MT1-MMP is the critical determinant of matrix degradation and invasion by ovarian cancer cells

Membrane-type 1 matrix metalloproteinase (MT1-MMP), a transmembrane metalloprotease that plays an important role in the invasion of many solid tumour types, promotes pericellular matrix degradation and may also stimulate tumour cell motility. As both these processes are key contributors to intraperitoneal ovarian tumour metastasis, we examined six ovarian cancer cell lines to determine whether MT1 is a critical mediator of invasive behaviour for this tumour type. Our results indicated that only those cell lines that expressed MT1 were capable of penetrating a type I collagen barrier, with the capacity for both matrix degradation and invasion reflecting endogenous MT1 expression level. Ectopic MT1 expression endowed an invasive phenotype upon cell lines lacking MT1 that were previously non-invasive, indicating the crucial role of this protease. Conversely, invasion was abolished by tissue inhibitor of metalloproteinase-2 (TIMP-2), a potent inhibitor of MT1, yet was minimally affected when other (secreted) MMPs were inhibited using TIMP-1 and the gelatinase inhibitor SB-3CT. Whereas collagen I degradation was strikingly accelerated by ectopic MT1 expression, cell motility remained unchanged. We conclude that MT1 is necessary for collagen I invasion by ovarian cancer cells, and that its requisite activity is the promotion of matrix degradation, with no impact on cell motility.

Keywords: MT1-MMP; ovarian cancer; invasion; motility; collagen I; E-cadherin

Epithelial ovarian cancer is a highly aggressive malignancy associated with a poor prognosis. These tumours have usually metastasised before clinical presentation, and fewer than 30% of patients diagnosed with metastatic disease survive beyond 5 years (Landis et al., 1999), as present therapeutic strategies are ultimately ineffective in preventing disease progression. In contrast to most other solid tumours, which metastasise through a multi-step haematological route, ovarian cancer cells metastasise via exfoliation from the primary tumour into the peritoneal cavity. Ovarian cancer cells preferentially bind collagen I and this ligand is highly effective in stimulating motility in these cells (Moser et al., 1996; Burleson et al., 2004). Indeed, during peritoneal metastasis, tumour cells attach preferentially at locations where the mesothelium is disrupted and the underlying collagen I-rich stromal matrix is exposed (Ghosh et al., 2002; Mochizuki et al., 2004).

Metastatic cancer cells have acquired the proteolytic mechanisms required for penetration of basement membrane and collagen I-rich stromal matrices, which are normally refractory to invasion by epithelial cells (Sivridis et al., 2004). Matrix metalloproteases (MMPs), key mediators of ECM degradation (Egeblad and Werb, 2002), are upregulated in invasive cancers including those of ovarian origin (Ghosh et al., 2002). Although the importance of MMPs in ovarian cancer metastasis is recognised (Stack et al., 1998; Ghosh et al., 2002), clinical trials that have evaluated the broad-range MMP inhibitors marimastat and tanomastat for cancer treatment, including ovarian, have yielded disappointing results (Coussens et al., 2002; Hirte et al., 2006). This may be partially attributed to the concomitant inhibition of critical physiological processes involved in combating tumour progression (Overall and Lopez-Otin, 2002). It is therefore necessary to identify which subsets of MMPs play a critical role in ovarian cancer metastasis, so that they can be specifically targeted. A central role for a transmembrane member of the MMP family, membrane-type 1 MMP (MT1), in cancer metastasis has emerged in association with the aggressive behaviour of several cancer types (Aznavorian et al., 2001; Seiki, 2003; Ueda et al., 2003; Sabeh et al., 2004; Cao et al., 2005), as reviewed by Sounni and Noel (2005). We therefore sought to determine whether this protease plays an essential role in regulating ovarian cancer cell invasive behaviour.

The potency of MT1 owes to the ability of its proteolytic activity to be focused, via its translocation to the leading edge of invasive cancer cells (Mori et al., 2002). Indeed, MT1 is an essential functional component of invadopodia (Chen and Wang, 1999; Artym et al., 2006). Although MT1 is known for its potent collagenolytic activity, it cleaves a range of additional ECM substrates and cell surface-associated receptors. Membrane-type 1 MMP also activates soluble MMPs including pro-MMP-2, a
gelatinase that cooperates with MT1, to degrade stromal and basement membrane barriers (Ohuchi et al., 1997; Seiki, 2003). The resulting proteolyzed matrix, in turn, promotes further tumour growth and invasion (Hornebeck and Maquart, 2003).

Although matrix degradation is an obligatory aspect of tumour cell invasion, cell motility is an additional important contributor. Membrane-type 1 MMP has been implicated in enhancing cell motility through cleavage of cell surface receptors, such as CD44 and syndecan-1, and through generation of haptotactic signals by cleavage of laminin and collagen IV (Gingras et al., 2001; Rozanov et al., 2001; Ueda et al., 2003; Takino et al., 2004). Consistent with these observations, MT1 overexpression in some cancer cell types appears to promote motility on collagen I and fibronectin matrices, although the requirement for its enzymatic activity in this process is controversial (Giannelli and Antonaci, 2000; Kajita et al., 2001; Endo et al., 2003; Hornebeck and Maquart, 2003; Koshikawa et al., 2005; cf. Cao et al., 2004). Collectively, these studies suggest that MT1 may coordinate two cell activities necessary for invasion – matrix degradation and cell motility.

Although the functionality of MT1 in many solid tissue cancers is well established, information on its role in ovarian cancer is limited. Membrane-type 1 expression by cells of orthotopically implanted human ovarian cancer biopsies has been linked to a more aggressive tumorigenicity (Drew et al., 2004), and inhibition of MT1 activity has been shown to block collagen invasion in one ovarian cancer cell line, DOV-13 (Ellerbrook et al., 1999; Ellerbrook et al., 2001). However, as different ovarian cancer cell lines display varying capacities for peritoneal tumour formation in vivo (Shaw et al., 2004), and may use different mechanisms for invasion, we sought to determine whether MT1 expression determines invasive behaviour within a diverse panel of human ovarian cancer cell lines. A polymerised collagen I matrix was used to assess invasion for several reasons: Collagen I plays a key involvement in ovarian cancer metastasis, being the most abundant matrix molecule in the submesothelial stroma, and is the preferred substrate for ovarian cancer cell attachment during peritoneal metastasis (Moser et al., 1996). Furthermore, the common use of Matrigel for invasion assays has been brought into question as it does not form a protease-dependent barrier and therefore does not adequately represent a basement membrane (Hotary et al., 2006).

Although motility is an important aspect of ovarian cancer peritoneal dissemination, the potential contribution of motility by MT1 in ovarian cancer cells has not been assessed. Therefore, the impact of MT1 expression on motility, in addition to collagen I degradation, was evaluated. As elevated E-cadherin is known to suppress invasive behaviour of cancer cells and to negatively regulate MMP expression, including that of MT1 (Ara et al., 2000; Takahashi et al., 2002; Nawrocki-Raby et al., 2003a,b; Hazan et al., 2004; Hlbek et al., 2004; Munshi and Stack, 2006), we also assessed cadherin expression in these cell lines.

Our results demonstrate that MT1 activity is the prime determinant of ovarian cancer cell invasion through a collagen I matrix, and that this effect occurs through enhancement of matrix degradation without impacting cell motility.

### MATERIALS AND METHODS

#### Cell culture

Human ovarian cancer cell lines HEY, SKOV-3 and HOC-7 (obtained from Dr Alexander Marks, University of Toronto, Toronto, ON, Canada), OVC4A429 (from Dr Robert Kerbel, Sunnybrook Hospital, Toronto, ON, Canada), OVCAR-3 and ES-2 (from American Type Culture Collection, Manassas, VA, USA) were maintained in α-minimal essential medium (α-MEM; Gibco, Invitrogen Corp., Mississauga, ON, Canada) supplemented with 10% foetal bovine serum (FBS; Cansera International Inc., Etobicoke, ON, Canada), 0.017% penicillin G and 0.01% gentamycin at 37°C and 5% CO₂.

#### RT-PCR

RNA was extracted from confluent cells grown on collagen I films using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada) and reverse transcribed using a First Strand cDNA Synthesis Kit (Invitrogen Corp.). Primers for MT1 were: forward 5’-ataacagtctgctagcagag-3’ and reverse 5’-aagactgtgctgctctggct-3’ (310 bp ampiclon). Primers for E-cadherin were: forward 5’-ctacctgtgctgctctggct-3’ and reverse 5’-gcagcttgtgctgctctggct-3’ (361 bp ampiclon). Primers for RPL13a, used as an endogenous control gene (Mogal A, 2006) were: forward 5’-cactgtgctgaacagaggtct-3’ and reverse 5’-gcagctgtgctgctctggct-3’ (319 bp ampiclon). An annealing temperature of 59°C was used for all primers. Samples were taken at various cycles to ensure comparisons were made during the log phase of amplification. For MT1, 26–30 cycles were used; for RPL13a, 26 cycles and for E-cadherin 32 cycles. Real-time PCR for MT1 was performed using the TaqMan® Gene Expression Assay system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions, using validated probes human MT1 (no. 4351182) and eucaryotic 18S endogenous control (no. 4319413E).

#### Western blotting

Confluent cells were lysed in 50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, pH 7.4, containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein was quantified by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Equal amounts of protein (10–20 μg) were separated by 12% SDS–PAGE and transferred onto PVDF membranes (Amersham Biosciences, Oakville, ON, Canada), which were probed for MT1 (1:1000, Ab815; Chemicon International Inc., Temecula, CA, USA), E-cadherin (1:1000, Chemicon), N-cadherin (1:2500, Signal Transduction Laboratories, Lexington, KY, USA) or β-tubulin (1:2000, T5168, Sigma-Aldrich). Secondary HRP-coupled antibodies (Amersham, Oakville, ON, Canada) were diluted 1:3000. Immunoreactive proteins were visualised using ECL Western Blotting Detection Reagents (Amersham Biosciences).

#### Gelatin zymography

Capacity for pro-MMP-2 activation was evaluated as a means of assessing MT1 activity. This assay was performed using cells seeded on 3D collagen I gels as is necessary for stimulation of this event (Ellerbrook et al., 1999; Ellerbrook et al., 2001; Zigrino et al., 2001). Vitrogen™ (eight volumes of 3 mg ml⁻¹) was diluted with 1 volume of 10× zMEM and one volume of 0.1 M NaOH, and the neutralised collagen I solution was polymerised at 37°C for 1 h (100 μl well⁻¹; 48-well plate). Cells (1×10⁴) were seeded atop the collagen I gels in 300 μl media that had been pre-conditioned for 24 h by human gingival fibroblasts (HGF), in order to provide an exogenous source of pro-MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2). Additional aliquots of cells were seeded into wells for later quantification, to verify equal cell numbers had been present during the assay, allowing a meaningful comparison of pro-MMP-2 activation in the conditioned media between the ovarian cancer cell lines. Following a 36-h incubation, media was collected, diluted in non-reducing sample buffer, and 20 μl aliquots were separated by 12% SDS–PAGE using gels co-polymerised with 0.016% gelatin (Sigma-Aldrich). Gels were washed twice for 10 min in 2.5% Triton X-100, rinsed in dH₂O, and incubated in enzyme activation buffer (50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, 5 mM CaCl₂, and 0.166% Brij 35) for 24–48 h at room temperature. Coomassie blue (0.5%) staining revealed proteins with gelatinolytic activity (gelatin clearance).
Transwell collagen invasion

Experiments were conducted in 8 µm transwell chambers (Costar, Corning Inc., Corning, NY, USA). Vitrogen™ (Cohesion, Palo Alto, CA, USA) was diluted in ice-cold PBS and neutralised with 0.01 N NaOH for a final concentration of 0.2 µg µl⁻¹, of which 100 µl was applied to each transwell membrane, polymerised by incubation at 37°C for 1 h, and dried to form a compact collagen I film on the membrane. The coated membranes were rinsed with PBS and equilibrated in serum-free α-MEM using pre-warmed solutions. Cells were resuspended in α-MEM containing 1% FBS, and 1 x 10⁵ cells in 100 µl were seeded into the upper wells. α-MEM (500 µl) supplemented with 10% FBS was used as a chemoattractant in the lower well. Where applicable, the MMP inhibitor GM6001 (Chemicon) was added at 25 µM, and TIMP-1 and TIMP-2 (obtained from Dr Chris Overall, University of British Columbia, Vancouver, BC, Canada) were used at 2.5 µg/ml. SB-3CT (MMP2/MMP-9 inhibitor IV, Chemicon), which selectively inhibits the gelatinases MMP-2 and MMP-9 (Kleifeld et al, 2001) was used at 6 µM. At the time of harvest (24–72 h), invaded cells were released from the bottom of the transwell insert using 200 µl of 0.08% trypsin (in a 24-well plate), collected by centrifugation, and invaded cells were quantified based on nucleic acid content using CyQUANT™ (Molecular Probes Inc. Eugene, OR, USA) according to the manufacturer’s instructions. Invasion experiments were performed three times, with data normalised to HEY cell invasion levels and combined for the three experiments.

Collagen I degradation

Chamber slides were coated with Vitrogen™ that had been neutralised with 0.01 M NaOH and diluted in PBS such that 200 µl containing 20 µg collagen I were added to each well. The collagen was subsequently polymerised at 37°C for 1 h and dried down overnight to create compact collagen I films. Transwells were coated with collagen as described above for transwell invasion. The collagen matrices were biotinylated under sterile conditions, using 20 µg/ml EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce, Rockford, IL, USA) in 50 mM sodium bicarbonate pH8.3 for 2 h, quenched using 50 mM Tris, pH7.5, and washed before addition of cells. For examination of pericellular collagen degradation, cells were seeded into chamber slide wells at low density (1 x 10⁵ cells well⁻¹) in α-MEM containing 5% FBS. To assess collagen removal from transwell pores, cells were seeded in transwell chambers as described above for transwell invasion. Following incubation for 18–72 h, matrix degradation experiments were terminated by fixation in 4% paraformaldehyde, permeabilised with 0.1% Triton X-100/PBS, blocked in 2% BSA/PBS, and stained with streptavidin Alexafluor-488 and rhodamine-phalloidin (Molecular Probes Inc.), each diluted 1:200 in 2% BSA/PBS, then visualised by confocal laser scanning microscopy (LSM 510, Carl Zeiss Inc., Toronto, ON, Canada).

Ectopic MT1 expression

Membrane-type I expression constructs encoding wild-type flag-tagged MT1 (MT1f) and catalytically inactive MT1 (MT1f-E240A) (Tam et al, 2002), generated by the lab of Dr M Sharon Stack (Wu et al, 2004), were obtained from Dr C Overall. Cells were transfected using Fugene-HD (Roche Applied Science, Laval, PQ, Canada) according to the manufacturer’s instructions. Additional control cells were transfected with pEGFP (Clontech, Mountain View, CA, USA) or left untransfected. Cells were seeded for migration, collagen degradation, or invasion assays 24–36 h post-transfection.

Cell migration

Cell migration on collagen was assessed using transwell membranes coated with unpolimerised collagen I (Vitrogen™) as described for the invasion assay, except the collagen I was neither neutralised nor incubated at 37°C before being dried onto the membrane. Cell seeding, application of chemoattractant, and quantification of migrated cells were performed as described for the transwell invasion assay, and experiments were terminated at 8–10 h. As with the invasion assays, the migration assays were initiated 24–48 h post-transfection (where applicable) to allow for cell recovery and ectopic expression of MT1. Migration experiments were repeated three times.

RESULTS

Characterisation of MT1 expression and activity

Four of the six ovarian cancer cell lines examined, HEY, OVCA429, ES-2, and HOCC-7, expressed MT1 as determined both by real-time PCR (Figure 1A) and Western blotting (Figure 1B). HEY cells expressed the highest levels of MT1, whereas expression was not detected in either the SKOV-3 or OVCAR-3 cells. To further...
confirm the expression of MT1, a well-established assay that is based on the capacity of MT1 to cleave and activate pro-MMP-2 was performed (Ellerbroek et al, 1999; Ellerbroek et al, 2001; Zigrino et al, 2001). The activation of pro-MMP-2 involves binding of a TIMP-2/pro-MMP-2 dimer to MT1, presenting the pro-MMP-2 to a second MT1 molecule, which cleaves the pro-domain from MMP-2 (Strongin et al, 1995). The pro-MMP-2 activation results paralleled the MT1 expression pattern; only the cell lines that expressed MT1 (HEY, OVCA429, ES-2, and HOC-7) were able to cleave pro-MMP-2, with HEY demonstrating the most extensive activity (Figure 1C).

Transwell collagen I invasion and matrix degradation correlate with MT1 expression

The relationship between MT1 expression and invasive capacity of ovarian cancer cell lines was assessed using a transwell collagen invasion assay. HEY cells had extensively penetrated the collagen I barrier by 24 h (Figure 2A). In comparison, OVCA429 and ES-2 cells required twice as long to accomplish moderate levels of invasion, whereas HOC-7 cells rarely invaded. SKOV-3 and OVCAR-3 cells, which do not express MT1, did not invade, even when incubated for up to 5 days (data not shown). Invasion was abolished (>95%) by the presence of GM6001 in the culture medium (Figure 2A), with similar effects obtained when OVCA429 and ES-2 cells were used (data not shown). These studies demonstrate an essential role of MMP-mediated proteolysis for invasion of these collagen I matrices.

To examine the relationship between transwell collagen invasion and matrix degradation, the ability of cells to clear the collagen plugs within pores of collagen-coated transwell membranes was assessed. Only those cell lines expressing MT1 removed collagen from the transwell pores (Figure 2B), consistent with the transwell invasion results. HEY cells showed extensive clearing of collagen from the pores by 24 h, whereas OVCA429, ES-2, and HOC-7 cells required a longer incubation to accomplish collagen degradation. By 55 h, HEY cells had cleared essentially all pores, OVCA429 cells had cleared 40–50% of pores and ES-2 and HOC-7 cells had cleared only 10–20% of pores. In contrast, SKOV-3 and OVCAR3 cells were unable to clear the pores. Incubation of HEY cells with GM6001 prevented collagen clearing (Figure 2B), with similar results obtained for OVCA429 and ES-2 cells.

Cell lines expressing endogenous MT1 generally show higher motility on collagen I

As MT1 promotes the motility of several cancer cell types on various matrices including collagen (Gingras et al, 2001; Rozanov et al, 2001; Takino et al, 2004), we evaluated this potential association within our panel of ovarian cancer cell lines. For these experiments, we assessed the ability of the cells to transverse transwell membranes coated with collagen I that was left unpolymerised, to eliminate the prerequisite of proteolysis. SKOV-3 and OVCAR-3 cells were significantly less motile than the MT1-expressing cell lines. However, within the MT1-expressing cell lines, there was not a clear correlation between the level of MT1 expression and motility. HEY cells, which express the highest levels of MT1, exhibited the same level of motility as OVCA429 and ES-2 cells, which express much lower levels of MT1 (Figure 2C).

Figure 2 Collagen I transwell invasion, matrix degradation and motility of ovarian cancer cell lines. (A) Collagen I transwell invasion capacity. Results from three independent experiments were normalised to HEY and data were pooled. Bars represent the mean ± s.e. (nd – HEY invasion was not assessed beyond 24 h as invasion was complete by this time). (B) Clearance/degradation of collagen I plugs from transwell invasion membranes. Confocal fluorescence microscopy was used (×16) to visualise the biotinylated collagen. Representative areas near the centre of the membrane are shown and arrows highlight transwell pores that have been cleared of their collagen I plugs, except for HEY, in which all pores were cleared. Inset shows an enlarged image that distinguishes cleared pores (black) from uncleared pores (grey). (C) Transwell migration through unpolymerised collagen I-coated transwells at 8–10 h. Results from three independent experiments were normalised to HEY and data were pooled. Bars represent the mean ± s.e.
Inhibition of MT1 activity prevents invasion

The contribution of MT1 to invasion was assessed using a variety of MMP inhibitors. HEY cells were chosen for these experiments, as they consistently exhibited the most invasive behaviour. Invasion was abolished by the broad-range MMP inhibitor GM6001, indicating the essential role of MMP-mediated proteolysis in this process (Figure 3A). Tissue inhibitor of metalloproteinase-1, which does not affect MT1 activity (Will et al., 1996), but is an effective inhibitor of all secreted MMPs (Hornebeck et al., 2005), did not affect invasion. Although the selective inhibition of the gelatinases, MMP-2 and MMP-9 with SB-3CT (Kleinfeld et al., 2001) resulted in a 20% reduction in invasion, this decrease was modest in comparison to the reduction attained with TIMP-2, which prevented invasion as effectively as GM6001 (>90% inhibition). In contrast to SB-3CT and TIMP-1, GM6001 and TIMP-2 are potent inhibitors of MT1 (Will et al., 1996). Collectively, these results indicate a critical requirement of MT1, and not other MMPs, for collagen I invasion.

Membrane-type 1 expression activates invasive behaviour by non-invasive cells

As our experiments indicated that inhibition of MT1 activity prevented invasive behaviour, we next examined whether MT1 was a limiting factor for ovarian cancer cell invasion. Both the non-invasive SKOV-3 and OVCAR-3 cells and the moderately invasive OVCA429 and ES-2 cell lines were transfected with either wild-type MT1 (MT1f) or catalytically inactive MT1 (E24A) expression constructs, and subjected to a transwell invasion assay. Comparably high levels of expression were achieved with both constructs, at levels vastly exceeding endogenous MT1 expression by the invasive OVCA429 and ES-2 cell lines (Figure 3C). Transfection efficiency exceeded 50% for each of the four cell lines transfected, as shown using EGFP expression (Figure 3D). Membrane-type 1 overexpression by the moderately invasive OVCA429 and ES-2 cells caused a marked acceleration in their invasion. Moreover, ectopic expression of MT1 in the non-invasive SKOV-3 and OVCAR-3 cell lines activated an invasive phenotype (Figure 3B). Although MT1 overexpression allowed all cell lines to penetrate the collagen barrier within 24 h, the extent of invasion was correlated with motility. As SKOV-3 and OVCAR-3 cells were less motile, a longer incubation (48 h) was required to achieve extensive invasion by the MT1-transfected cells (Figure 3B). In contrast to MT1, overexpression of the E240A mutant was without effect, indicating the requirement for MT1 catalytic activity in the enhanced invasion.

Membrane-type 1 promotes ovarian cancer cell invasion solely through enhanced matrix degradation and not cell motility

Cell invasion can be partitioned into matrix degradation and cell motility. As MT1 has been implicated in both, we examined the impact of its overexpression on these processes independently to determine the mechanism through which MT1 promoted invasion. SKOV-3 and OVCAR-3 cells were transfected as described in the previous experiment. To assess matrix degradation, cells were seeded in chamber slides pre-coated with polymerised collagen that was biotinylated to allow visualisation. After 24 h, cells and matrices were fixed and analysed by confocal microscopy. Overexpression of MT1 resulted in a striking enhancement of collagen degradation, as shown by the cleared areas in Figure 4A. In comparison, non-transfected cells, and cells transfected with E240A, did not exhibit collagen clearance. Similar results were obtained when OVCA429 and ES-2 cells were transfected with these constructs (data not shown).

To determine whether MT1 also promoted the migration of ovarian cancer cells, as demonstrated in other cancer cell types
MT1 promotes ovarian cancer cell invasion

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Figure 4 MT1 enhances invasion solely through its promotion of matrix degradation; not by affecting cell motility. (A) Degradation of biotinylated collagen I matrices by MT1-transfected SKOV-3 and OVCAR-3 cells after a 24 h incubation. Matrices were not degraded by cells expressing the E240A catalytically inactive MT1 mutant, nor by control untransfected (UT) cells. Cells were visualised via rhodamine phalloidin staining of actin (red) and biotinylated collagen matrices were visualised using streptavidin AF488 (green), using confocal microscopy (× 40). (B) Overexpression of MT1 or E240A did not affect cell motility on collagen. Cells were seeded in transwell chambers pre-coated with 20 µg unpolymerised collagen and allowed to migrate for 8–10 h. Results shown are the mean ± s.e. for three independent experiments.

Figure 5 Expression of E- and N-cadherin by ovarian cancer cell lines. (A) RT–PCR for E-cadherin. RPL13a amplification verified equal amounts of cDNA template. (B) Western blot analysis for E- and N-cadherin. Blots were reprobed with β-tubulin to verify equal loading.

Invasive capacity does not relate to E-cadherin expression

E-cadherin is considered to be a tumour suppressor, as its expression is associated with increased cell–cell adhesion and decreased motility. The inability of SKOV-3 and OVCAR-3 cells to invade 3-D collagen gels may relate to their E-cadherin expression (Kokenyesi et al, 2003). That MT1 expression is upregulated through β-catenin signalling may explain the reported inverse correlation between E-cadherin and MT1 expression (Ara et al, 2000; Takahashi et al, 2002; Nawrocki-Raby et al, 2003a, b; Hazan et al, 2004; Hlubek et al, 2004; Munshi and Stack, 2006). Therefore, we analysed cadherin expression to determine whether E-cadherin was associated with the absence of MT1 and cell invasion in SKOV-3 and OVCAR-3 cells. The expression of N-cadherin was also assessed, as this cadherin sub-type has been associated with motile carcinoma cells that have undergone epithelial-mesenchymal transition (Derycke and Bracke, 2004; Hazan et al, 2004).

Both RT–PCR and Western blot analysis of cell lysates revealed that E-cadherin expression was restricted to HOC-7 cells, whereas the other five cell lines expressed N-cadherin (Figure 5A). These findings demonstrate that E-cadherin expression is not a factor contributing to the inability of the SKOV-3 and OVCAR-3 cell lines to invade and migrate on collagen. However, E-cadherin expression may explain the lower motility and invasiveness of the MT1-expressing HOC-7 cells relative to the other invasive MT1-expressing cells.

DISCUSSION

Our results establish a critical role for MT1 in ovarian cancer cell invasion and are the first to reveal its expression in a panel of cancer cell lines exhibiting different capacities for invasion. Endogenous MT1 expression and activity were positively correlated with invasiveness in the six ovarian cancer cell lines examined. Importantly, the levels of invasion indicated by our in vitro assays mirrored the reported abilities of the cell lines to form peritoneal tumours in vivo (Shaw et al, 2004). HEY, OVC429, and ES-2 cell lines expressed the highest levels of MT1, and were consistently shown to be the most invasive in the in vitro assays used. These three cell lines also reproducibly formed invasive intraperitoneal tumours in mice (Shaw et al, 2004). Conversely, we found SKOV-3 and OVCAR-3 cells did not express MT1 and were incapable of invading polymerised collagen I matrices in vitro. Consistent with these results, SKOV-3 and OVCAR-3 seldom

(Gingras et al, 2001; Rozanov et al, 2001; Cao et al, 2004; Takino et al, 2004), the transfected cells were subjected to a transwell collagen migration assay. In contrast to its striking effect on collagen degradation, MT1 expression did not enhance cell migration (Figure 4B). Consistent with these observations, treatment of the MT1-expressing cells (HEY, 429, and ES-2) with GM6001 did not alter their motility (data not shown). Together, these data demonstrate that ovarian cancer cell motility is not promoted by MT1 proteolytic activity or expression.
translating therapeutic potential of MT1-1 mRNA and protein, pro-MMP-2 cleavage, and collagen I degradation generally suggests that potential variations in events required for MT1 activation and invasion by DOV-13 cells (Ellerbroek et al, 2001). Thus, although the cell lines that express endogenous MT1 are more motile than those that lack MT1 expression, this relationship is correlative and not causal; MT1 does not appear to contribute to their superior motility. Rather, MT1 is likely regulated in parallel with the pathways that promote a motile phenotype.

Although cell motility was not altered by manipulation of MT1 expression or activity, migratory capacity is an important factor in determining the extent of invasion. The importance of migratory capacity in ovarian cancer metastasis is perhaps best illustrated by the fact that therapeutic strategies aimed at preventing tumour cell motility reduce peritoneal metastasis of ovarian cancer cells (Hashimoto et al, 2005). Motility and invasion of numerous cancer cell types is prevented by expression of the tumour-suppressor E-cadherin. Through its sequestration of the transcriptional co-activator β-catenin, E-cadherin inhibits Tcf/LEF transcriptional activation of numerous targets that contribute to a motile, invasive phenotype, including MT1 expression (Ara et al, 2000; Takahashi et al, 2002; Nawrocki-Raby et al, 2007).
The pattern of E-cadherin expression in ovarian cancer appears to be distinct from that of most other epithelial cancers. Primary ovarian carcinomas have upregulated E-cadherin and are more epithelial in character than the N-cadherin-expressing ovarian surface epithelium (mesothelium) from which they are presumed to arise. Subsequently, E-cadherin expression decreases and correlates with progression to metastatic disease, in a manner similar to other carcinomas (Auersperg et al, 2002; Yuecheng et al, 2006) indicating ovarian cancer metastasis is likely promoted by the de-differentiation that accompanies E-cadherin downregulation. It has been suggested that the inability of the SKOV-3 and OVCAR-3 ovarian cancer cell lines to invade collagen I may be due to their E-cadherin expression (Kokenyesi et al, 2003). However, our studies clearly show that of the cells used in the present study, only HOC-7 expressed E-cadherin, whereas the other cell lines expressed N-cadherin. The idea proposed by Kokenyesi et al, (2003) is consistent with the behaviour of the HOC-7 cells, which have an epithelial morphology, grow in tight clusters, and migrate as a sheet in wound-healing assays (K Sodek, unpublished data) (Artymiuk et al, 2005) and suggests that a high degree of cell–cell adhesion and consequent migratory activity is needed for the invasion of collagen I. Furthermore, a comparison of the cell lines used in our study and those used by Kokenyesi et al (2003) suggests that the ability of the cell lines to invade collagen I may be due to differences in E-cadherin expression and function.

Expression of MT1 by cancer cells in ovarian tumours has been associated with poor patient survival (Davidson et al, 1999; Sakata et al, 2000; Davidson et al, 2002). As with other cancers, MMP inhibitors have yielded disappointing results in ovarian cancer clinical trials, likely because they have been administered at late stages of disease and are broad-range, inhibiting MMPs that have anti-tumorigenic/anti-angiogenic activities (Overall and Lopez-Otin, 2002). Alternatively, it has been recently reported that the inhibitor concentrations required to inhibit MMP catalytic activity are far above the physiological levels achieved (Hotary et al, 2006). Furthermore, the MMP inhibitors marimastat and tanomastat, which have been administered in ovarian cancer trials, are selective for MMPs other than MT1 (Hidalgo and Eckhardt, 2001). The development of specific inhibitors of MT1 has proven a difficult task, as its structure is similar to other MMPs yet, more restrictive, thus inhibitors that bind MT1 also tend to bind other MMPs, especially the gelatinases (Yamamoto et al, 1998). However, the further development of a new class of peptidomimetic mercaptosulphide MMP inhibitors that have high selectivity for MT1 and the gelatinases holds promise for design of a specific MT1 inhibitor (Hurst et al, 2005; Fisher and Mobashery, 2006). An additional attractive approach is to establish and target the pathways that govern MT1 expression.

As cell motility is clearly related to the extent of invasion of ovarian cancer cells, it is important to determine factors synchronising this aspect of invasive behaviour with matrix degradation. The combination of gene expression profiling, proteomics and bioinformatical approaches as applied to these cell lines are necessary to better understand potential pathways through which MT1 expression and cell motility are regulated, and are likely to reveal new therapeutic targets for ovarian cancer.

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