Human migrating extravillous trophoblasts express a cell surface peptidase, carboxypeptidase-M

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We previously reported that a cell-surface aminopeptidase, dipeptidyl peptidase IV, is expressed on extravillous trophoblasts (EVT) and suggested the involvement of its enzyme activity in EVT migration. In this study, we examined the expression of another cell-surface peptidase, carboxypeptidase-M (CP-M), at human embryo implantation sites, which catalyses biologically active peptides at extracellular sites. CP-M was immunohistochemically detected on syncytiotrophoblast, but not on cytotrophoblasts in floating chorionic villi (9–12 weeks of gestation). At villus-anchoring sites, CP-M was weakly detected on some EVT in the distal part of the cell column. CP-M was clearly expressed on EVT in the trophoblastic shells and in the maternal vessels. In the decidua, almost all interstitial trophoblasts expressed CP-M. Flow cytometry and RT–PCR showed that CP-M expression was induced on the outgrown EVT in primary villous explant culture. The CP-M induction on cultured EVT under 20% O₂ concentration was significantly higher than that under 1% O₂ concentration. In invasion assays, migration of JEG-3 cells, a CP-M-/endovascular extravillous trophoblast/hypoxia/invasion cell line, was significantly enhanced by an inhibitor of CP-M, DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA). These findings indicate that CP-M is a differentation-related molecule for human EVT and suggest that CP-M expression on EVT is partially regulated by tissue oxygen concentration.

Key words: carboxypeptidase M/endovascular extravillous trophoblast/hypoxia/invasion

Introduction

Trophoblast invasion is one of the most important steps of implantation and placentation. In the cell column, extravillous trophoblasts (EVT) are considered to transform to an invasive phenotype and invade the maternal decidual tissues from the distal part of the column. Although the mechanisms of their invasion resemble the events that occur during malignant tumour cell invasion (Aplin, 1991; Tabibzadeh and Babaknia, 1995), EVT invasion is confined spatially to the uterus and temporally to early pregnancy. EVT invasion was reportedly regulated by several molecules such as matrix metalloproteases (MMP) and serine proteinases, which degrade the extracellular matrix to make decidua preferable for EVT migration (Bischof et al., 1995). In addition, cell adhesion molecules including integrins were demonstrated to play an important role in the EVT invasion processes (Damsky et al., 1994).

Previously, we reported that membrane-bound peptidases, neutral endopeptidase (NEP)/CD10 and dipeptidyl peptidase IV (DPPIV)/CD26 were expressed in the chorion laeve which contains EVT (Imai et al., 1994). These peptidases are expressed on the cell surface and can degrade several biologically active peptides at extracellular sites. Kenny et al. (1989) speculated that cell-surface peptidases regulate growth and differentiation of many cellular systems by modulating the activity of peptide factors and their access to target cells. Recently, we found that DPPIV was expressed on EVT in the decidua early in pregnancy and that its enzyme activity was involved in EVT invasion (Sato et al., 2002).

In this study, we observed that carboxypeptidase-M (CP-M), another membrane-bound peptidase, was expressed on EVT early in pregnancy. CP-M is a zinc-dependent proteinase that catalyses the removal of carboxy-terminal basic amino acids, such as arginine and lysine, from peptides. Several biologically active peptides such as bradykinin and dynorphin A have been reported to be naturally occurring substrates for CP-M (Skidgel et al., 1989). The removal of carboxy-terminal arginine or lysine results in the modulation or inactivation of peptide hormone activity, and can also change the physical properties of the proteins (Skidgel, 1988). CP-M has been reported to be expressed in various organs such as syncytiotrophoblast in the placenta, lungs, kidney, intestine, brain, peripheral nerve and so on (Skidgel, 1988; Skidgel et al., 1989; Nagae et al., 1992). However, the physiological role of CP-M in these tissues has not yet been thoroughly clarified.

Previously, we reported that human luteal cells in the corpora lutea of both the menstrual cycle and early pregnancy express CP-M. CP-M was rapidly expressed on the cell surface of granulosa cells during the LH surge, suggesting the involvement of CP-M in the initial luteinization process of the granulosa cell differentiation (Yoshioka et al., 1998). In the present study, to investigate the role of this molecule in EVT differentiation, we examined the precise expression profiles of CP-M on EVT-lineage cells at embryo implantation sites by immunohistochemical study with samples obtained from women early in pregnancy. Using primary villous explant culture, we also investigated the effect of oxygen concentration on CP-M expression in EVT, which had been proposed to regulate EVT differentiation early in pregnancy (Genbacev et al., 1997, 2001; Caniggia et al., 2000). Furthermore, the role of CP-M in EVT invasion was explored by invasion assays using a human choriocarcinoma cell line, JEG-3, bearing CP-M.
Materials and methods

Reagents
Mouse anti-human CP-M monoclonal antibody (clone 1C2, IgG1) was purchased from Novocastra Laboratories Ltd (UK). The mouse anti-human cytokeratin 7 mAb (clone OV-TL12/30, IgG1) and the mouse anti-human vimentin mAb (clone V9, IgG1) were obtained from Dako (Denmark). The mouse anti-human melanoma cell adhesion molecule (MCAM/CD146 antigen, clone 5-E6, IgG1) was purchased from Alexis Biochemicals (USA). The fluorescein isothiocyanate (FITC)-conjugated mouse anti-human cytokeratin 8, 18 mAb (clone SD3, IgG1) was obtained from Ylem SL (Italy). The FITC-conjugated sheep anti-human von Willebrand factor polyclonal antibody was purchased from The Binding Site Ltd (UK). The FITC-conjugated sheep antibody raised against rat IgG was also purchased from The Binding Site Ltd and used for negative control staining. FITC-conjugated and non-conjugated mouse IgG1 negative control (clone DAK-GO1; Dako) were used for negative control staining. Murine anti-trinitrophenol (TNP) mAb (IgG1) was used as blocking mAb for double staining in immunohistochemistry (Tsujiura et al., 1990). The FITC-conjugated rabbit anti-mouse immunoglobulins (DakoTpat, Denmark) and rhodamine-conjugated goat anti-mouse immunoglobulins (Santa Cruz Biotechnology, Inc., USA) were used as secondary antibodies for immunohistochemistry or flow cytometry. An inhibitor of CP-M, di-mercaptopimethyl-3-guanidino-ethlytiopropanic acid (MGTA), was obtained from Calbiochem (USA).

Tissue samples
Tissue samples at implantation sites of human conceptus were obtained from five patients who underwent therapeutic hysterectomy for cervical intraepithelial neoplasia or uterine myoma during normal pregnancy at 9 (n = 3), 10 (n = 1) and 12 (n = 1) weeks of gestation. The gestational age was calculated from the date of the last menstrual period and, if necessary, was adjusted by ultrasonographic measurements of the gestational sac and/or fetal crown-rump length. For human chorionic villous explant culture, first trimester human placental tissues were obtained from 14 patients (6–8 weeks of gestation) who had undergone legal abortions. Informed consent for the use of these tissues in this study was obtained from all donors. Analysis of these samples was approved by the Ethical Committee of Kyoto University Hospital.

Immunohistochemistry
Immunofluorescence staining was performed as reported previously with slight modification (Sato et al., 2002). Each specimen was embedded in OCT compound (Tissue-Tek; Miles Inc., USA), snap-frozen in liquid nitrogen and stored at −80°C. Frozen tissues were sliced into 7 μm thick sections using a cryostat microtome (Cryocut 1800; Reichert-Jung, Germany), immediately air-dried on Neoplane (Nissin EM, Japan)-coated glass slides, and fixed in acetone at −20°C for 5 min. The slides were incubated with anti-human CP-M mAb (5 μg/ml) or negative control mAb (5 μg/ml) for 60 min at room temperature. Antibodies were diluted with Roswell Park Memorial Institute (RPMI) 1640 culture medium (Gibco BRL, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) and 0.1% NaN3. After washing in phosphate-buffered saline (PBS), the slides were incubated with rhodamine-conjugated goat anti-mouse immunoglobulins (diluted 1:40) for 30 min at room temperature in the dark.Slides were then incubated with anti-TNP mAb (20 μg/ml) to block the reactivity of goat anti-mouse secondary antibodies for 30 min. After washing, the slides were reacted with FITC-conjugated mouse anti-human cytokeratin 8, 18 mAb (10 μg/ml) or FITC-conjugated mouse negative control mAb (10 μg/ml), FITC-conjugated sheep anti-human von Willebrand factor antibody (100 μg/ml) or FITC-conjugated sheep anti-rat IgG immunoglobulin (100 μg/ml) for 30 min. The slides were mounted with an anti-fade agent (Perma Fluor Aqueous Mounting Medium; Immunon, USA) and then examined under a confocal laser scanning microscope (Carl Zeiss Inc., Germany).

To assess CP-M expression profiles on the EVT at implantation sites in normal pregnancy, several frozen sections derived from two to four individual sample blocks from each case were subjected to immunohistochemical examination. EVT were classified into six subtypes according to their locations such as: (i) proximal site of the cell column; (ii) distal site of the column; (iii) trophoblastic shell; (iv) interstitial trophoblasts in the shallow portion of the decidua; (v) interstitial trophoblasts in the deep portion of the decidua; (vi) endovascular trophoblasts. The staining intensity of CP-M expression was further classified into four grades (−, absence of staining; ±, weakly positive; +, moderately positive; ++, highly positive). To reduce bias in the visual assessment, the staining of syncytiotrophoblast in the only sample derived from chorionic tissues at 10 weeks of gestation was defined as showing moderately positive intensity and was used as the standard control to assess fluorescence intensity throughout the immunohistochemical analyses. This assessment was carried out by two individuals.

Human chorionic villous explant culture
Villous explant cultures of placental tissues obtained from legal abortions (6–8 weeks of gestation, n = 14) were performed using the method described (Aplin et al., 1999) with some modifications. Briefly, the placental tissues were placed in ice-cold RPMI and processed within 2 h of collection. The tissues were washed with sterile RPMI and aseptically dissected to remove decidual tissue. Small fragments of placental villi were teased apart, soaked in culture medium (RPMI supplemented with 10% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin) and 10 pieces of villous fragments were placed in each of four 35 mm collagen type I-coated dishes (Iwaki brand, Japan). After overnight incubation under 20% O2/5% O2/5% CO2/5% N2 conditions to allow the explants to adhere to the dishes, 2 ml of culture medium was added. These culture dishes were then placed in either 20% O2 or 1% O2 (37°C, 1% O2/5% CO2/94% N2) environment. After 48 h incubation, the cultured dishes were gently washed with PBS. The explant tissues on the dishes were fixed with 0.5% paraformaldehyde for 15 min at 4°C, then immunostained with anti-human CP-M (5 μg/ml) or mouse negative control mAb (5 μg/ml) followed by staining with FITC-conjugated rabbit anti-mouse antibody. The stained dishes were examined under a confocal laser scanning microscope (Carl Zeiss Inc., Germany) without mounting.

In some cases (n = 7), villous explant cultures were performed in 10 cm dishes coated with collagen type I. The explanted villous tissues were incubated for 48 h under the 20% O2 or 1% O2 conditions described above. After culturing, the dishes were gently washed with PBS and incubated with 0.05% trypsin (Difco Lab., USA) and 0.05% EDTA for 5 min. The detached cells were collected and filtered through a 40 μm mesh to remove the contaminated villous tissues and selectively obtain the spreading cells from the attached villous tissues. The collected cells were further cultured on 35 mm collagen type I-coated dishes under the same oxygenic conditions or were cultured on collagen type I-coated 8-well chamber slides (Lab-Tek; Nunc Inc., USA). After 4 h incubation, the attached cells were washed thoroughly to remove non-attached cells including tissue debris. These isolated EVT on 35 mm collagen type I-coated dishes were dissolved in TRIzol (Gibco BRL, USA) to extract total RNA, converted with trypsin and RNA or subjected to flow cytometry as described below. The isolated EVT on the 8-well chamber slide were immunostained by anti-human cytokeratin 7 antibody (5 μg/ml), anti-human MCAM antibody (5 μg/ml), anti-human vimentin antibody (5 μg/ml) or negative control antibody (5 μg/ml), followed by FITC-conjugated rabbit anti-mouse Ig secondary antibody as described above to examine the contamination of stromal cells.

Cell lines and culture conditions
BeWo and JEG-3, continuous cell lines established from human choriocarcinomas (Pattillo and Gey, 1968; Kohler and Bridson, 1971), were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the American Type Culture Collection (Rockland, USA), respectively. The cells were maintained as monolayers under 20% O2 conditions. For passage, the cells were dispersed with 0.05% trypsin and 0.05% EDTA solution, and replated on culture dishes.

RT–PCR analysis
Total RNA were extracted from the isolated EVT that had been cultured under 20% O2 conditions or under 1% O2 conditions, JEG-3 and BeWo cells using TRIzol as recommended by the manufacturer. Five micrograms of total RNA were reverse-transcribed with random primers using a commercial kit (First Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech UK Ltd, UK). The resulting cDNA mixtures were subjected to 30 cycles of PCR amplification with oligonucleotides from the human CP-M cDNA as primers (sense primer 5′-GCCGTAGATGATGTGTTTTC-3′; position 636–655; antisense primer 5′-TGGTGTAGTTGGTGGTTT-3′; position 1332–1351) (Tan et al., 1989) or
with human S26 primers (sense primer 5'-GGTCCGTGCCTCCAAGATGA-3'; position 8–27; antisense primer 5'-TAAATCGGGGTGGGGGTGTT-3'; position 308–327) (Vincent et al., 1993). After PCR amplification, 10 μl of each PCR product was electrophoresed on a 1% agarose gel, and amplified bands were detected by ethidium bromide staining. After cloning the PCR product, its sequence was verified.

Flow cytometry
JEG-3 and BeWo cells were dispersed by 0.05% trypsin and 0.05% EDTA, sedimented and incubated at 4 °C for 30 min with 10 μl of anti-human CP-M mAb (100 μg/ml) or negative control mAb (100 μg/ml). The cells were washed twice with Hank’s’ balanced salt solution (HBSS), and incubated with 20 μl of FITC-conjugated rabbit anti-mouse immunoglobulins (diluted 1:40) for 30 min at 4°C in the dark. After washing by HBSS, the stained cells were analysed by FACScanlibr (Becton Dickinson Immunocytometry Systems, Japan).

Cultured cells isolated from human villous explant culture were also analysed by FACScanlibr after immunostaining using anti-human CP-M mAb (10 μl, 100 μg/ml) or negative control mAb (10 μl, 100 μg/ml) to detect CP-M expression on isolated EVT.

The negative level of relative fluorescence intensity was defined from the histogram of control group with anti-TNP mAb staining in each experiment. The majority of negative area was adjusted within 10^3–10^4 fluorescence intensities. Under this condition, the mean relative fluorescence intensity for CP-M staining was calculated from the 5000 viable cells that were gated as JEG-3 cells, BeWo cells, or isolated EVT.

Invasion assay
The invasion assay was carried out as previously described with slight modifications (Katsuragawa et al., 1997). JEG-3 or BeWo cells were cultured on cell culture inserts (6.4 mm in diameter; Becton Dickinson Labware, USA) containing polyethylene terephthalate membranes with 8 μm diameter pores, which were placed in each well of a 24-well tissue culture plate (Becton Dickinson Labware). The upper surface of the filters was coated with cold Matrigel (10 μg; Collaborative Research Co., USA) and air-dried aseptically. Prior to use, the inserts were rehydrated with 100 μl of warm RPMI 1640 for 1 h. The JEG-3 or BeWo cells (1x10^5 in 200 μl of RPMI 1640 containing 1% FBS) were added to the top of the filter, and 800 μl of medium was added to the culture medium in the upper chamber. After a 18 h culture for JEG-3 or a 24 h culture for BeWo cells, non-invaded cells and Matrigel on the upper surface of the filter were thoroughly removed with cotton swabs. Cells remaining on the lower surface of the filter, which had migrated through the Matrigel and the filter, were fixed in methanol for 10 min at room temperature, and stained with haematoxylin. For quantification, the cells on the lower surface of the filter were counted under a microscope in five pre-determined fields at ×200 magnification. The assay was performed in triplicate chambers.

Figure 1. The immunoreactive localization of carboxypeptidase-M (CP-M) on extravillous trophoblasts (EVT) around cell columns. (A–C) A villus-anchoring site at 10 weeks of gestation. (D–I) Larger magnification of the area indicated in D–F. (A, D and G) Green-stained by FITC using anti-cytokeratin 8, 18 mAb. (B, E and H) Red-stained by rhodamine using anti-CP-M mAb. (C, F and I) Combined images of fluorescein isothiocyanate- and rhodamine-staining. At the proximal part of the cell column of anchoring villi, CP-M was hardly detected on EVT that expressed cytokeratin. In the distal part of the cell column, CP-M was weakly expressed on some EVT. On almost all interstitial trophoblasts in decidua tissues, CP-M was weakly to highly expressed. CP-M was highly detected on EVT in the trophoblastic shell (B). Column = cell column; Shell = trophoblastic shell; IntTB = interstitial trophoblast. Bars = 100 μm.
These experiments were repeated nine times for JEG-3 cell invasion and six times for BeWo cell invasion.

**Proliferation assay**

JEG-3 or BeWo cells (1 × 10⁵ in 200 μl of RPMI with 1% FBS) were plated in each well of a 96-well plate. After 24 h of incubation in the presence of MGTA (0, 10⁻⁵ or 10⁻⁴ mol/l), viable cell numbers in the wells were evaluated by WST assay (Premix WST-1, Cell Proliferation Assay System; Takara Biomedicals Co. Ltd, Japan) according to the manufacturer’s instruction. In this assay, the tetrazolium salt, WST, is metabolized to a coloured formazan salt by mitochondrial enzyme activity in viable cells. Briefly, after the remaining cells were washed with culture medium, they were incubated in RPMI 1640 with 500 μg/ml WST. After 30 min, WST reduction was determined using an automated enzyme-linked immunosorbent assay plate reader (Molecular Devices, USA) at an optical density of 450 nm. The experiments were repeated five times.

**Statistical analysis**

The absorbance of the WST-1 assay and the invaded cell numbers in the invasion assay were expressed as means ± SEM and their differences were analysed by one-way analysis of variance, followed by Scheffe’s F-test. The difference in mean relative fluorescence intensity on flow cytometry was analysed by two-tailed paired t-test. Differences were considered to be significant at P < 0.05.

**Results**

**Immunohistochemical localization of CP-M**

As previously reported, this enzyme was detected in syncytiotrophoblast (Skidgel et al., 1989). The intensity of staining of CP-M expression on syncytiotrophoblast appeared unchanged among the specimens obtained from normal pregnant women (9–12 weeks of gestation). In the floating villi, CP-M was hardly detected on cytotrophoblasts. CP-M was also hardly detected on the proximal part of the cell column of anchoring villi (Figure 1). In the distal part of the cell column, CP-M was weakly or highly expressed. The moderate or intense expression of CP-M was detected on trophoblastic shells in all specimens (Figures 2A–C). Endovascular EVT also moderately or highly expressed CP-M (Figures 2D–I).

Figure 2. The immunoreactive localization of carboxypeptidase-M (CP-M) on extravillous trophoblasts (EVT) in the trophoblastic shells and maternal vessels (9 weeks of gestation). (A–C) Chorionic villi and trophoblastic shell. (D–F) Endovascular trophoblasts in a spiral artery whose basal layer was relatively conserved. (G–I) A spiral artery whose structure was largely destroyed by invading EVT. (A and D) Green-stained by fluorescein isothiocyanate (FITC) using anti-cytokeratin 8, 18 mAb. (B and E) Red-stained by rhodamine using anti-CP-M mAb. (C and F) Combined images of A and B, or D and E, respectively. (G) A merged image with green-staining for cytokeratin 8, 18 and red-staining for CP-M. (H) Green-stained by FITC using anti-von Willebrand factor as a marker for endothelial cells. (I) A merged image with green-staining for von Willebrand factor and red-staining for CP-M. (B) CP-M was clearly expressed on EVT in the trophoblastic shell. (E) CP-M was also expressed on endovascular trophoblasts that invaded a spiral artery (arrows). (G–I) CP-M was highly expressed on the endovascular trophoblasts that were destroying the wall of a spiral artery (arrows). Arrowheads showed an adjacent intact vessel. IVS = intervillous space; Shell = trophoblastic shell; EndTB, endovascular trophoblasts. Bars = 50 μm.
These expression profiles of CP-M on EVT are summarized in Table I.

Table I. Carboxypeptidase-M (CP-M) expression on extravillous trophoblasts (EVT)

<table>
<thead>
<tr>
<th>Cell column</th>
<th>Trophoblastic shell</th>
<th>Interstitial trophoblasts</th>
<th>Endovascular trophoblasts</th>
</tr>
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<tbody>
<tr>
<td>Proximal</td>
<td>Distal</td>
<td>Shallow</td>
<td>Deep</td>
</tr>
<tr>
<td>9 weeks of gestation (n = 3)</td>
<td>7/8</td>
<td>1/8</td>
<td>2/8</td>
</tr>
<tr>
<td>10 weeks of gestation (n = 1)</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>12 weeks of gestation (n = 1)</td>
<td>4/5</td>
<td>1/5</td>
<td>5/5</td>
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</table>

EVT were classified into six subtypes according to their locations such as (i) proximal site of the cell column (n = 27); (ii) distal site of the column; (iii) trophoblastic shell (n = 21); (iv) interstitial trophoblasts in the shallow portion of the decidua; (v) interstitial trophoblasts in the deep portion of the decidua; (vi) endovascular trophoblasts (n = 31). The staining intensity of CP-M expression was classified into four grades (−, absence of staining; ±, weakly positive; +, moderately positive; ++, highly positive). The staining intensity of syncytiotrophoblasts in the only sample derived from chorionic tissues at 10 weeks of gestation was defined as moderately positive intensity and used as the standard control to assess fluorescence intensity.

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**Expression of CP-M on outgrown villous tips detected by immunocytochemistry and flow cytometry**

During 48 h culture on collagen type I-coated dishes, cytokeratin 7-positive cells were outgrown from the explanted villous tips as previously reported (Aplin et al., 1999). Under 20% O₂ conditions, CP-M was clearly expressed on these outgrown cells (Figure 3A and B). On the other hand, CP-M expression was very weak on these outgrown cells under 1% O₂ conditions (Figure 3C and D).

Under both oxygenic conditions, cytokeratin 7 and MCAM were detected in >95% of the isolated outgrown cells, whereas vimentin-positive cells comprised <5% (data not shown). Flow cytometry also confirmed that CP-M expression on isolated outgrown cells from explant culture under 20% O₂ conditions was more intense than that on those cultured under 1% O₂ conditions (Figure 4A). The differences in mean relative intensity were 30.2 ± 3.91 versus 22.4 ± 4.34 (P < 0.05, n = 7).

**The mRNA expression of CP-M on outgrown cells from explanted villous tips detected by RT–PCR**

In PCR products derived from RNA isolated from outgrown cells from explanted villous tips, specific bands compatible with 716 bp were detected by ethidium bromide staining (Figure 4B). The amplified fragments were extracted from the gels, cloned and verified by sequencing as previously described (Higuchi et al., 1995). The analysed sequence was identical to that of CP-M (Tan et al., 1989).
CP-M expression on JEG-3 cells and BeWo cells

Flow cytometry showed that CP-M was expressed on the cell surface of JEG-3 cells. However, CP-M was not detected on the BeWo cell surface (Figure 5).

Invasion assay

In the presence of MGTA, the invaded cell number of JEG-3 cells was significantly enhanced in a dose-dependent manner (A, n = 9). On the contrary, there was no significant alteration of the invaded cell number observed in BeWo cells by MGTA treatment (B, n = 5). *P < 0.01.

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Invasion assay

In the presence of MGTA, the invaded cell number of JEG-3 cells was significantly enhanced in a dose-dependent manner (Figure 6A). On the contrary, there was no significant alteration of the invaded cell number observed in BeWo cells by MGTA treatment (Figure 6B).

Proliferation assay

By WST-1 assay, there was no significant difference in viable cell number in either JEG-3 or BeWo cells with or without MGTA (data not shown).

Discussion

The immunohistochemical analysis showed that immunoreactive CP-M is expressed on syncytiotrophoblasts, but not on cytotrophoblasts, in the floating chorionic villi early in normal pregnancies. At villus-anchoring sites, CP-M was hardly detected on EVT in the proximal part of the cell column, but weakly detected on some EVT in the distal

Figure 4. The cell surface and mRNA expressions of carboxypeptidase-M (CP-M) on the isolated extravillous trophoblasts (EVT) by flow cytometry (A) and RT-PCR (B). (A) Typical histogram of flow cytometry analysis of the isolated EVT cultured under 20% O2 and 1% O2 concentrations. The CP-M expression on the cell surface was detected in the majority of isolated EVT. The CP-M expression was lower in EVT cultured under 1% O2 concentrations. x-Axis: relative fluorescence intensity; y-axis: cell number. (B) PCR products using primers for CP-M (arrows, 716 bp) and S26 (arrow heads, 320 bp) were detected by ethidium bromide staining. Lane 1, molecular markers. Lanes 2–7, PCR products derived from cDNA of the isolated EVT cultured under 20% O2 (lanes 2–4) or 1% O2 (lanes 5–7) concentration in the independent three experiments. Lane 8, negative control PCR product without cDNA.

Figure 5. The cell surface expression of carboxypeptidase-M (CP-M) on JEG-3 (A) and BeWo (B) cells detected by flow cytometry. CP-M was expressed on JEG-3 cells (A), whereas it was hardly detected on BeWo cells (B). x-axis: relative fluorescence intensity; y-axis: cell number.

Figure 6. Invasion assay using JEG-3 (A) and BeWo (B) cells in the absence or presence of an inhibitor for carboxypeptidase-M (CP-M), dl-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (MGTA). In the presence of MGTA, the invaded cell number of JEG-3 cells was significantly enhanced in a dose-dependent manner (A, n = 9). On the contrary, there was no significant alteration of the invaded cell number observed in BeWo cells by MGTA treatment (B, n = 5). *P < 0.01.
part. In the decidual tissue, almost all interstitial trophoblasts expressed CP-M. Notably, CP-M was clearly expressed on EVT in the trophoblastic shells and in the maternal vessels. These expression profiles indicate that CP-M expression is induced on human trophoblasts during their differentiation process toward EVT.

CP-M was reported to be expressed on tissue macrophages. Since this molecule is not expressed on monocytes, it has been widely accepted as a differentiation marker for monocyte–macrophage lineage cells (Rehli et al., 2000). In addition, we previously reported that CP-M expression is rapidly induced on luteinizing human granulosa cells during corpus luteum formation, when granulosa cells dramatically transform into large luteal cells that produce abundant progesterone. Thus, CP-M was proposed to be a new differentiation marker for the granulosa–large luteal cell lineage (Yoshioka et al., 1998). CP-M was reported to metabolize several biologically active peptides such as bradykinin and dynorphin A. However, key substrates that are concerned with function or differentiation for macrophages and large luteal cells have not yet been clarified. The immunohistochemical results showed that CP-M is expressed on syncytiotrophoblast, confirming the previous report that CP-M is present on the microvilli fraction in the placenta (Skidgel et al., 1989). Since immunoreactive CP-M was not detected on cytotrophoblasts, CP-M expression is considered to be induced during the differentiation process from cytotrophoblasts into syncytiotrophoblast. This study also showed that the CP-M expression rapidly increases on EVT from the distal part of the cell column to trophoblastic shells or interstitial trophoblasts. These changes suggest that some substrates for CP-M are involved in trophoblast differentiation and/or function. Although we could not determine substantial substrates concerning trophoblast differentiation in this study, it can be concluded that CP-M is a differentiation-related molecule for human EVT as well as villous trophoblasts. In contrast to CP-M, DPP IV was detected on cytotrophoblasts in the chorionic villi and the EVT at the proximal sites of the cell column, but its expression on EVT diminished from the distal part of the cell column to invading interstitial trophoblasts and EVT in the shell in early pregnancy (Sato et al., 2002). This indicates that physiological switching of expression of the membrane-bound peptidases occurs during the differentiation process of EVT. These findings support the concept that membrane-bound peptidases play some roles in cell differentiation and/or function in the reproductive system (Fujigawa et al., 1999).

Using primary villous tissue culture, we investigated whether CP-M expression can be induced on EVT in vitro. Outgrown cells from the villous tips were mainly cytokeratin 7-positive cells, which is an excellent cell marker for trophoblasts (Aplin et al., 1999). We also observed that MCAM/CD146 antigen, a specific marker for EVT (Shih and Kurman, 1996), was expressed on these outgrown cells. Thus, these outgrown cells were considered mainly to consist of EVT. In these outgrown cells, immunoreactive CP-M was clearly detected by immunocytochemical staining. After a separation procedure, vimentin-positive cells comprised <5% of the isolated cells, showing that contamination by mesenchymal cells from villous stromal tissues was limited. The mRNA expression of CP-M was detected in isolated EVT by RT–PCR. The cell surface expression of CP-M was also confirmed by flow cytometry. Based on these findings, we conclude that CP-M expression can be induced on EVT in vitro and we consider this molecule useful as one of the differentiation markers to analyze the differentiation mechanism of EVT.

Recently, local oxygen tension has received attention as an important regulator for EVT differentiation early in pregnancy (Genbacev et al., 1997, 2001; Caniggia et al., 2000). Therefore, we examined the effects of oxygen concentration on EVT differentiation around the cell column using this marker. In the villous explant culture under 20% O₂ conditions, the cytokeratin 7-positive outgrown cells from villous tips clearly expressed CP-M. However, CP-M expression on these EVT was weak under hypoxic conditions. This tendency was also corroborated by flow cytomteric analysis in isolated EVT. This indicates that oxygen concentration an important factor regulating CP-M expression on EVT in vitro and suggests that local oxygen concentration is deeply implicated in EVT differentiation around the cell column in vivo (Genbacev et al., 1997). In this regard, high expression of CP-M on EVT in the trophoblastic shells and endovascular region are compatible with the above in-vitro findings, because these sites are nearly in contact with maternal arterial blood and the local oxygen concentration is estimated to be high. Although there is no definitive evidence concerning tissue oxygen concentration at implantation sites, it has been speculated that oxygeonic tension in maternal decidual tissue is higher than that at embryonic sites during early pregnancy (Rodesch et al., 1992), suggesting a gradient of oxygen tension from the cell column to decidual tissue. This may agree with the increased expression of CP-M on interstitial trophoblasts in the decidua.

Next, to speculate on the physiological role of CP-M in EVT, we examined the effects of inhibitor for CP-M on the function of a CP-M-bearing human choriocarcinoma cell line, JEG-3 cells. The invasion assay demonstrated that the administration of MGTA enhanced the number of invaded JEG-3 cells. Since this inhibitor did not affect the proliferation of JEG-3 cells, the stimulatory effects of the inhibitor in the invasion assays must have been due to enhancement of the invasive property itself, rather than enhanced cell proliferation. The concentration of MGTA used in this study was reported to be sufficient for inhibition of CP-M activity. However, it was also known to inhibit other carboxypeptidases such as carboxypeptidase-D (Hadhkar et al., 2001). To address this issue, we examined the effects of MGTA using an other human choriocarcinoma cell line, BeWo cells, that did not express CP-M and observed that MGTA did not affect the number of invading BeWo cells. Although it is indirect evidence, this result suggests that CP-M enzyme activity is involved in the regulation of JEG-3 cell invasion. It has been reported that bradykinin, enkephalin hexapeptides such as Lys⁶- or Arg⁶-enkephalins and dynorphin A are representative substrates for CP-M. Unfortunately, our preliminary examination failed to show any distinct effects of the above peptide factors on JEG-3 cell invasion. Possibly, undefined substrates for CP-M may contribute to promoting JEG-3 cell invasion. Recently, arginine that is removed from the carboxy-terminal of various peptides by carboxypeptidase was reported to be used for substrate for NO synthesis, suggesting that CP-M can promote local NO production (Hadhkar et al., 2001). This may contribute to maternal vessel dilatation around endovascular trophoblasts to supply sufficient blood circulation within intervillous spaces. Taking these characteristics of CP-M into consideration, it would be necessary to identify substantial substrates to determine the precise physiological roles of CP-M in EVT function and/or differentiation.

In conclusion, the present study showed that CP-M expression is induced on human trophoblasts during their differentiation process into EVT in vivo by immunohistochemical examination and that this induction can be reproduced in vitro using primary villous culture. These findings indicate that CP-M is a differentiation-related molecule for human EVT and suggest that some substrates for CP-M are involved in trophoblast differentiation and/or function. CP-M enzyme activity was shown to affect JEG-3 cell invasion. Although JEG-3 cells are derived from cancer cells and do not necessarily reflect the normal function of trophoblasts, this showed the possibility that CP-M expressed on EVT is involved in trophoblast invasion. The present study also showed that CP-M expression on EVT is partially regulated.
by oxygen concentration in vitro, supporting the current concept that local oxygenic concentration is one of the important factors regulating EVT differentiation in vivo early in pregnancy. The clarification of substantial substrates for CP-M at implantation sites will contribute to further understanding of the mechanisms of EVT differentiation in vivo early in pregnancy.

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