Expression of matrix metalloproteinase-26 and tissue inhibitor of metalloproteinase-4 in human normal cytotrophoblast cells and a choriocarcinoma cell line, JEG-3

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The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) is critical for embryo implantation. Disturbance of this balance may lead to tumour metastasis. To understand the roles of MMP-26 and TIMP-4 in physiological and pathological invasion, the expression of these proteins in normal human cytotrophoblast cells and in a malignant choriocarcinoma cell line, JEG-3, was investigated. MMP-26 and TIMP-4 proteins were detected in the cytoplasm of these cells. The expression levels of MMP-26 mRNA and protein in JEG-3 cells were significantly higher than those in the cytotrophoblasts; conversely, the expression levels of TIMP-4 mRNA and protein were much lower in JEG-3 cells than those in cytotrophoblasts (P < 0.01). Enzyme inhibition studies demonstrated that TIMP-4 was a potent inhibitor of MMP-26 with an IC₅₀ value of 0.4 nmol/l. This study confirms that MMP-26 is an epithelial enzyme and suggests that MMP-26 and TIMP-4 may play a role in tissue-remodelling processes associated with placentation and tumour progression, and that a higher MMP-26 to TIMP-4 ratio may promote cancer invasion.

Key words: choriocarcinoma JEG-3/matrix metalloproteinases/MMP-26/normal cytotrophoblasts/TIMP-4

Introduction

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that may participate in many physiological and pathological processes. Because of their ability to dissolve connective tissue components such as collagens, laminins, fibronectin and proteoglycans, MMPs may be one of the most important classes of molecules used by invading cells to facilitate invasive growth and spread (Sang et al., 1996; McCawley et al., 2000). The activated MMPs can be inhibited by tissue inhibitors of metalloproteinase (TIMPs). The activation and inhibition of MMPs is well regulated under normal physiological conditions (Douglas et al., 1997). However, in a number of pathological situations the loss of strict regulatory mechanisms may lead to tumour metastasis (Murray et al., 1999).

Implantation of the embryo is a little understood aspect of reproductive biology. There are striking similarities between the behaviour of invasive placental cells and that of invasive cancer cells (Fisher et al., 1993; Murray et al., 1999). The development of the placenta requires a series of complex, co-ordinated interactions between fetal-derived trophoblast cells and the maternal uterus (Benirschke et al., 1995). The invasion of trophoblast cells into the maternal endometrium is one of the key events in human placentation. The invasive mechanisms of cytotrophoblasts have been investigated for many years. Matrix-degrading enzymes such as MMPs may help cytotrophoblasts to cross basement membranes and invade. Implantation requires co-operation between the developing embryo and maternal tissue and the invasion of the cytotrophoblasts is highly controlled. In contrast, tumour progression and metastasis are frequently uncontrolled and occur without the help or restraint of the host tissue into which the tumour invades. The key to understanding the invasive cascade is the relationship between the extracellular matrix (ECM) and the ECM receptors (integrins) and ECM-degrading enzymes including MMPs and their inhibitors (TIMPs) (Nagase et al., 1999). The balance between the elements is critical for maintaining health and is frequently perturbed in a number of disease states. The loss of strict regulation of trophoblast invasion leads to choriocarcinoma. In fact, there is ample evidence that MMPs are important mediators of tumour cell invasion (Macdougall et al., 1995).

MMPs and TIMPs therefore play an important role in tissue remodelling processes such as embryo implantation, tumour invasion and tumour metastasis. To date 23 different MMPs and four kinds of TIMPs have been cloned. MMP-26, MMP-28 and TIMP-4 have been recently identified and cloned (Greene et al., 1996; de Coignac et al., 2000; Park et al., 2000; Uria et al., 2000; Lohi et al., 2001; Marchenko et al., 2001). MMP-26 (endometase or matrilysin-2) was cloned from fetal cDNA, human endometrial tumour cDNA library and human placenta cDNA. MMP-26 is 43, 41, 41 and 39% identical to human metalloelastase, stromelysin, collagenase-3 and matrilysin respectively (Park et al., 2000). A study of MMP-26 mRNA steady state levels reveals, among the tissue examined, a specific expression in human
placenta and uterus. It is also widely expressed in malignant tumours from different sources, as well as in diverse tumour cell lines. This protein hydrolyzes type IV collagen, fibronectin, fibrinogen and gelatin, indicating that MMP-26 is a potent enzyme with a wide substrate specificity. MMP-26 is also able to activate progelatinase B. These data suggest that MMP-26 may play a role in some of the tissue-remodelling events associated with embryo implantation and tumour progression. In addition, a previous study has shown that over-expression of TIMP-4 in human breast cancer cells inhibits invasion in vitro and tumour growth and metastasis in vivo (Greene et al., 1996). However, detailed function and expression patterns of MMP-26 and TIMP-4 in normal placenta and choriocarcinoma have not been reported. The aim of the present study is to determine if MMP-26 and TIMP-4 may be responsible for the invasive behaviour of human normal cytotrophoblasts and choriocarcinoma cells. Therefore, we studied the expression patterns of MMP-26 and TIMP-4 in cultured normal human cytotrophoblast cells and in a choriocarcinoma cell line, JEG-3. This study may help with future investigations of the roles of MMP-26 and TIMP-4 in human normal pregnancy and pregnancy disease.

Materials and methods

Indirect immunohistochemistry

Human choric onc villi tissues were obtained from patients undergoing therapeutic termination of pregnancy at early gestation. Informed consent was provided by the patients and the study was approved by the local ethics committee. Week 6 human placental villi were cut into pieces. Frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides and stored at −80°C until use. The slides with villi sections were placed at room temperature for 15 min, then fixed for 30 min in freshly prepared 4% paraformaldehyde (PFA; Sigma, Deisenhofen, Germany) containing 0.2% Triton X-100. After rinsing several times in 0.01 mol/l phosphate-buffered saline (PBS; pH 7.4), the slides were incubated in 5% bovine serum albumin (BSA) to block non-specific antibodies for 45 min at room temperature. The BSA solution was then aspirated with filter paper, and the slides were incubated with primary antibody against MMP-26 or TIMP-4 (Li et al., 1997; Park et al., 2000; Hurst et al., 2001) diluted 1:100 in PBS at 4°C overnight. After rinsing in PBS, sections were incubated with biotin-conjugated secondary mouse anti-rabbit antibodies (Dako, Hamburg, Germany). After another rinse in PBS, sections were incubated with alkaline phosphatase streptavidin IgG. Colorimetric detection was then performed by a standard, indirect streptavidin–biotin immunoreaction method with the Dako universal LASB kit according to the manufacturer’s instructions. Parallel experiments were performed using rabbit preimmune IgG as a negative control.

In-situ hybridization

In-situ hybridization was performed as previously described (Wang et al., 2001). Briefly, slides with villi sections (10 μm) were treated with proteinase K, prehybridized and hybridized overnight with digoxigenin-labelled antisense transcripts from a MMP-26 (Hurst et al., 2001) or TIMP-4 cDNA insert (Leco et al., 1997). The TIMP-4 antisense probe is a 600 bp fragment from nucleic acid 13–612. The MMP-26 antisense probe is a 233 bp fragment from nucleic acid 97–329. After hybridization, RNase treatment and three stringent washes were performed. Sections were incubated with mouse anti-digoxigenin antibodies (Boehringer) followed by incubation with biotin-conjugated secondary mouse anti-rabbit antibodies (Dako). The colorimetric detection was performed by a standard, indirect streptavidin–biotin immunoreaction method with the Dako universal LSAB kit. Parallel experiments were performed using sense probes as a negative control.

Cell line and cell culture

The choriocarcinoma cell line JEG-3 was a generous gift from Dr Piao, Yunshang and Dr Wang, Yanling (State Key Laboratory of Reproductive Biology, Institute of Zoology, CAS, Beijing). JEG-3 cells were plated at 1–2×10^5 cells with 1 ml serum-free FD (Ham’s F-12/DMEM; Gibco BRL, Gaithersburg, MD, USA) medium supplemented with 2 mmol/l sodium pyruvate and 2 mmol/l glutamine in fibronectin (Gibco)-coated 24-well dishes (Vinod et al., 2000).

The human cytotrophoblast cells were isolated and maintained as previously described (Li et al., 1991, 1996; Xu et al., 2000). Briefly, human choriocarcin villi tissues were obtained from patients who underwent therapeutic termination of pregnancy at early gestation. Informed consent was provided by the patients and the project was approved by the local ethics committee. The time of gestation was defined according to the first day of the last menstrual period and further examined morphologically by means of a stereomicroscope. Tissues were minced separately and digested with 0.25% Trypsin (Sigma) and 15 IU/ml DNase I (Sigma) at 4°C for 1 h. Trypsinization was stopped by addition of two volumes of FD medium (1:1; Gibco). After washing, the dispersed cells were filtered through a nylon sieve to remove the gross villous core residues. The filtered cell suspension (1–2 ml) was then added slowly to the top of a BSA gradient (prepared by sequential addition of 3 ml of 3, 2 and 1% BSA in FD medium to a 15 ml centrifuge tube). The cells were sedimented for 1 h at unit gravity, and the cytotrophoblast cells were collected from the bottom of the tube. The purified cytotrophoblast cells were plated at 1–2×10^5 cells with 1 ml of serum-free medium supplemented with 1 ng/ml epidermal growth factor, 10 μg/ml insulin, 0.1% BSA, 1.75 mmol/l HEPES and 2 mmol/l glutamine in fibronectin-coated 24-well dishes. The cells began to attach within 2 h of plating. They spread and showed a monolayer epithelial cell morphology after 24 h under serum-free culture conditions. Immunocytochemistry studies revealed that >98% of the cells exhibited positive staining for cytokeratin and GnRH and were vimentin negative, consistent with their identification as cytotrophoblast cells.

Immunocytochemistry

Polyclonal and monoclonal antibodies against MMP-26 and TIMP-4 were produced and characterized as described in our previous reports (Park et al., 2000; Hurst et al., 2001). The final concentrations of the purified IgG used were 1–10 μg/ml. Cytotrophoblast cells were cultured in chamber slides (Nunc, Roskilde, Denmark). After 48 h in culture, cytotrophoblast cells were fixed for 30 min in freshly prepared 4% PFA (Sigma) containing 0.2% Triton X-100. After rinsing several times in 0.01 mol/l PBS (pH 7.4), the slides were incubated in 5% BSA to block non-specific reactions for 45 min at room temperature. Then the BSA solution was aspirated with filter paper, and the slides were incubated with the primary antibody against MMP-26 or TIMP-4 diluted 1:100 in PBS at 4°C overnight. After rinsing in PBS, the slides were incubated with biotin-conjugated secondary mouse anti-rabbit antibodies (Dako, Hamburg, Germany). After another rinse in PBS, sections were incubated with alkaline phosphatase streptavidin IgG. Colorimetric detection was then performed by a standard, indirect streptavidin–biotin immunoreaction method with the Dako universal LASB kit according to the manufacturer’s instructions. Parallel experiments were performed using rabbit preimmune IgG as a negative control. Similar experiments were performed using the JEG-3 cells.

Gelatin zymography

The presence of gelatinolytic MMPs in media was detected by gelatin zymography. The harvested culture media were standardized according to the protein content of cell lysates, which was measured according to a previously described method (Bradford, 1976). Thus, 10–20 μl medium, equivalent to 6 μg protein of cell lysates, was loaded to each lane for zymography. The medium was mixed with 5:1 (v/v) with sample buffer and then applied to gels for electrophoresis without boiling under non-reducing conditions in 15% acrylamide gel co-polymerized with 1 mg/ml gelatin (Sigma). After electrophoresis, the gels were washed at room temperature for 1 h in 2.5% Triton X-100, 50 mmol/l Tris–HCl at pH 7.5, to remove sodium dodecyl sulphate (SDS) and incubated for 2–3 days in buffer (150 mmol/l NaCl, 5 mmol/l CaCl2 and 50 mmol/l Tris–HCl, pH 7.6) at 37°C. Thereafter, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) isopropanol alcohol, 10% glacial acetic acid for 60 min and destained in 10% (v/v) methanol, 5% (v/v) glacial acetic acid. The gelatinolytic activities were detected as clear bands on a uniform blue background.

Western blot analysis

Proteins obtained from cell lysates were boiled in SDSβ-mercaptoethanol sample buffer and ~10 μg samples were loaded onto each lane of the 12%
MMP-26 and TIMP-4 in cytotrophoblast and choriocarcinoma

Figure 1. TIMP-4 (A) and MMP-26 (B) immunostaining in the placental villi (blue stain). White arrows indicate syncytiotrophoblasts (STR). Black arrows indicate cytotrophoblasts (CTR). The red arrow indicates a vessel. Cells labelled with blue indicate TIMP-4 or MMP-26 gene expression. Positive TIMP-4 and MMP-26 protein expression occurred in the STR and CTR. No detectable background staining was observed under the same conditions for the preimmune rabbit IgG. Original magnification ×40.

Figure 2. In-situ hybridization analysis of TIMP-4 (C) and MMP-26 (D) expression in the placental villi (brown). White arrows indicate syncytiotrophoblasts (STR). Black arrows indicate cytotrophoblasts (CTR). Cells labelled with brown indicate TIMP-4 or MMP-26 gene expression. Positive TIMP-4 and MMP-26 gene expression occurred in the STR and CTR. No detectable background staining was observed under the same condition for the sense probe. Original magnification ×40.

Table I. Sequence and other information on the primers used for RT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Fragment size (bp)</th>
<th>Position on cDNA</th>
<th>Diagnostic restriction enzyme cut: fragments sizes produced (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-26</td>
<td>Sense</td>
<td>GGGACTTTGTTGAGGGCTAT</td>
<td>198</td>
<td>91–110</td>
<td>SphI: 139 and 59</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CTGCGGAGATGAGGTTT</td>
<td>271–288</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-4</td>
<td>Sense</td>
<td>CAAGCCAGTTCTCCTTC</td>
<td>310</td>
<td>777–794</td>
<td>PsiI: 24 and 286</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGACATTCGCCATTTTC</td>
<td>1069–1086</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

acrylamide–PAGE gels. The proteins were separated by electrophoresis and the proteins in the gels were blotted onto nylon membrane by electrophoretic transfer in 25 mmol/l Tris, 192 mmol/l glycine buffer, pH 8.3. Blots were blocked in 10% milk for 1 h. Primary antibodies against MMP-26 and TIMP-4 were diluted 1:2000 (to ~20 ng RNA). PCR cycles were as follows: 95°C for 30 s, 57°C for 1 min, and 72°C for 1.5 min. The MMP-26 and TIMP-4 PCR products and β-actin cDNA fragment (25 ng) (198, 310 and 564 bp respectively) were labelled using a random primer DNA labelling system (Gibco) in the presence of 32P-dCTP and purified using nick columns according to the manufacturer’s instructions.

**RT–PCR**

RNA isolated from cytotrophoblast cells or JEG-3 was subjected to RT–PCR. RNA was reverse transcribed by oligo(dT) priming and AMV reverse transcriptase (Promega Corporation, Madison, WI). PCR amplification was carried out with 5 µl of the RT product from cytotrophoblast cells or JEG-3 (~20 ng RNA). PCR cycles were as follows: 95°C for 5 min followed by 35 cycles of 94°C×30 min, 53°C×30 min and 72°C×30 min. PCR products (10 µl) were visualized under ultraviolet light on 1.5% agarose gels containing 1 µg/ml ethidium bromide. To confirm the amplification of the required cDNA sequence using the PCR primers and conditions described, PCR products were digested with a restriction enzyme (Table I) as directed by the manufacturer (Promega). Primer sequences, annealing temperatures, diagnostic restriction enzymes and the DNA fragment sizes produced are given in Table I.

**Northern blot analysis**

Cytotrophoblast cells or JEG-3 cultured for 48 h were homogenized into a TRizol solution and total RNA was isolated using methods described by Chomczynski and Sacchi (Chomczynski et al., 1987). Total RNA was quantified by absorbance at 260 nm and by ethidium bromide staining after electrophoresis of agarose gels. Samples of total RNA (10 µg) were transferred to Hybond-N membranes (Amer sham, Braunschweig, Germany) by capillary transfer as previously described (Sambrook et al., 1989). The total RNA was cross-linked to the membranes by UV irradiation (0.12 J.cm⁻²).

The MMP-26 and TIMP-4 PCR products and β-actin cDNA fragment (25 ng) (198, 310 and 564 bp respectively) were labelled using a random primer DNA labelling system (Gibco) in the presence of 32P-dCTP and purified using nick columns according to the manufacturer’s instructions. Membranes were incubated in prehybridization buffer (1 mol/l NaCl, 50 mmol/l Tris, 2.2 mmol/l sodium pyrophosphate, 10 g/l SDS, 1×Denhardt’s reagent, 10 mg/l denatured salmon sperm DNA, pH 7.5) for 3 h at 65°C. Labelled probe (25 ng, specific activity ~1 Ci/µg), denatured by placing in boiling water for 5 min and then snap-cooling on ice, was added and hybridization was carried out overnight at 65°C. After hybridization, the membranes were washed for 15 min twice in wash buffer 1 (1 g/l SDS, 2×SSC) then twice in wash buffer 2 (1 g/l SDS, 0.2×SSC) at 65°C. After autoradiography at ~70°C with X-ray film, probes were removed from the membranes as previously described (Sambrook et al., 1989).

The relative amounts of RNA loaded onto each lane and transferred to the
Figure 3. Expression of MMP-26 and TIMP-4 protein in JEG-3 cells cultured for 48 h. Slides with JEG-3 were labelled with FITC-conjugated secondary antibody and propidium iodide (PI) respectively, as Materials and methods [PI staining of the nucleus (red), immunolabelling of matrix proteins (green)]. The yellow colour represents the overlap of green and red. Positive staining for MMP-26 (a,b) and TIMP-4 (c,d) was observed. Samples labelled by rabbit preimmune IgG, instead of the primary antibody, served as a control (e). Original magnification 100×.

Figure 4. Immunofluorescence confocal micrographs of the production of MMP-26 and TIMP-4 by human cytotrophoblast cells cultured for 48 h. Cells were fixed and double-stained as described in Materials and methods [PI staining of the nucleus (red), immunolabelling of cellular proteins (green)]. The yellow colour represents the overlap of green and red. Positive staining for MMP-26 (a,b) and TIMP-4 (c,d) was observed. Samples labelled by rabbit preimmune IgG, instead of the primary antibody, served as a control (e). Original magnification 50×.

Figure 5. Western blots of MMP-26 (A) and TIMP-4 (B). Medium and placental villi were used as negative and positive controls respectively. Experimental conditions are as described under Materials and methods. Lanes: cytotrophoblast cells (CTB) and JEG-3 cells. Mean ± SEM (n = 3–4) fold increase in the ratio of protein expression (C).

membranes were determined by probing the blots with 32P-labelled cDNA for human β-actin signals and quantified by densitometry. The relative concentrations of the signals for the mRNAs on the autoradiograms were calculated as the ratio of mRNA signal to β-actin signal.

IC50 determination of human TIMP-4 against human MMP-26
The recombinant human MMP-26/endometase/matrilysin-2 protein was produced as described in our previous report (Park et al., 2000). The recombinant full length TIMP-4 protein was kindly provided by Professor Hideaki Nagase at the Kennedy Institute of Rheumatology, Imperial College School of Medicine, London, UK. The enzyme and the inhibitor were pre-incubated at 25°C for 5 h. The enzymatic assay was initiated by adding the fluorescence quenched peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (Bachem Bioscience Inc., USA). The final concentrations were: enzyme 3 nmol/l, substrate 1 µmol/l, inhibitor 0–2 nmol/l. The activity of MMP-26 in the absence of an inhibitor is the relative 100% activity. The TIMP-4 concentration at 50% MMP-26 activity is the value of the IC50. Fluorescent assays were performed at λexcitation = 328 nm and λemission = 393 nm using
Production of MMP-26 and TIMP-4 in cultured cells

Production of MMP-26 and TIMP-4 protein in cytотrophoblast cells and JEG-3 was also detected by Western blot (Figure 5). The cell lysate results showed that both cytотrophoblast cells and choriocarcinoma cells were able to produce MMP-26 and TIMP-4. The protein expression level of MMP-26 was significantly higher \((P < 0.01)\) in the JEG-3 cells than in the cytотrophoblast cells (Figure 5a,c), but the steady-state concentration of TIMP-4 protein was significantly lower \((P < 0.01)\) in JEG-3 cells than in cytотrophoblast (Figure 5b,c).

Gelatin zymography

Cytотrophoblast cells and JEG-3 were cultured for 48 h. Samples of the medium were subjected to zymographic analysis and two bands of gelatinolytic activity that had relative molecular masses of 29 and 25 kDa were detected (Figure 6a). These molecular masses correspond to the known sizes of pro-MMP-26 and MMP-26 respectively. The proteinase activities of MMP-26 were stronger in JEG-3 cells (Figure 6a,c) than in cytотrophoblast cells \((P < 0.01)\). Furthermore, the gelatinolytic activity of MMP-26 was relatively weak because MMP-26 is reported to be a bad enzyme for degrading gelatin. Gels incubated in the presence of EDTA (Figure 6b) showed no bands of gelatinolytic activity, demonstrating that all the gelatinolytic activities were those of metalloproteinases.

MMP-26 and TIMP-4 RT–PCR

RNA was isolated from cytотrophoblast and JEG-3 cells. MMP-26 and TIMP-4 transcripts (198 and 310 bp respectively) were detected in RNA from the cytотrophoblast cells and JEG-3 by RT–PCR (Figure 7). These results were compatible with the expected sizes of DNA fragments and indicated the specificity and repeatability of this study. MMP-26 (Figure 7a) and TIMP-4 (Figure 7b) were expressed both in the cytотrophoblast cells and JEG-3 cells. The identity of all PCR products was confirmed by restriction digestion (data not shown).

mRNA encoding MMP-26 and TIMP-4 in cultured cells

Cytотrophoblast cells from human early placenta and JEG-3 cells were cultured for 48 h. mRNAs encoding MMP-26 and TIMP-4 were detected in RNA from the cytотrophoblast cells and JEG-3 by Northern blot analysis (Figure 8). Cytотrophoblast cells showed significantly less expression of MMP-26 mRNA \((P < 0.01)\) than did JEG-3 cells (Figure 8A,D), whereas cytотrophoblast cells showed significantly more expression of TIMP-4 mRNA \((P < 0.01)\) than did JEG-3 cells (Figure 8B,D).

IC\(_{50}\) determination of human TIMP-4 against human MMP-26

The inhibition kinetic studies of purified recombinant human TIMP-4 against recombinant human MMP-26 were performed as described in the Materials and methods and the results are shown in Figure 9. TIMP-4 is a slow and tight-binding inhibitor of MMP-26 with an IC\(_{50}\) value of 0.4 nmol/l.

Discussion

Effective fetal–maternal interactions during early placentation are critical for a successful pregnancy (Kikkawa, 1996). Optimal placental perfusion requires the controlled invasion of trophoblast cells deep
into the decidua to the spiral arteries. Trophoblast stem cells, also referred to as cytotrophoblast cells, reside in chorionic villi of two types, floating and anchoring villi. During early placentation, cytotrophoblast cells in the floating villi proliferate and differentiate by fusing to form the multinucleate syncytiotrophoblast layer. Cytotrophoblast cells in anchoring villi either fuse to form the syncytiotrophoblast layer, or break through the syncytiotum at selected sites and form multilayered columns of non-polarized extravillous trophoblast cells, which physically connect the embryo to the uterine wall. The extravillous trophoblast cells invade into the uterine wall as far as the first third of the myometrium and its associated spiral arteries, where they disrupt the endothelium and the smooth muscle layer and replace the vascular walls. The invasive activity of the extravillous trophoblast cells is at a maximum during the first trimester of gestation, peaking at ~10–12 weeks and declining thereafter. Insufficient invasion results in fetal intrauterine growth restriction, maternal hypertension and proteinuria. In contrast, unrestricted invasion is associated with premalignant conditions, such as invasive mole, and with malignant choriovitallia.

Invading trophoblastic cells undergo striking and rapid changes in cellular functions that are temporally and spatially regulated along the invasive pathway. Tumour invasion and trophoblastic invasion share the same biochemical mediators: the MMPs and their inhibitors. MMPs are a family of enzymes capable of digesting the extracellular matrices of the host tissues. Human cytotrophoblastic cells produce MMPs and are constitutively invasive. Tissue inhibitors of metalloproteinases inhibit cytotrophoblastic invasion in vitro, indicating that MMPs are causally related to trophoblast invasion in the endometrium. In contrast to tumour invasion of a host tissue, trophoblastic invasion during implantation and placentation is controlled stringently in both time and space. MMPs and their inhibitors may play a role in the pathogenesis of gestational trophoblastic diseases. Previous studies suggest that much less MMP-1 and MMP-2 is expressed by the extravillous trophoblasts of first-trimester placenta than by choriocarcinoma cells and extravillous trophoblast of partia. Furthermore, the expression of TIMP-1 is significantly less in choriocarcinoma than in the syncytiotrophoblast of normal placenta. The expression of MMP-9 in some cells is crucial for invasion and MMP-9 and TIMP-1 are more associated with the cancer phenotype than other types of MMP and TIMP (Miyagi et al., 1995; Yudate et al., 1996; Vegh et al., 1999; Bischof et al., 2000a,b).

In the current study, the expression levels of MMP-26 and TIMP-4 genes and proteins in human cytotrophoblast and JEG-3 cells were detected by immunochemistry, zymography, Western blot, RT–PCR and Northern blot. Our results showed that both human cytotrophoblast cells and a choriocarcinoma cell line, JEG-3, expressed MMP-26 and TIMP-4 at the transcriptional and translational levels. Importantly, both the expression level and proteolytic activity of MMP-26 were significantly less in human cytotrophoblast cells than in JEG-3. In contrast, the gene and protein expression levels of TIMP-4 were much higher in human cytotrophoblast cells than in JEG-3. In addition, the proteolytic activity of MMP-26 is much weaker than that of MMP-2 and -9 in cytotrophoblast cells by gelatin zymography. The differential expression patterns of MMP-26 and TIMP-4 in normal and malignant cells suggest that MMP-26 and TIMP-4 levels may contribute to both the strict control of the invasive process of cytotrophoblast cells and the uncontrolled invasive ability of choriocarcinoma cells. More specifically, the much higher protein expression ratio of MMP-26 to TIMP-4 in JEG-3 cells than in normal cytotrophoblasts indicates a possible mechanism for the uncontrolled invasive behaviour of malignant cells. Conversely, the lower ratio of MMP-26 to TIMP-4 allowed the limited and controlled invasion of normal cells.

The localization of MMP-26 and TIMP-4 in the same types of cells indicated a possible interaction between MMP-26 and TIMP-4 at the same location. We have reported the IC_{50} values of TIMP-4 against five major MMPs, MMP-1, -2, -3, -7 and -9, to be 19, 3, 45, 8 and 83 nmol/l respectively (Liu et al., 1997). Our current studies determined the IC_{50} value of TIMP-4 against MMP-26 to be 0.4 nmol/l, demonstrating that TIMP-4 is the most potent against MMP-26 compared with other MMPs tested. This result suggests that TIMP-4 is a physiological inhibitor of MMP-26 in cellular systems. Since TIMP-4 inhibits human breast cancer invasion (Liu et al., 1997), it may also inhibit cytotrophoblast and JEG-3 cell invasion.

Although MMP-26 can degrade ECM proteins by itself, it is not a very powerful enzyme in digesting ECM proteins. MMP-26 may not be the major direct invasive power during the invasive process. The most important role of MMP-26 in cell invasion may be played through activating pro-MMP-9 (Uria et al., 2000). MMP-26 may play...
Figure 9. IC50 determination of human TIMP-4 (I) against human MMP-26 (E). The enzyme and inhibitor were pre-incubated at 25°C for 5 h. The enzymatic assay was initiated by adding the fluorescence quenched peptide substrate (S) Meta-Pro-Leu-Gly-Leu-Arg-Ala-Arg-NH2. The final concentrations are: [E] = 3 mmol/l, [S] = 1 mmol/l, [I] = 0–2 nmol/l. The activity of MMP-26 in the absence of an inhibitor is the relative 100% activity. The TIMP-4 concentration at 50% MMP-26 activity is the value of the IC50. The IC50 determined was 0.4 nmol/l.

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