

The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form

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SUMMARY

We have assessed the effect of fibronectin and laminin-1 on the expression of molecules involved in the activation pathway of MMP-2, a key proteinase in tissue remodelling. HT1080 fibrosarcoma cells cultured on fibronectin were shown to activate endogenous MMP-2, to a level comparable with that elicited by treatment with phorbol ester. In contrast, the MMP-2 expressed by HT1080 cells cultured on laminin-1 was mainly in the pro- (inactive form). Culture of the cells on peptide fragments of fibronectin derived from the central cell binding domain also promoted MMP-2 activation, indicating that signals via fibronectin binding to integrin receptors may be involved. HT1080 cells cultured on immobilised antibodies to the $\alpha 5$ and $\beta 1$ integrin subunits secreted levels of active MMP-2 similar to those observed for full length fibronectin, whereas cells cultured on an antibody to the $\alpha 6$ integrin subunit secreted mainly proMMP-2. The data demonstrate that the activation of MMP-2 by HT1080 cells is regulated by the nature of the extracellular matrix, and that signals via the $\alpha 5 \beta 1$ integrin receptor may be involved in the fibronectin induced up-regulation of MMP-2 activation.

We then assessed the effect of fibronectin on the

components of the putative MT1-MMP/TIMP-2 'receptor' complex implicated in MMP-2 activation. Levels of TIMP-2 protein expressed by HT1080 cells did not vary detectably between cells cultured on fibronectin or laminin-1. However, the expression of MT1-MMP protein was up-regulated when the cells were cultured on fibronectin, which could be attributed to an increase in levels of a truncated 45 kDa form. Parallel studies using gelatin zymography demonstrated that the up-regulation of the production of the 45 kDa band was concomitant with MMP-2 activation. Inhibitor studies revealed that the truncation of MT1-MMP to a 45 kDa form is MMP mediated, although not inhibited by TIMP-1. In vitro, the 45 kDa form could be generated by cleavage of membrane-bound native MT1-MMP with several recombinant MMPs, including both active MT1-MMP and MMP-2. The implication that either MMP-2 or MT1-MMP can process MT1-MMP to 45 kDa, raises the possibility that truncation of MT1-MMP represents a self-regulatory end-point in the activation pathway of MMP-2.

Key words: Fibronectin, MMP-2 activation, MT1-MMP, $\alpha 5 \beta 1$ integrin

INTRODUCTION

Cell-matrix interactions affect a diverse range of cellular functions including cell differentiation, migration, proliferation and survival. Information provided by the extracellular matrix (ECM) can control processes of embryonic growth and differentiation, tissue remodelling and repair. It follows that localised proteolytic degradation of the ECM, and the release of matrix fragments and matrix bound growth factors and cytokines will have profound effect on signals reaching the cell from the ECM (for review see Werb, 1997).

The matrix metalloproteinases (MMPs) have been implicated in normal matrix remodelling events such as mammary gland involution (Werb et al., 1996), and in pathological conditions, including tumour invasion and metastasis (Chambers and Matrisian, 1997). MMP-2 (gelatinase A) is associated with tumour tissues, and the appearance of active MMP-2 is closely correlated with tumour metastasis (Sato and Seiki, 1996). In common with all the MMPs, MMP-2 is synthesised as a latent proenzyme, requiring proteolytic removal of the propeptide for activation. The physiological mechanism by which proMMP-2 is activated is

under intense study, and recent evidence suggests that this event is triggered by an interaction between proMMP-2 and cell surface bound MT-MMPs (Sato et al., 1994; Strongin et al., 1995; Butler et al., 1998). From this new subclass of MMPs, MT1-MMP is the best characterised, and is overexpressed in several tumour tissues in which activated MMP-2 is found, including lung and colon (reviewed by Sato and Seiki, 1996). Our laboratory has demonstrated that MT1-MMP performs the first proteolytic 'clip' in the removal of the MMP-2 propeptide, leading to MMP-2 autolysis and the production of fully active enzyme (Atkinson et al., 1995; Will et al., 1996). As MT1-MMP is an ECM degrading enzyme in its own right (Pei and Weiss, 1996; d'Ortho et al., 1997; Ohuchi et al., 1997) active MT1-MMP and active MMP-2 at the cell surface provide a powerful combination for the localised remodelling of the ECM (d'Ortho et al., 1998).

Although MT1-MMP mediated activation of proMMP-2 can be stimulated *in vitro* by concanavalin A or by phorbol esters, little is known of the factors that influence this process *in vivo*. ECM macromolecules influence cellular expression of MMPs, a process which can be mediated via integrin receptors (Werb et al., 1989; Larjava et al., 1993; Arner and Tortorella, 1995). MMP-1 expression is elevated in response to collagen type I culture substrates (Riikonen et al., 1995; Langholz et al., 1995). Increases in MMP-2 activation were reported following culture of several cell types on or within collagen type I gels (Azzam and Thompson, 1992; Gilles et al., 1997; Haas et al., 1998). Vitronectin receptor ($\alpha v\beta 3$) ligation also stimulates MMP-2 expression, as demonstrated by Sefror et al. (1992) who observed that treatment of a melanoma cell line with an anti- $\alpha v\beta 3$ integrin antibody increased MMP-2 expression and stimulated the invasion of the cells through Matrigel.

The aim of this study was to investigate the regulation by matrix macromolecules of both MMP-2 activation and MT1-MMP expression, using HT1080 fibrosarcoma cells as a model. We report here that fibronectin promotes proMMP-2 activation by HT1080 cells. Culture of HT1080 cells on fibronectin, or on fragments of fibronectin encompassing the RGD integrin binding site, up-regulated processing of proMMP-2 to the active form. In marked contrast, culture of these cells on laminin-1 did not promote MMP-2 processing. The potential for fibronectin integrin receptors to signal MMP-2 activation was examined. We investigated the effects of the ECM substrates fibronectin and laminin-1 on the expression of TIMP-2 and MT1-MMP by HT1080 cells and found that the truncation of MT1-MMP protein to a 45 kDa form increased when the cells were cultured on fibronectin.

MATERIALS AND METHODS

Reagents

Human plasma fibronectin was purchased from Bioproducts, Hertfordshire, UK, and murine laminin-1 was from Sigma Chemical Company. Culture reagents were from Sigma or Gibco BRL with the exception of fetal calf serum (FCS) which was obtained from Globepharm, Surrey, UK. All chemicals were purchased from Sigma, ICN or Pierce. Radiochemicals were purchased from Amersham Life Sciences. The MMP inhibitor CT1746 (N1-(1-(S)-carbamoyl-2,2-dimethylpropyl)-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)-propyl]succinamide) was a gift from Dr A. Docherty (Celltech Research, Slough, UK). The 120 kDa and 110 kDa fibronectin

fragments were prepared by thermolysin digestion of human fibronectin as described (Borsi et al., 1986). The fibronectin fragment Fn III 6-10 was a gift from Dr S. Aota (National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland) and was prepared as detailed by Danen et al. (1995). Recombinant human proMMP-2 and proMMP-3 were purified from media conditioned by the relevant transfected mouse myeloma cells: proMMP-2 (Murphy et al., 1992b); proMMP-3 (Murphy et al., 1992a). Recombinant proMMP-13 (Knäuper et al., 1996) was a kind gift from Dr V. Knäuper (University of East Anglia, Norwich, UK). Recombinant MT-MMP ($\Delta 502-559$ Δ TM-MT1-MMP; d'Ortho et al., 1997) was kindly provided by Dr H. Will, InVitek GmbH, Berlin-Buch, Germany. Recombinant human tissue inhibitors of metalloproteinases (TIMPs -1 and -2) were expressed in mouse myeloma cells (Murphy et al., 1991; Willenbrock et al., 1993) and MMP inhibitory activity was assayed as described by Murphy et al. (1981).

Preparation of extracellular matrix and antibody substrates

Culture plates were coated with human plasma fibronectin, fragments of fibronectin, or laminin-1 following the method of Tremble et al. (1994). ECM was added to culture plates at 30 μ g/ml in phosphate buffered saline (PBS) and incubated overnight at 4°C. The solution was then aspirated, the wells washed with PBS and blocked with 1% (w/v) heat-denatured bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Wells were washed with PBS and used the same day. The following monoclonal antibodies to integrin subunits were coated on culture plastic at 100 μ g/ml: anti- $\alpha 5$, mAbs 16 and 11 (Akiyama et al., 1989; LaFlamme et al., 1992); anti- $\beta 1$, mAb 13 (Akiyama et al., 1989) and anti- $\alpha 6$, GoH3, (TCS Biologicals, Botolph Claydon, Buckinghamshire, UK). The antibody-coated wells were washed with PBS and blocked with BSA according to the protocol above.

Cell culture

The HT1080 human fibrosarcoma cell line was purchased from the European Collection of Animal Cell Cultures, Wiltshire, UK. A second HT1080 line that is *N-ras* transformed (Paterson et al., 1987) was a gift from Dr C. Marshall, Institute of Cancer Research, London, UK. MMP-2 activation by cells cultured on fibronectin was compared in the two lines; both cell lines responded similarly. The *N-ras* transformed cells were used routinely for this study as their constitutive expression of the gelatinases was greater. HT1080 cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. A third HT1080 cell line, stably transfected with wild-type MT1-MMP using the HCMV/gpt vector pGW1HG (Green et al., 1994) was a gift from Dr J. Clements, British Biotech Pharmaceuticals Ltd, Oxford, UK. These cells were used for the preparation of MT1-MMP enriched cellular membranes and maintained in a selection medium of DMEM supplemented with 10% FCS, 4 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, HT supplement, 20 μ M mycophenolic acid and 2 mM xanthine.

Preparation of conditioned media

Subconfluent cultures of HT1080 cells were trypsinised and centrifuged, firstly in serum containing medium, followed by two washes in serum free medium. Cells were seeded onto uncoated 24-well tissue culture wells (Costar) or substrate-coated wells in DMEM supplemented with 0.1% (w/v) BSA and cultured for 48 hours. In some experiments, phorbol 12 myristate 13-acetate (PMA) was added after the first 2 hours in culture, along with MMP inhibitors. At harvest, cells were trypsinised and the number of cells per well determined by counting the cells with a Neubauer haemocytometer.

TIMP-2 complexed with MMP-2 was purified from the conditioned medium by adsorption to gelatin-Sepharose (Butler et al., 1998). Bound material (about 10-100 ng) was eluted using Laemmli reducing sample buffer and analysed for TIMP-2 by western blotting (see below).

Preparation of cell lysates

HT1080 cells were cultured on dishes coated with ECM or monoclonal antibodies, or treated with PMA as described above. After 48 hours in culture, the conditioned medium was harvested for analysis by zymography and the cell monolayers washed with cold PBS before lysis. Cells were lysed as described by Lohi et al. (1996), using a lysis buffer of 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl, 1% (v/v) Triton-X-100, 0.02% (w/v) azide, with the protease inhibitors pepstatin A (1 µg/ml), phenylmethylsulfonyl fluoride (PMSF; 100 µM), trans-epoxysuccinyl-l-leucylamido (4-guanidino)-butane (E-64; 1 µg/ml), and EDTA (10 mM). The cell lysates were cleared by centrifugation and total protein estimated using the bicinchoninic acid assay (Sigma).

Zymography

Conditioned media from HT1080 cultures were analysed for gelatin degrading activity by electrophoresis under non-reducing conditions on SDS-polyacrylamide gels containing 0.5 mg/ml denatured type I collagen (Heussen and Dowdle, 1980). The volume of conditioned medium loaded per lane was standardised on the basis of the cell counts obtained at harvest. Gels were incubated overnight at room temperature in 100 mM Tris-HCl, pH 7.9, 30 mM CaCl₂ and 0.02% (w/v) sodium azide. White zones of lysis indicating gelatin degrading activity were revealed by staining with Coomassie brilliant blue.

Isolation of RNA and northern blot analysis

HT1080 cells were cultured in DMEM 0.1% (w/v) BSA on plastic or ECM protein coated dishes. Cells were cultured for 24 hours prior to lysis and extraction of total cellular RNA by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA samples were separated on an agarose 2.2 M formaldehyde gel, transferred to a Nylon membrane (Boehringer Mannheim) and probed with digoxigenin-labeled riboprobes as described (Atkinson et al., 1995).

Western blot

Lysate proteins (25 µg protein per lane) or gelatin-Sepharose eluates (10 µl) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by electroblotting and probed with either a polyclonal antibody to TIMP-2 (Ward et al., 1991) or a polyclonal antibody to human MT1-MMP, affinity purified as described (d'Ortho et al., 1998). Bound antibody was detected using a peroxidase conjugated secondary antibody followed by chemiluminescence detection.

Preparation of cell membranes

HT1080 cell membranes enriched in the 60 kDa form of MT1-MMP were prepared from HT1080 cells stably transfected with wild-type MT1-MMP (described above). Processing of MT1-MMP to the 45 kDa form was prevented by culturing the cells in the presence of CT1746 inhibitor for 48 hours prior to harvest (Butler et al., 1998). To remove excess CT1746 at harvest, the cells were washed with ice-cold DMEM (containing inhibitors) with pH adjusted to 9.0. Cells were scraped, centrifuged and the pellet washed twice with DMEM pH 9.0, using 25 ml solution/175cm² cell monolayer per wash. The membranes were resuspended in buffer (20 mM Tris-HCl, pH 7.8, 10 mM CaCl₂, 0.025% (v/v) Brij 35, 0.02% (w/v) sodium azide) containing the protease inhibitors pepstatin A (1 µg/ml), E-64 (1 µg/ml) and PMSF (100 µM). The amount of protein in the membrane preparation was estimated by the bicinchoninic acid assay to be 2.6 mg/ml and the amount of active MT1-MMP present in the membrane preparation was estimated by a quenched fluorescent peptide assay, assuming a k_{cat}/K_m value of $1.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Butler et al., 1998).

MMP-2 cleavage of membrane bound MT1-MMP

Recombinant proMMPs-2 and -3 were activated essentially as described by Murphy et al. (1991). ProMMP-13 was activated by incubation with 2 mM APMA for 1 hour at 37°C and $\Delta\text{TM-MT1-}$

MMP was activated as described (Butler et al., 1998). MT1-MMP enriched cell membrane preparation (10 µl) was incubated alone or with active MMPs for 4 hours at 37°C. The cleavage reaction was stopped by the addition of Laemmli reducing sample buffer (Laemmli and Favre, 1973). The molecular mass of the resulting MT1-MMP fragments were estimated by SDS-polyacrylamide gel electrophoresis followed by western blot analysis as described above.

RESULTS

A comparison of the effects of fibronectin and laminin-1 substrates on HT1080 fibrosarcoma processing of proMMP-2 to the active form

HT1080 cells cultured on plastic constitutively synthesised and secreted MMP-2 and MMP-9 (gelatinase B) as detected by gelatin zymography (Fig. 1A). The substrates plasma fibronectin and laminin-1 both supported cell adhesion and spreading. Cells cultured on laminin-1 expressed mainly latent, proMMP-2, molecular mass 66 kDa (Fig. 1A, lane 2). In contrast, cells cultured on fibronectin (Fig. 1A, lane 1) displayed an apparent increase in processing of proMMP-2 from the 66 kDa latent form, via an intermediate at 62 kDa, to the fully active 59 kDa protein. This increase in MMP-2 processing induced by fibronectin was similar to the levels of processing induced by PMA (Fig. 1A, lane 3), a known stimulator of MMP-2 activation for these cells (Lohi et al., 1996). Increased processing of MMP-2 by cells cultured on fibronectin was also evident when secreted proteins were labelled with [³⁵S]methionine, immunoprecipitated with an antibody to MMP-2 and analysed by autoradiography (data not shown).

MMP-9 secretion was up-regulated by PMA treatment (Fig. 1A, lane 3); however, neither culture of the cells on fibronectin nor on laminin-1 affected MMP-9 expression (Fig. 1A, lanes 1 and 2). Activated MMP-9 was not detected in this system; activation of MMP-9 is known to proceed via different mechanisms to MMP-2 (Murphy et al., 1992c).

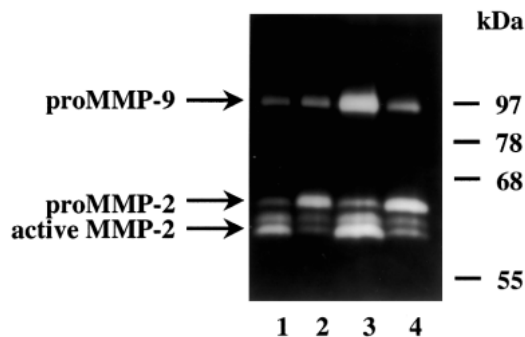


Fig. 1. Increased processing of proMMP-2 to the active form by HT1080 cells cultured on fibronectin in comparison with laminin-1. HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1, or on culture plastic with or without PMA for 48 hours. Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence of PMA (lane 3) or in the absence of PMA (lane 4). Arrows indicate the electrophoretic mobility of recombinant proMMP-9 and recombinant pro- and active MMP-2. Molecular mass markers are indicated on the right.

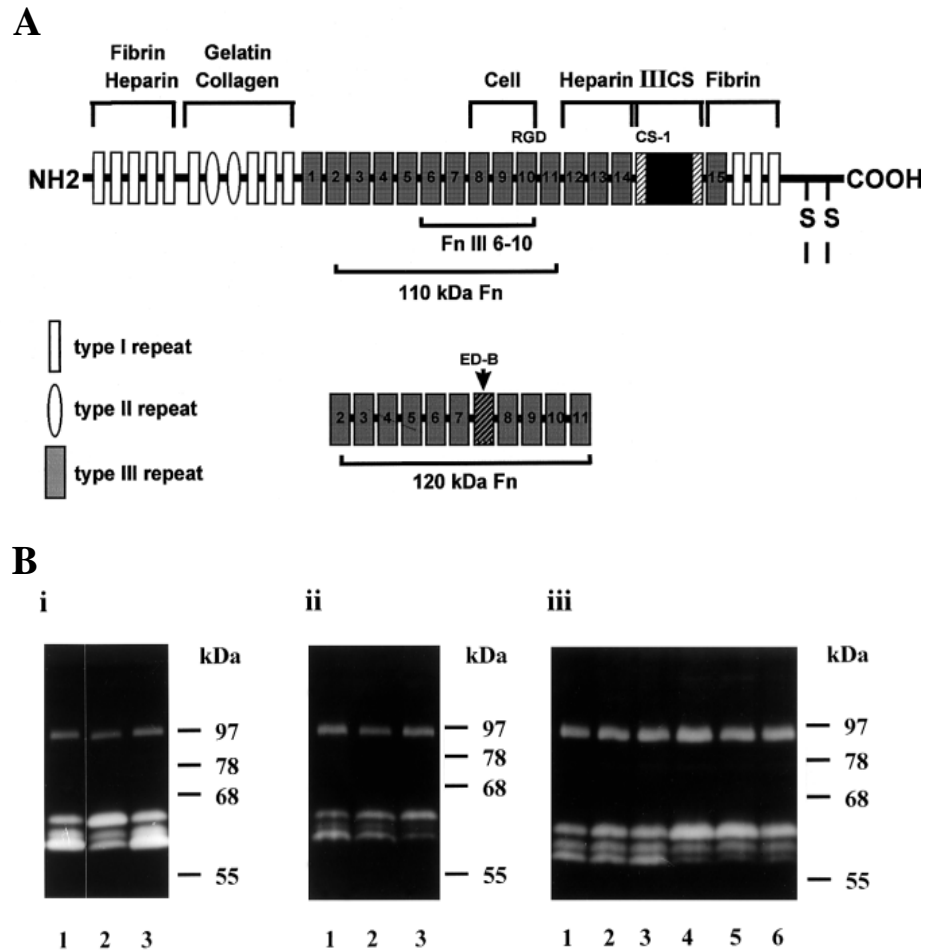


Fig. 2. Processing of proMMP-2 to the active form is up-regulated by fragments of fibronectin comprising the $\alpha 5\beta 1$ integrin binding site and by a monoclonal antibody to $\alpha 5$ integrin. (A) Schematic diagram of intact plasma fibronectin and the fibronectin fragments used in this study. (B) HT1080 cells were cultured under serum free conditions for 48 hours on fibronectin or fibronectin fragments, or on immobilised antibodies to integrin subunits. Samples of conditioned media were analysed for gelatin degrading activity by zymography. (i) Cells were cultured on fibronectin (lane 1), on laminin-1 (lane 2) or on the 120 kDa fragment (lane 3). (ii) Cells were cultured on fibronectin (lane 1), on Fn III 6-10 (lane 2) or on laminin-1 (lane 3). (iii) Monoclonal antibodies to integrin subunits were used as substrates. Cells cultured on fibronectin (lane 1), on the anti- $\alpha 5$ antibody mAb 16 (lane 2), on anti- $\beta 1$ antibody mAb 13 (lane 3), on anti- $\alpha 5$ antibody mAb 11 (lane 4), or on anti- $\alpha 6$ antibody GoH3 (lane 5). Cells cultured on laminin-1 (lane 6). Molecular mass markers are indicated on the right.

As the activation of MMP-2 is a cell membrane-mediated process, one way in which fibronectin might effect an up-regulation of MMP-2 processing is by binding MMP-2 close to the cell surface. Solid phase studies have demonstrated the binding of a C-terminal domain fragment of MMP-2 to fibronectin (Wallon and Overall, 1997); however, using the ELISA method of Allan et al. (1995), we were unable to demonstrate binding of the full length pro- and active MMP-2 to fibronectin (data not shown). It is unlikely, therefore, that fibronectin acts to 'trap' MMP-2 close to the cell surface.

Ligands that promote cell attachment via the $\alpha 5\beta 1$ integrin up-regulate proMMP-2 activation by HT1080 cells

To determine whether specific regions of fibronectin have the potential to affect MMP-2 activation, defined peptide fragments of fibronectin were coated to culture plastic. The 120 kDa chymotryptic fragment of fibronectin comprising the fibronectin type III repeats 2-11 of the central cell binding domain (CCBD; Fig. 2A) supported HT1080 adhesion and spreading. MMP-2 activation on this fragment (Fig. 2Bi, lane 3) was equivalent to that observed for full length fibronectin (Fig. 2Bi, lane 1). A second fragment of 110 kDa, which lacked the alternatively spliced ED-B domain (Fig. 2A) also induced MMP-2 activation (data not shown), indicating that regions in the CCBD other than the ED-B domain effect changes in MMP-2 activation.

The CCBD contains an RGD sequence in fibronectin type III repeat 10, which is of key importance for cell attachment via integrin receptors (for review, see Mohri, 1996). We cultured HT1080 cells on fibronectin type III repeats 6-10 (Fn III 6-10; Fig. 2A), which comprises the RGD sequence and subregions in type III repeats 8 and 9 that act synergistically with the RGD sequence for full adhesion activity (Danen et al., 1995). Despite poor cell spreading on this fragment, the activation of MMP-2 (Fig. 2Bii, lane 2) appeared greater than that observed for cells cultured on a laminin-1 substrate, on which the cells were well spread (Fig. 2Bii, lane 3). This indicated to us that Fn III 6-10 may contain the information capable of signalling MMP-2 activation.

We mediated direct interactions with the integrin receptors that bind the CCBD, by using antibodies to integrin subunits as substrates for cell adhesion. We confirmed that the HT1080 cell adhesion to fibronectin was completely inhibited by mAb16, a monoclonal antibody to the $\alpha 5$ integrin subunit, (results not shown; Yamada et al., 1990). Since mAb16 supports cell adhesion and spreading and mimics the action of the fibronectin ligand (Akiyama et al., 1989) we chose to culture HT1080 cells on this antibody. MMP-2 activation was up-regulated when the cells were cultured on this antibody (Fig. 2Biii, lane 2), with a processing profile comparable to that when cells were cultured on fibronectin (Fig. 2Biii, lane 1). In contrast, the monoclonal mAb 11 to the $\alpha 5$ integrin subunit, which promotes receptor cross-linking but does not mimic

ligand action (LaFlamme et al., 1992) did not up-regulate MMP-2 processing when used as a substrate for HT1080 adhesion (Fig. 2Biii, lane 4). MAb 13 to the $\beta 1$ integrin subunit also promoted MMP-2 activation when it was used as a culture substrate (Fig. 2Biii, lane 3).

HT1080 cell adhesion to laminin-1 is via the $\alpha 6\beta 1$ integrin and can be completely inhibited by the addition of a monoclonal antibody to the $\alpha 6$ subunit, GoH3 (von der Mark et al., 1991). As the processing of MMP-2 by HT1080 cells on a laminin-1 substrate is poor (Fig. 1, lane 2), we speculated that the $\alpha 6$ subunit is not involved in promoting MMP-2 processing. HT1080 cells were cultured on a substrate of GoH3 antibody, on which the cells spread well, but they failed to process MMP-2 to the levels seen when fibronectin or mAb16 was used as a substrate (Fig. 2Biii, lane 5).

Culture of HT1080 cells on fibronectin or laminin-1 does not influence the expression of TIMP-2 protein

The activation of MMP-2 is exquisitely regulated by the levels of TIMP-2 at the extracellular surface (Strongin et al., 1995; Butler et al., 1998). We analysed the effect of culture substrate on HT1080 TIMP-2 expression. TIMP-2 complexed to MMP-2 in the conditioned medium was concentrated by gelatin-Sepharose chromatography and analysed by western blot using a polyclonal antibody to TIMP-2 (Ward et al., 1991). Although the levels of TIMP-2 synthesised by HT1080 cells varied between experiments, within experiments no difference was noted in the levels of TIMP-2 secreted when cells were cultured on fibronectin as compared with laminin-1 (Fig. 3A). Western blot of cell lysates showed no obvious differences in the levels of cell associated TIMP-2 between the substrates (Fig. 3B).

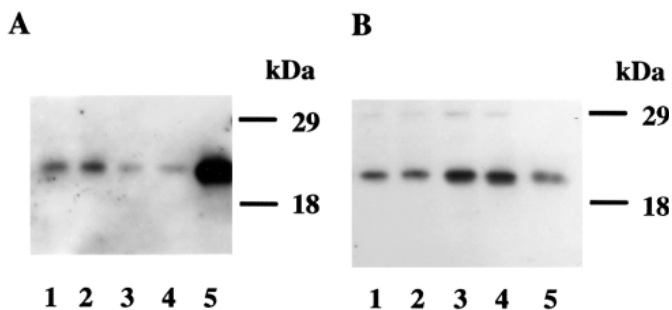


Fig. 3. TIMP-2 protein expression by HT1080 cells cultured on fibronectin or laminin-1. HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1 for 48 hours. (A) Western blot analysis of secreted TIMP-2. TIMP-2/MMP-2 complexes were purified from the conditioned media by binding to gelatin-Sepharose. Bound proteins were eluted with reducing sample buffer and separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to TIMP-2. Two separate experiments are depicted. Experiment 1: cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2). Experiment 2: cells cultured on fibronectin (lane 3), cells cultured on laminin-1 (lane 4). Recombinant TIMP-2 (lane 5). (B) Western blot analysis of HT1080 cell lysates. Cells were lysed in a buffer containing Triton X-100. Lysate proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with a polyclonal antibody to TIMP-2. Two separate experiments are depicted. Cells cultured on fibronectin (lanes 1, 3), cells cultured on laminin-1 (lanes 2, 4). Recombinant TIMP-2 (lane 5).

Messenger RNA levels for MT1-MMP expressed by HT1080 cells are not altered by culture on fibronectin or laminin-1 substrates

Fibronectin substrates were examined for their potential to regulate MT1-MMP expression at the messenger RNA level. RNA prepared from HT1080 cells was analysed by northern blot and a single band of MT1-MMP was detected (Fig. 4), consistent with the 4.5 kilobase transcript reported by Sato et al. (1994). Culture of the cells on fibronectin or laminin-1 did not alter the steady state levels of MT1-MMP mRNA (Fig. 4).

Culture of HT1080 cells on fibronectin up-regulates the processing of MT1-MMP protein to a 45 kDa form

MT1-MMP protein in HT1080 cell lysates was analysed by western blot using a polyclonal antibody to MT1-MMP developed and affinity purified as described (d'Ortho et al., 1998). This antibody detected major bands of MT1-MMP at molecular mass 60 kDa and 45 kDa and a faint doublet at 63 kDa (Fig. 5A). Levels of the 60 kDa form of MT1-MMP were unaffected by the different culture conditions used in this study. However, culture of the cells on fibronectin increased the total MT1-MMP protein level; more specifically the 45 kDa band was up-regulated. By densitometric scanning the total MT1-MMP protein produced by the cells cultured on fibronectin was 1.6 times that detectable from cells cultured on laminin. Densitometric scanning also revealed that cells cultured on fibronectin produced at least 3 times more 45 kDa MT1-MMP than cells cultured on laminin. PMA also up-regulated the 45 kDa band. Strikingly, where increases in the 45 kDa band were detected, there was a concomitant increase in the processing of MMP-2 to the active form, as detected by zymography (Fig. 5B).

Processing of MT1-MMP to the 45 kDa form is mediated by matrix metalloproteinase(s)

To investigate whether MMPs are involved in the processing of MT1-MMP to 45 kDa, HT1080 cells were cultured on fibronectin in the presence of inhibitors of metalloproteinase

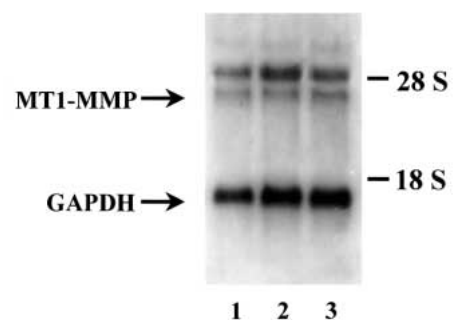
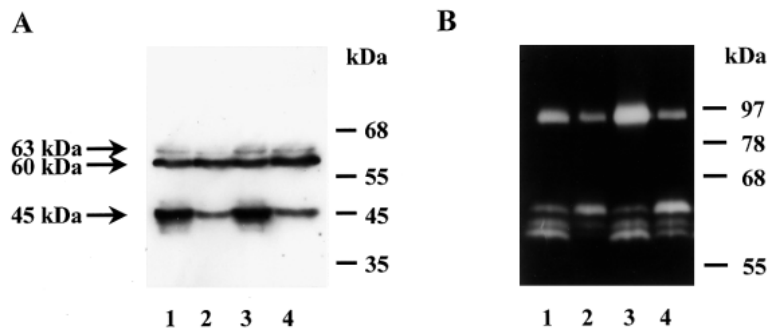


Fig. 4. HT1080 fibrosarcoma mRNA levels for MT1-MMP are not altered by culture on fibronectin or laminin-1. Northern blot of RNA from HT1080 cells cultured on fibronectin, laminin-1 or on plastic. Cells were cultured for 24 hours prior to extraction of total RNA. Samples (5 μ g/lane) were separated on agarose gels, transferred to a Nylon membrane and MT1-MMP mRNA detected by hybridisation with a digoxigenin-labelled riboprobe. The MT1-MMP probe reveals the 28S ribosomal RNA (uppermost band of upper doublet). The position of 18S ribosomal RNA is shown. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe was used as an internal standard. Cells were cultured on plastic (lane 1) on laminin-1 (lane 2); or cultured on fibronectin (lane 3).

Fig. 5. The processing of MT1-MMP protein to a 45 kDa form by HT1080 fibrosarcoma cells is increased by culture on fibronectin. HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1, or on culture plastic with or without PMA for 48 hours.

(A) Western blot analysis of HT1080 cell lysates. Cells were lysed in a buffer containing Triton-X-100. Lysate proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence of PMA (lane 3) or in the absence of PMA (lane 4). (B) The conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence of PMA (lane 3) or in the absence of PMA (lane 4). Molecular mass markers are indicated on the right.



activity for 46 hours prior to harvest for zymography and western blot. The hydroxamate inhibitor CT1746 is a general metalloproteinase inhibitor when used at the concentration employed here. Inclusion of CT1746 in a culture of HT1080 cells on fibronectin completely abrogated the generation of the 45 kDa band (Fig. 6A, lane 2), indicating the requirement for metalloproteinase activity in the processing of MT1-MMP to 45 kDa. Exogenously added TIMP-2 also inhibited this processing of MT1-MMP, further defining the processing activity as an MMP (Fig. 6A, lane 3). TIMP-1, even at high concentrations (150 nM), was ineffective as an inhibitor of processing of MT1-MMP to 45 kDa (Fig. 6A, lane 4). The conditioned media from this experiment were examined by zymography. Both CT1746 and TIMP-2 inhibited MMP-2 activation, but TIMP-1 had no effect (Fig. 6B).

To investigate which MMPs are capable of processing MT1-MMP to the 45 kDa form, we used HT1080 cells stably transfected with wild-type MT1-MMP to prepare cell

membranes. The membranes were enriched in the 60 kDa active form of MT1-MMP by culturing the cells in the presence of CT1746 prior to harvest (to inhibit processing to the 45 kDa form); however, care was taken to wash away the CT1746 at harvest (see Methods). The amount of active MT1-MMP present in the membrane preparation was estimated at 23 pmol/mg of membrane protein by a quenched fluorescent peptide assay. Based on this value, recombinant active MMPs were added at a 1:1 molar ratio with native MT1-MMP, with the exception of recombinant soluble Δ TM-MT1-MMP, which was added at a 5:1 molar ratio to native MT1-MMP. Incubation at 37°C for 4 hours resulted in the generation of the 45 kDa form of MT1-MMP, with active preparations of MMP-2, and Δ TM-MT1-MMP capable of cleaving native MT1-MMP to the 45 kDa form (Fig. 7, lanes 3 and 4). In addition, active MMP-3 and MMP-13 cleaved MT1-MMP to 45 kDa in vitro (Fig. 7, lanes 5 and 6), indicating that MT1-MMP has a cleavage site that is susceptible to several members of the MMP family. We cannot detect MMP-3 or MMP-13 protein expression in HT1080 cells (data not shown) and so it is unlikely that these proteinases are involved in HT1080 processing of MT1-MMP to 45 kDa. However, they may play a role in other cell systems, particularly as MMP-13 is activated by membrane associated MT1-MMP/MMP-2 (Knäuper et al., 1996). A second band of processed MT1-MMP at 37 kDa was observed in all membrane preparations incubated with active MMPs, but this band was not detected in the lysates prepared directly from cells in culture. Incubation of the membrane preparation alone also resulted in the generation of the 45 kDa band, but to a lesser extent than when active MMPs were added, demonstrating intrinsic MT1-MMP processing activity in the membrane preparation.

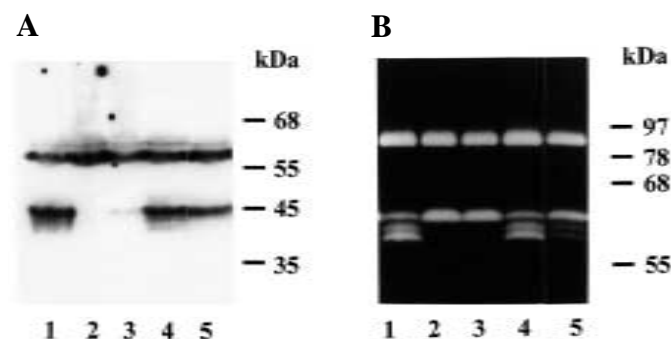


Fig. 6. Cellular processing of MT1-MMP to the 45 kDa form is mediated by a matrix metalloproteinase. HT1080 cells were cultured on fibronectin under serum free conditions, with or without protease inhibitors for 48 hours. The conditioned medium was harvested for zymography and the cells lysed for western blot. (A) Western blot analysis. HT1080 cell lysates were prepared and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Cells cultured on fibronectin (lanes 1-4), alone (lane 1), or in the presence of metalloproteinase inhibitor CT1746 at 1 μ M (lane 2), or recombinant MMP inhibitors TIMP-2 and TIMP-1 at 150 nM (lanes 3 and 4, respectively). Cells cultured on laminin-1 (lane 5). (B) The conditioned medium was analysed by gelatin zymography. Lanes 1-5, see legend for A. Molecular mass markers are indicated on the right.

DISCUSSION

Fibronectin matrices influence cellular functions including adhesion, migration, and differentiation via interactions with cell surface integrin receptors. These interactions are of importance in early wound repair (Greiling and Clark, 1997) and tumour development where several peptide and antibody inhibitors of fibronectin/integrin function effectively inhibit metastasis (Akiyama et al., 1995).

We have demonstrated in this report that fibronectin up-regulates the activation of MMP-2 by HT1080 fibrosarcoma cells, and that fibronectin induces a change in the levels of

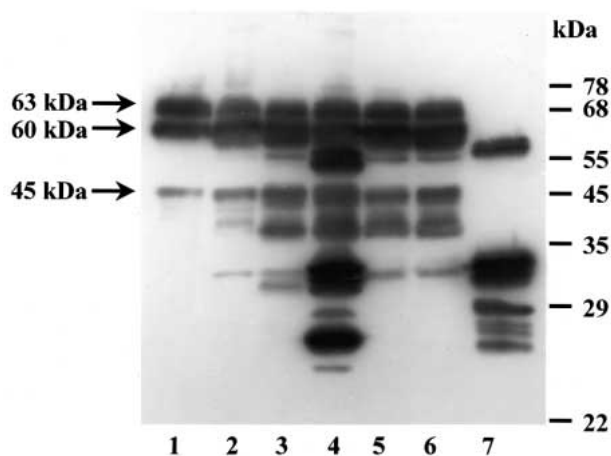


Fig. 7. In vitro cleavage studies: incubation of membrane bound native MT1-MMP with active MMPs. Cell membranes enriched in the 60 kDa form of MT1-MMP were prepared from cultures of MT1-MMP transfected HT1080 cells grown in the presence of CT1746 inhibitor. Membrane preparations were incubated alone, or in the presence of active MMPs for 4 hours at 37°C, Laemmli sample buffer added and the protein mixture separated by 10% SDS-polyacrylamide gel electrophoresis, electroblotted to nitrocellulose and probed with the polyclonal antibody to MT1-MMP. Cell membranes incubated alone (lane 2), or in the presence of active MMP-2 (lane 3), active Δ TM-MT1-MMP (lane 4), active MMP-3 (lane 5), active MMP-13 (lane 6). Cell membranes, unincubated (lane 1). Recombinant active Δ TM-MT1-MMP alone (lane 7). Molecular mass markers are indicated on the right.

processed MMP-2 which is similar to that induced by phorbol ester. We compared the up-regulation of MMP-2 activation in response to fibronectin with activation on laminin-1. Processing of MMP-2 by cells on laminin-1 appeared to be quite low. Differences in MMP-2 activation were observed without major changes in cell shape, because the cells spread well on both fibronectin and laminin-1.

In our study, to induce an up-regulation of MMP-2 processing it was necessary to coat the fibronectin to culture plastic; soluble fibronectin added to the culture medium did not affect MMP-2 processing (data not shown). It has previously been shown that cells do not respond to soluble fibronectin, except at high concentrations, partly due to the need for a conformational change induced by substrate adsorption that opens up the molecule to expose the cell adhesive domain (Yamada and Kennedy, 1984; Fukai et al., 1995). Reich et al. (1995) reported that soluble laminin added to HT1080 cultures for 6 hours increased MMP-2 mRNA and protein expression, but no comment was made as to whether MMP-2 activation was affected. In our study, soluble laminin-1 added to cultures of HT1080 for 24 or 48 hours did not alter MMP-2 protein expression (data not shown), or MMP-2 activation.

To determine whether specific domains of fibronectin signal MMP-2 activation, we cultured HT1080 cells on polypeptide fragments of fibronectin. We noted that the processing of MMP-2 on the 120 kDa fragment of fibronectin was similar to that observed for full length fibronectin. Werb et al. (1989) reported that rabbit synovial fibroblasts respond to the 120 kDa fragment, but not to full length fibronectin, up-regulating the synthesis of MMP-1, -3 and -9. Our data differs from that study

on several points. Firstly, in our study, full length fibronectin as well as the 120 kDa fragment signalled changes in MMP-2 activation; secondly, HT1080 synthesis of MMP-9 did not appear to be affected by fibronectin substrates and thirdly, although rabbit synovial fibroblasts express MMP-2, there was no activation of MMP-2 apparent when these cells were cultured on fibronectin (Werb et al., 1989). In a later study with rabbit synovial fibroblasts, Huhtala et al. (1995) demonstrated that the reason full-length fibronectin did not signal MMP synthesis was due to opposing signals from $\alpha 5 \beta 1$ integrin binding to the CCB and $\alpha 4 \beta 1$ integrin binding to the alternatively spliced CS-1 peptide region of fibronectin (Fig. 2A), as the latter down-regulates MMP synthesis. As HT1080 cell adhesion to fibronectin can be completely inhibited by an antibody to the $\alpha 5$ integrin subunit (Yamada et al., 1990) it is unlikely that the CS-1 region is involved in the regulation of MMP-2 activation by HT1080 cells.

We decided to concentrate on the CCB of fibronectin and to culture HT1080 cells on smaller fragments comprising the RGD site and synergy site. However, we encountered a problem with poor cell spreading on fragment Fn III 6-10; nevertheless, MMP-2 activation was observed. To circumvent this problem of altered cell shape, cells were cultured directly on a substrate of anti-integrin antibodies. HT1080 cells spread well on antibodies to the $\alpha 5$, $\alpha 6$ and $\beta 1$ integrin subunits. Adhesion to the anti- $\alpha 5$ integrin subunit monoclonal antibody mAb 16 promoted MMP-2 processing, but interestingly, monoclonal mAb 11, also to the $\alpha 5$ integrin subunit failed to support MMP-2 activation. Both antibodies are known to promote integrin receptor clustering; however mAb 16, in contrast to mAb 11, also mimics the action of the fibronectin ligand (Miyamoto et al., 1995). Fibronectin receptor occupancy would therefore appear to be important in the signalling of MMP-2 activation. The lack of processing of MMP-2 when cells were cultured on laminin-1 was also observed when the cells were cultured on an antibody to the laminin receptor $\alpha 6$ integrin subunit. However, mAb 13 against the $\beta 1$ subunit of $\alpha 5 \beta 1$ also promoted MMP-2 activation. This result is consistent with the fact that mAb 13 can mimic receptor occupancy. Collectively, our data with fibronectin peptides and integrin antibodies indicate that the mechanism by which fibronectin up-regulates MMP-2 processing by HT1080 cells may involve signalling via the $\alpha 5 \beta 1$ integrin.

Several reports have described the regulation of MMP-2 and MMP-9 expression in response to treatment with anti-integrin antibodies (Larjava et al., 1993; Seftor et al., 1992). Kubota et al. (1997) demonstrated that soluble anti- $\alpha 2$ and anti- $\alpha 3$ integrin antibodies induced proMMP-2 secretion and activation by human rhabdomyosarcoma cells. Studies with two glioblastoma cell lines showed that MMP-2 expression was increased by treatment with anti- $\alpha 3 \beta 1$ or anti- $\alpha 5 \beta 1$ integrin antibodies (Chintala et al., 1996). Clearly, increases in MMP-2 expression and activation may be signalled by several of the integrin receptors and the response to individual integrins is cell type specific. Similarly, MMP induction by ECM molecules is dependent upon the cell type. In our laboratory, HT1080 cells respond to full length fibronectin by increasing MMP-2 activation whereas MMP-2 activation by human foreskin fibroblasts (which also express $\alpha 5 \beta 1$ integrin) is not affected by culture on full-length fibronectin or the 120kDa fragment (data not shown). This differential response may

indicate diverging intracellular signalling pathways following integrin ligation in these two cell types, and future studies may elucidate the mechanisms involved.

As the experiments described here were conducted over 24–48 hours, we have not ruled out the possibility that fibronectin induces the expression of an endogenous cytokine or growth factor, that in turn signals changes to MMP-2 activation. It has been demonstrated for early passage fibroblasts that the induction of MMP-1 activity by PMA proceeds via an interleukin-1 autocrine loop (West-Mays et al., 1995), and similarly, the RGD peptide-induced expression of MMPs -1, -3 and -9 by rabbit chondrocytes is augmented by an interleukin-1 autocrine loop (Arner and Tortorella, 1995). Evidence suggests that growth factors synergise with extracellular matrix/integrin mediated signalling pathways (Schwartz et al., 1995). Ligand-mediated integrin clustering leads to the accumulation of growth factor receptors (Plopper et al., 1995; Miyamoto et al., 1996). Furthermore, $\beta 1$ integrin receptor occupancy results in the enhancement of growth factor receptor tyrosine phosphorylation and the transiently enhanced activation of mitogen-activated protein kinases (Miyamoto et al., 1996). The signalling mechanisms involved in fibronectin up-regulation of MMP-2 activation and the possibility that an endogenous growth factor or cytokine is required will be the subject for future investigations.

In order to effect increases in proMMP-2 activation, we speculated that the fibronectin matrix may be signalling changes to other molecules involved in the activation cascade. Interactions between the C-terminal domain of proMMP-2 and a membrane-bound component are known to be important (Strongin et al., 1995; Ward et al., 1994). MMP-2 has been reported to bind $\alpha v \beta 3$ integrin on the surface of $\beta 3$ integrin transfected cells and to purified $\alpha v \beta 3$ integrin in solid phase studies (Brooks et al., 1996, 1998), a process apparently mediated by the C-terminal domain of MMP-2. It is unlikely, however, that MMP-2/ $\alpha v \beta 3$ interactions play a role in MMP-2 activation in the current study, for immunolocalisation studies with several anti- αv or $\alpha v \beta 3$ integrin antibodies have indicated that the HT1080 cells used in these experiments apparently do not express $\alpha v \beta 3$ integrin (A. Messent and J. Gavrilovic, unpublished observations). Membrane bound MT1-MMP is known to initiate MMP-2 activation (Sato et al., 1994; Atkinson et al., 1995; Will et al., 1996). It has been hypothesised that MT1-MMP and TIMP-2 form a 'receptor' complex, that binds MMP-2 via its C terminus (Strongin et al., 1995; Imai et al., 1996; Butler et al., 1998). Evidence for this trimolecular complex has been provided by cross-linking experiments (Strongin et al., 1995) and a model for MMP-2 activation has been described in which proteolysis of MMP-2 bound in the complex requires an adjacent MT1-MMP molecule that is TIMP-2 free and therefore catalytically active (Butler et al., 1998). This model predicts that the balance of TIMP-2 and MT1-MMP is of critical importance in determining the activation status of MMP-2. In agreement with this model, addition of low concentrations of TIMP-2 to cell membranes increases MMP-2 activation, presumably by increasing the concentration of MT1-MMP/TIMP-2 complex available for MMP-2 binding. Conversely, high concentrations of TIMP-2 inhibit MMP-2 activation (Strongin et al., 1995; Butler et al., 1998), probably because all MT1-MMP molecules form MT1-MMP/TIMP-2 complexes, leaving no

free MT1-MMP molecules available to initiate proteolysis (Butler et al., 1998). We postulated, therefore, that the increased activation of MMP-2 by HT1080 cells cultured on fibronectin in comparison with cells cultured on laminin-1 could reflect a variation in either TIMP-2 or MT1-MMP status.

The data indicated that the secretion of TIMP-2 was similar when HT1080 cells were cultured on fibronectin or laminin-1 matrices. Furthermore, western blot analysis of cell lysates indicated that the amount of TIMP-2 associated with the cells was similar for both matrices. Using solid phase studies, we also found that TIMP-2 does not bind fibronectin, thus ruling out the possibility that a fibronectin matrix might act to trap TIMP-2 (and therefore MMP-2) close to the cell (data not shown). Collectively, these observations suggested that the effect of fibronectin on the activation of MMP-2 by HT1080 cells was unlikely to be due to alterations in TIMP-2 status.

We also investigated the expression of MT1-MMP by HT1080 cells. It has been shown that the culture of several cell types on collagen type I gels or within collagen lattices induces MT1-MMP mRNA expression and a corresponding increase in MMP-2 activation (Gilles et al., 1997; Haas et al., 1998). In the current study MT1-MMP mRNA levels were similar when cells were cultured on fibronectin or laminin-1 substrates. To determine whether post-translational regulation of MT1-MMP is affected by fibronectin, MT1-MMP protein was analysed by western blot using a newly described antibody to MT1-MMP. Two major immunoreactive bands were detected at 60 kDa and 45 kDa, and a faint doublet at 63 kDa. This profile is very similar to that reported by Lohi et al. (1996), who detected the doublet at 63 kDa and major bands at 60 and 43 kDa in stimulated HT1080 cells using two polyclonal antibodies to MT1-MMP. The 60 kDa band in the current study corresponds most likely to the active form of MT1-MMP (Pei and Weiss, 1996; Will et al., 1996; S. Atkinson, G. Butler, G. Murphy, unpublished results). Although the level of the 60kDa form was not affected by the various culture conditions employed, it was clear that the overall MT1-MMP protein levels increased when the cells were cultured on fibronectin, as the 45 kDa band was up-regulated. Similarly, PMA treatment up-regulated the expression of the 45 kDa band, as has been noted by Lohi et al. (1996). Increases in the 45 kDa band were noted to be concomitant with the activation of MMP-2, an observation that agrees with the report by Lohi et al. (1996). It is yet to be demonstrated whether there is a direct link between MMP-2 activation and MT1-MMP processing to 45 kDa, and what role the 45 kDa band plays in MMP activation mechanisms. According to the study by Lohi et al. (1996) the truncation of MT1-MMP to the 43kDa form involves the loss of the N terminus, since antibodies raised to peptides from the C-terminal hemopexin domain and the intracellular domain of MT1-MMP detect the 43kDa form in western blot. Allowing for the loss of the N terminus, simple calculations would predict that MT1-MMP is cleaved within the catalytic domain. We postulate that this would render the molecule inactive, which would represent an important regulatory step in MT1-MMP activation cascades.

To unravel the sequence of events involved in the fibronectin stimulation of MT1-MMP processing to 45 kDa more information is required on the nature of MT1-MMP processing and on the identity of the protease(s) involved. We added protease inhibitors to HT1080 cells cultured on fibronectin.

Inhibition of MT1-MMP processing to 45 kDa by the hydroxamate inhibitor CT1746 and by TIMP-2 indicated that the protease was an MMP. Interestingly, TIMP-1, even at high concentrations, failed to inhibit processing to 45 kDa. Our laboratory has demonstrated that MT1-MMP activity is efficiently inhibited by TIMP-2 and TIMP-3, but not by TIMP-1 (Will et al., 1996). Our TIMP-1 data would therefore imply that MT1-MMP processing to 45 kDa is autolytic. However TIMP-1 also failed to inhibit MMP-2 autolysis from the intermediate to the fully active form, a surprising result, as TIMP-1 is an effective inhibitor of MMP-2 (Murphy et al., 1992b). It is possible that MMP-2 bound in an MT1-MMP/TIMP-2 complex is not susceptible to TIMP-1 inhibition (suggested by the kinetic study of Willenbrock et al., 1993).

As the TIMP-1 inhibition data are difficult to interpret, both MT1-MMP and MMP-2 must be considered as candidate proteases for HT1080 MT1-MMP processing to 45 kDa. MMP-2 is constitutively expressed in most cell types and, as noted above, the presence of active MMP-2 correlates well with the appearance of the 45 kDa band. We demonstrated by an in vitro study that both MT1-MMP and MMP-2 cleave 60 kDa MT1-MMP to the 45 kDa form. Furthermore this cleavage site proved to be susceptible to several members of the MMP family in vitro. These results raise the intriguing possibility that the end step in the activation pathway of MMP-2 involves the cleavage of the activator MT1-MMP. To fully understand the effects of fibronectin on MT1-MMP processing to 45 kDa, further work is needed to define the exact enzymes involved.

In summary, we have shown that the culture of HT1080 cells on fibronectin up-regulates the activation of MMP-2 and that signals via $\alpha 5\beta 1$ integrin are likely to be involved. We chose to study MT1-MMP and TIMP-2, which are key components of MMP-2 activation, to determine whether a fibronectin substrate alters their expression or processing by HT1080 cells. We observed that proteolytic processing of active 60 kDa MT1-MMP to a 45 kDa product was concomitant with MMP-2 activation and that this processing was up-regulated by fibronectin. We demonstrated that MT1-MMP processing is MMP mediated. Work is underway to determine the N-terminal sequence of the 45 kDa band and to assess whether it is catalytically inactive. We speculate that proteolysis of active MT1-MMP to a 45 kDa form may represent an end-point in the activation pathway of MMP-2.

The up-regulation of MMP-2 activation by fibronectin may be of particular importance in wound healing where fibronectin is laid down in the provisional matrix forming a conduit for inwardly migrating cells (Greiling and Clark, 1997). During cancer progression fibronectin is also a prominent component of the host stroma and may modulate MMP-2 activation, essential for tumour invasion.

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