulted in increased, biotinylated 60 and 43–45 kDa MT1 MMP at the cell surface, compared to untreated cells (Fig. 4, top panel). These differences in levels of cell surface MT1 MMP could not be detected immediately after MβCD treatment but only after the total 90 min experimental period. In agreement with previous

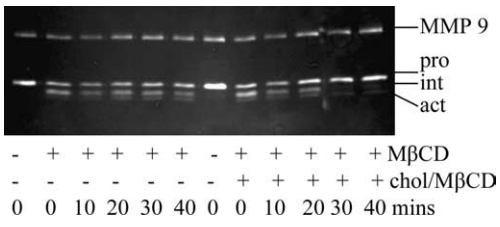

Fig. 3. Cholesterol repletion reverses MMP 2 activation induced by MβCD. HT1080 cells were incubated for 30 min at 37 °C with or without 5 mg/ml MβCD followed by incubation with cholesterol/MβCD complexes or medium only as described for the times shown. Cells were washed after each time point and further incubated for 2 h in DMEM. Cell supernatants were analysed by gelatin zymography.

reports using folimycin [19,20], we conclude that this was due to inhibition of the constitutive turnover of MT1 MMP by endocytosis resulting in an accumulation of active MT1 MMP. Cholesterol depletion is used to distinguish between caveolae-dependent and other endocytic pathways [28], but MβCD can inhibit both caveolae and clathrin-dependent endocytosis [29]. Recently, we reported that MT1 MMP is endocytosed via each of these pathways [1] but it is unclear whether both the active form and its degraded forms are internalised through the same, both or individual pathways. Separation techniques for caveolae enriched fractions, in which MT1 MMP is detected, isolate low-density fractions that include caveolin negative lipid rafts [30]. Alternative protocols are required to confirm that the majority of MT1 MMP is associated with caveolae and it is likely that the association varies between cell types and between different forms of MT1 MMP.

Inhibition of endocytosis is however unlikely to account for the total effects of cholesterol depletion on MMP 2 activation. EGF receptors associate with mainly non-caveolar lipid rafts such that EGF binding is inhibited [31]. It may be that a proportion of MT1 MMP is associated with lipid rafts in a manner that precludes its activation or alternatively that does not allow TIMP 2 binding and thus prevents formation of the MMP 2-activation complex. Recent work, published whilst this manuscript was in preparation [32], suggests that lipid raft association has no effect on MT1 MMP mediated activation of MMP 2. Cytoplasmic domain deleted MT1 MMP but not wild type MT1 MMP, expressed in MCF 7 breast carcinoma cells, was present in caveolin enriched membrane domains. Despite this both enzymes activated MMP 2. Others agree with our own observations, i.e., that endogenous MT1 MMP in

HT1080 cells is associated with caveolin enriched membrane fractions prepared from non-detergent cell extracts [1,14]. Our data here demonstrate induced MMP 2 activation when cholesterol is depleted, a process known to disrupt caveolae. It is possible, therefore, that in HT1080 cells, MT1 MMP or the other components of the trimolecular activation complex may have an association with caveolin-1 resulting in negative regulation as has been described for EMMPRIN/CD147 [33]. Caveolae endocytosis is reported to be the major trafficking pathway for MT1 MMP in human endothelial cells, however, in these cells, no association between caveolin 1 and MT1 MMP was demonstrated [13].

3.5. MMP 2 activation induced by cholesterol depletion requires the ERK signalling pathway

Depletion of cholesterol is reported to modulate signal transduction pathways in a variety of cell types [16–18,34–36]. We assessed the effects of inhibitors of a number of signal transduction pathways on MMP 2 activation induced by MβCD. Activation was consistently inhibited by U0126, an inhibitor of mitogen activated protein kinase kinase (MEK1/2) activation (Fig. 5A, lanes 7 and 8), but not by an inhibitor of stress activated protein kinase 2a (SAPK 2a/p38 MAPK) (SB203580) (Fig. 5A, lanes 11 and 12). Surprisingly, cells exposed to SB203580 alone responded with an induction of MMP 2 activation (Fig. 5A, lanes 9 and 10), indicative of an alternative processing mechanism, however SB203580 did not significantly increase activation induced by MβCD. Another inhibitor of MEK activation (PD 98059) also inhibited MMP 2 activation induced by MβCD but was less effective than UO126 (data not shown). Both inhibitors of MEK activation led to a reduction of 43–45 kDa MT1 MMP (Fig. 5A, lower panel) but in the presence of SB203580, the amount of 43–45 kDa MT1 MMP was comparable to that seen with cyclodextrin alone (Fig. 5A, lower panel).

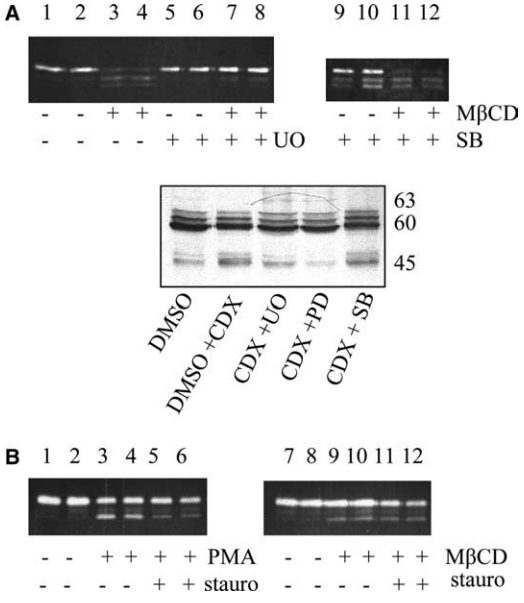

Fig. 5. MMP 2 activation induced by cholesterol depletion requires the ERK signalling pathway. (A) Serum depleted HT1080 cells were pre-incubated for 30 min at 37 °C with vehicle control, DMSO, or inhibitors as shown. Media were removed and replaced with or without 5 mg/ml Mβ CD and inhibitors for a further 30 min at 37 °C. Cells were incubated for a further 2 h at 37 °C in DMEM alone or with inhibitors. Cell supernatants were analysed by gelatin zymography (upper panel) and cell lysates by Western blotting for MT1 MMP (lower panel). (B) Serum depleted HT1080 cells were pre-incubated for 30 min at 37 °C with or without 100 nM staurosporine as shown. Media were removed and replaced with 20 ng/ml PMA or 5 mg/ml MβCD plus or minus staurosporine as shown, then incubated at 37 °C for a further 30 min. Finally, cells were washed and incubated with DMEM with or without inhibitor for 2 h at 37 °C. Cell supernatants were analysed by gelatin zymography for MMP 2 activation.

3.6. Cholesterol depletion in HT1080 cells induces ERK activation

ERK1/2 is the downstream target for active MEK1/2 and so it was of interest to determine whether cholesterol depletion has an effect on the activity of ERK in HT1080 cells. MβCD induced ERK activation (Fig. 6, lane 5), which was totally abolished by the presence of U0126 and severely reduced by PD98059 (Fig. 6, lanes 6 and 7). Interestingly, SB203580 induced ERK activation even in cells incubated without cyclodextrin (Fig. 6, lane 4), thus MMP 2 activation induced by SB203580 alone (Fig. 5A, lanes 9 and 10) is coincident with ERK phosphorylation, but we have yet to demonstrate a causal link between the two.

Caveolin binding motifs are found within the catalytically active enzyme domain of many signalling molecules, suggesting that caveolin 1 is a general inhibitor of kinases. In support of this, activation of the ERK signalling cascade in response to cholesterol depletion and caveolin downregulation is widely reported [37]. We found that the ERK pathway was activated in HT1080 cells in response to MβCD treatment, coincident with MMP 2 activation, and that a specific inhibitor of MEK-1 inhibited the activation of both. Activation of the ERK signalling cascade has previously been observed with respect to MMP 2 activation in addition to MT1 MMP expression and cell migration and invasion [38–42]. A new study [43] supports these findings, since MMP 2 activation induced by type 1 collagen was inhibited with PD 98059 and induced by the overexpression of a constitutively active form of MEK-1. The formation of homophilic MT1 MMP complexes at the cell surface is required for MMP 2 activation [44,45], thus oligomerisation of MT1 MMP in response to cholesterol depletion may be sufficient to initiate signal transduction leading to activation of MMP 2. Alternatively, increased TIMP 2 binding to MT1 MMP might initiate an ERK signalling cascade culminating in MMP 2 activation. More work is needed to substantiate these hypotheses.

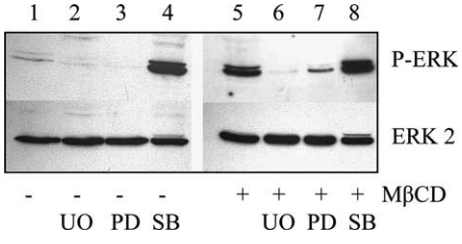

Fig. 6. Cholesterol depletion induces ERK activation. HT1080 cells were pre-incubated for 30 min at 37 °C with signalling inhibitors as shown, then incubated with or without 5 mg/ml MβCD for 30 min at 37 °C in the presence of the inhibitors. Cell lysates were prepared immediately after cholesterol depletion and analysed by Western blotting for phosphorylated ERK. The blots were then reprobed for total ERK 2.

Components of a number of other signal transduction pathways are associated with lipid rafts including protein kinase C (PKC) isoforms and phosphatidylinositol 3-kinase (PI 3K). Cholesterol depletion can also modulate the activity of these pathways with resulting effects on gene transcription [36]. We therefore investigated whether they have a role in MMP 2 activation induced by cholesterol depletion. PKC mediated signalling pathways appear not to be involved in MMP 2 activation induced by cholesterol depletion in HT1080 cells (Fig. 5B, right panel). However, staurosporine partially inhibited PMA stimulated MMP 2 activation (Fig. 5B, left panel), thus phorbol ester induced MMP 2 activation differs from that induced by cholesterol depletion. A difference in dependence on PKC activation was also found with respect to the shedding of the interleukin 6 receptor (IL6R) induced by cholesterol depletion, compared to that induced by PMA [27]. Likewise, inhibitors of PI 3K, Wortmannin and LY294002, had no effect on MMP 2 activation induced by MβCD (data not shown).

Cholesterol depletion affects cell morphology with alterations to the actin cytoskeleton that have far-reaching effects on many intracellular processes, including endocytosis. Disruption of the actin cytoskeleton by both physiological and pharmacological means is sufficient to induce MMP 2 activation [46]. We observed that MβCD induced HT1080 cell shape change and that UO126 appeared to partially abrogate this response (data not shown), indicating that ERK signalling to cytoskeletal proteins was inhibited. Folimycin, on the other hand, had no effect on cell morphology but reproducibly and efficiently induced activation of MMP 2. Undoubtedly, the effects of cholesterol depletion on the actin cytoskeleton contributed to MMP 2 activation in our experiments. Concanavalin A and Cytochalasin D also induce MMP 2 activation in cell culture accompanied by cell shape change. The former additionally crosslinks molecules on the cell surface and inhibits internalisation of MT1 MMP but our attempts to determine whether the Cytochalasin D effect is mediated by inhibition of MT1 MMP endocytosis have been inconsistent (J. English, S. Atkinson, unpublished observations). It is likely that the morphological changes in response to cholesterol depletion have profound effects on signal transduction, growth factor receptor activation and membrane dynamics in addition to the inhibition of MT1 MMP internalisation and recycling.

Overall, our data imply that in unstimulated human fibrosarcoma cells the cholesterol content of cell membranes is critical for the retention of MMP 2 in an inactive form. The data reported here suggest that this is unlikely to be a direct effect of cholesterol per se but on a requirement for cholesterol by the cell for the maintenance of endocytic pathways and a dynamic actin cytoskeleton in addition to regulation of signal transduction pathways. MMP 2 activation induced by cholesterol depletion is thus largely due to disruption of continuous constitutive recycling of MT1 MMP resulting in the protection and accumulation of fully active MT1 MMP on the cell surface. Our biotinylation experiments indicate that the accumulation of MT1 MMP at the cell surface is not an immediate effect of cholesterol depletion, suggesting that the induced MMP 2 activation is not simply the result of redistribution of functional MT1 MMP from lipid raft localisation. Further work is required to establish whether cholesterol depletion releases MT1 MMP from negative regulation associated with lipid raft localisation without an immediate increase in surface MT1 MMP.

Secondary signal transduction effects via ERK are clearly involved in this cholesterol depletion response, although the precise mechanisms, which could include activation of numerous growth factor receptors in addition to cytoskeletal reorganisation, remain unresolved. However, the short-term nature of these experiments is likely to rule out an effect on MT1 MMP transcription. Future studies will be directed towards understanding the association of MT1 MMP and/or MMP 2 and TIMP 2 with lipid enriched membrane microdomains that this work has identified as an additional layer of regulation of pericellular proteolysis.

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