Cyclic AMP enhances the expression of an extravillous trophoblast marker, melanoma cell adhesion molecule, in choriocarcinoma cell JEG3 and human chorionic villous explant cultures

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Human trophoblasts consist of two main cell lineages, villous trophoblasts (VT) and extravillous trophoblasts (EVT). To identify the molecules which are involved in EVT differentiation, we have raised a monoclonal antibody (mAb) designated CHL1, by immunizing a mouse against human chorion laeve which is composed of EVT. By immunohistochemical analysis, the CHL1 antigen was found to be expressed on the majority of EVT but not on VT in addition to its expression on endothelial and myometrial cells. A subsequent cDNA panning method revealed that the CHL1 antigen was identical to melanoma cell adhesion molecule (MCAM, Mel-CAM, S-endo 1 or MUC18/CD146), which has been previously reported as one of the EVT markers. MCAM expression on JEG3 cells, a human choriocarcinoma-derived cell line, was significantly enhanced when they were co-cultured with isolated human decidual tissue. Various cytokines and growth factors that were reportedly present in decidual tissue failed to increase MCAM expression in JEG3 cells, but decidua-induced MCAM expression in JEG3 cells was attenuated by the addition of protein kinase A inhibitor H89. In addition, cAMP, which is known to stimulate differentiation of VT, enhanced MCAM expression in JEG3 cells. Its promoting effect on MCAM expression was also observed in human chorionic villous explant cultures. These findings suggest that a cAMP-dependent intracytoplasmic signalling pathway is involved in the differentiation mechanism of human EVT.

Key words: CD146/cDNA panning/differentiation/extravillous trophoblasts/MCAM

Introduction

During early placentation, human trophoblasts differentiate into two main cell lineages: villous and extravillous cells. Villous trophoblasts (VT) cover the chorionic villi of the placenta, and their function is to transport oxygen and nutrients from the mother to the fetus. In contrast, extravillous trophoblasts (EVT) invade the maternal endometrium and migrate up spiral arterioles in the uterine muscle layer. This process leads to the development of vessels with low resistance and high capacity, thereby enhancing blood flow to the intervillous space to meet the increasing fetal demands for nutrient and gas exchange (Pijnenborg et al., 1983). These physiological changes are required for a successful pregnancy and failure of spiral artery transformation by EVT has been documented in pre-eclampsia and intrauterine growth retardation (Robertson, 1976). EVT invade and migrate through maternal decidua and degrade extracellular matrix (ECM) of the arterial wall during arterial transformation, a process resembling tumour invasion (Aplin, 1991). With this in mind, the roles of adhesion molecules, including integrins and ECM degrading enzymes such as metalloproteinases, on EVT invasion have been extensively investigated (Librach et al., 1991; Damsky et al., 1994). However, unlike tumour invasion, EVT invasion is strictly regulated. For example, EVT invade the maternal decidua and myometria but do not exceed one-third of the myometrial layer. In addition, the invasiveness of EVT observed in early pregnancy ceases as the pregnancy proceeds (Pijnenborg et al., 1983). Although previous investigations proposed the involvement of integrins and proteinases in the initiation of EVT invasion, the mechanisms that control EVT invasion in the deep portion have not yet been clarified.

To investigate regulatory mechanisms for EVT invasion, we have been focusing on molecules which are expressed on human EVT. Previously, we reported that CD9, a cell surface molecule which is known to be associated with β integrins, was predominantly expressed on human EVT. CD9 was also expressed on a choriocarcinoma-derived cell line BeWo in association with integrin α5β1 and it was shown to regulate BeWo cell invasion (Hirano et al., 1999a,b). In addition, a membrane-bound peptidase, dipetidyl peptidase-IV/CD26, was shown to be expressed on EVT and it could regulate the invasive property of another choriocarcinoma cell line, JEG3 (Sato...
mouse mAb (unrelated mAb, IgG1) (Tsujimura et al., 1990) was used as the negative control. Rhodamine-conjugated goat anti-mouse immunoglobulin polyclonal antibody (pAb) (Santa Cruz Biotechnology Inc., USA) was used as the secondary antibody.

**Immunofluorescence localization**

Placental tissues were processed for immunofluorescence double staining as previously described (Fujiwara et al., 1993) with a slight modification. Frozen tissues of 7 μm thickness were air-dried on Neoprene-coated glass slides (Nishin EM, Japan), and fixed in acetone at −20°C for 5 min. The slides were incubated with CHL1 mAb (5 μg/ml, diluted in culture medium), S-Endo1 mAb (5 μg/ml), or anti-TNP mAb (5 μg/ml) for 40 min at room temperature. They were then washed in phosphate-buffered saline (PBS), and incubated with the rhodamine-conjugated secondary antibody (diluted 1:40) for 40 min at room temperature in the dark. After an incubation with anti-TNP mAb (20 μg/ml) to block non-specific binding, the slides were reacted with FITC-conjugated anti-human cytokeratin mAb (diluted 1:20) for 40 min. The slides were then washed and examined with a confocal laser scanning microscope (Carl Zeiss Inc., Germany) equipped with filters to view the rhodamine and isothiocyanate fluorescence.

**cDNA library construction**

A cDNA expression library was constructed as previously described with a slight modification (Higuchi et al., 1999). Poly(A)+ RNA was prepared from human chorion laeve, and cDNA was synthesized by priming with Nor I oligo (dT) primer using a SuperScript Choice system (Invitrogen, The Netherlands) and ligated with Bsi XI adapter (Invitrogen). After digestion with Nor I restriction enzyme, the cDNA was ligated into a Bsi XI- and Nor I-digested pME18S mammalian cell expression plasmid vector, which carries a strong chimeric promoter of SV40 and SRz (Takebe et al., 1988). The ligated plasmid DNA was transformed into Escherichia coli DH 5α by electroporation (Cell Porator System, Invitrogen). The cDNA library obtained contained 1×10⁶ clones.

**Expression cloning of cDNA encoding the CHL1 antigen**

CO87 cells were obtained from the American Type Culture Collection and grown in Dulbecco’s minimum essential medium/10% calf serum. The cDNA library was introduced into subconfluent COS7 cell cultures in 10 cm dishes by the chimeric promoter of SV40 and SRz (Takebe et al., 1988). The chorion laeve from fetal membranes and subsequent screening analysis revealed that the CHL1 antigen is identical to the melanoma cell adhesion molecule (MCAM/CD146). MCAM is reported to be a membrane-bound glycoprotein which mediates cell–cell adhesion and has been shown to be expressed in a variety of normal human tissues and malignant neoplasms (Lehmann et al., 1987; Shih et al., 1994). In human placenta, it was reported that the expression of MCAM is confined to the EVT (Shih and Kurman, 1996) and its role in implantation as well as placental development being suggested (Shih et al., 1998). However, the key factors which regulate the expression of MCAM in human EVT remain unclear. Since MCAM is considered an excellent marker for EVT, we further examined factors which regulate MCAM expression as this may provide information on the differentiation mechanisms of human EVT.

**Materials and methods**

**Tissues**

Placental tissues were obtained from first-trimester pregnancies (6–12 weeks gestation, n = 6), including three cases of legally terminated pregnancies and three cases of hysterectomies for cervical intra-epithelial neoplasia during normal pregnancy. Term placenta (n = 5) and fetal membranes (n = 5) were obtained from normal deliveries. For RNA isolation, tissues were immediately frozen in liquid nitrogen and stored at −80°C. For immunohistochemistry, each specimen was embedded in OCT compound (Tissue-Tec, Miles Inc., Diagnostic Division, USA), snap-frozen in liquid nitrogen, and stored at −80°C. Informed consent for the use of these tissues in this study was obtained from all donors.

**Production and selection of mAb**

The production and selection of mAb were performed as described previously (Fujiwara et al., 1993; Higuchi, et al., 1999). The chorion laeve from fetal membrane was manually separated and cut into small pieces using scissors, and was then injected into the peritoneal cavity of 8-week-old BALB/c mice four times every 4 weeks. On the 5th day after the last immunization, the spleen cells of the mice were fused with X63Ag8 myeloma cells and cultured in 96-well microtitre plates. Supernatants from the growing hybridomas were screened by indirect immunofluorescence staining on frozen sections of fetal membrane and term placenta. Hybridomas of interest were cloned, expanded, and injected i.p. into female mice previously treated with pristane (2,6,10,14-tetramethylpentane) (T.Higuchi et al., 2002). These findings suggested that it is important to analyse molecules expressed on EVT when we investigate the function of the EVT, and thus we attempted to identify novel molecules that are expressed on human EVT.

Previously, we have raised monoclonal antibodies (mAb) against ovarian cells and identified several molecules that are involved in folliculogenesis and corpus luteum formation (Yamada et al., 1998a;b; Higuchi et al., 1999). In the present study, we have raised mAb against human chorion laeve, which contains EVT expressing CD9 and dipeptidyl peptidase-IV. Among the mAb that reacted with the antigens on EVT, one mAb, named CHL1, was selected since expression of the CHL1 antigen in trophoblasts was confined to cells of extravillous lineage. Next, to identify a cDNA clone which encodes the CHL1 antigen, we constructed a cDNA library derived from human chorion laeve and subsequent panning analysis revealed that the CHL1 antigen is identical to the melanoma cell adhesion molecule (MCAM/CD146).

For the immunochemistry and flow cytometry, an anti-trinitrophenyl (TNP) monoclonal antibody (mAb) was prepared from human chorion laeve, which contains EVT expressing the CHL1 antigen, we constructed a cDNA library derived from human chorion laeve and subsequent panning analysis revealed that the CHL1 antigen is identical to the melanoma cell adhesion molecule (MCAM/CD146). MCAM is reported to be a membrane-bound glycoprotein which mediates cell–cell adhesion and has been shown to be expressed in a variety of normal human tissues and malignant neoplasms (Lehmann et al., 1987; Shih et al., 1994). In human placenta, it was reported that the expression of MCAM is confined to the EVT (Shih and Kurman, 1996) and its role in implantation as well as placental development being suggested (Shih et al., 1998). However, the key factors which regulate the expression of MCAM in human EVT remain unclear. Since MCAM is considered an excellent marker for EVT, we further examined factors which regulate MCAM expression as this may provide information on the differentiation mechanisms of human EVT.

**Antibodies**

To confirm specific immunoreactivity of the CHL1 mAb with MCAM, a commercially available mouse anti-human CD146/MCAM mAb (S-Endo1, IgG1; Alexis Biochemicals, USA) (Bardin et al., 1996) was used in immunohistochemistry. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human cytokeratin 8/18 mAb (SD3, IgG1; YLEM, Italy) was used in the immunohistochemical study as a marker for epithelial cells and trophoblasts. For the immunocytochemistry and flow cytometry, an anti-trinitrophenyl (TNP) mAb (unrelated mAb, IgG1) (Tsujimura et al., 1990) was used as the negative control. Rhodamine-conjugated goat anti-mouse immunoglobulin polyclonal antibody (pAb) (Santa Cruz Biotechnology Inc., USA) was used as the secondary antibody.

**Immunoprecipitation and sodium dodeyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)**

Cell surface biotinylation was carried out as follows. COS7 cells transfected with CHL1 cDNA were washed with Hanks’ balanced salt solution (HBSS) and incubated in a solution containing 150 mmol/l NaCl, 10 mmol/l HEPES, 0.2 μg/ml NHS-LC-Biotin (Pierce Chemical Co., USA), 0.2 mmol/l CaCl₂ and
MgCl₂, pH 8.0, for 30 min at 4°C. The reaction was stopped by the addition of 40 mmol/l glycine, and cells were washed with HBSS.

Cells were then lysed with HEPEs-buffered saline (HBs; 10 mmol/l HEPEs, 150 mmol/l NaCl, pH 7.0) containing 10 mmol/l CHAPS, 10 µg/ml chymostatin, and 20 µg/ml antipain, and kept for 30 min at 4°C. The lysates were cleared of insoluble material by centrifugation, and the supernatant was immunoprecipitated with CHL1 mAb or TNP mAb at a concentration of 5 µg/ml followed by the addition of Dynabeads. The beads were washed with washing buffer (HBs containing 10 mmol/l CHAPS) and then boiled with SDS-PAGE sample buffer. Immunoprecipitates recovered were subjected to SDS-PAGE with 50 µmol/l dithiothreitol, and then electrotransferred to an Immobilon membrane (Bedford, USA). The membrane was blocked with 5% BSA in Tris-buffered saline, and biotinylated proteins were incubated with 100 ng/ml horseradish peroxidase–streptavidin (Pierce Chemical Co.) followed by detection with an ECL Western blotting kit (Amersham–Pharmacia, USA).

RNA isolation and Northern blot analysis
Total RNA from human fetal membrane, placenta, or cultured JEG3 were isolated by the TRIzol method (Invitrogen). Northern blot analysis was performed as previously reported (Higuchi et al., 1995). In brief, 10 µg total RNA were electrophoresed on a 1.0% agarose–formaldehyde gel and then transferred to nylon membranes. The membranes were incubated with a prehybridization solution (Rapid Hyb; Amersham–Pharmacia) for 30 min at 65°C and hybridized for 2 h at 65°C with the labelled probes in the same solution. After hybridization, the filters were washed according to the manufacturer’s protocol, and then subjected to autoradiography. To ensure even loading, the membranes were washed and rehybridized with a human ribosomal protein S26 cDNA probe, the expression level of which remains virtually constant in most tissues.

Cell lines and culture condition
JEG3, continuous cell lines established from a human choriocarcinoma, were obtained from the American Type Culture Collection (USA) and maintained in Roswell Park Memorial Hospital (RPMI) 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

To examine the effects of decidual factors, 1×10⁵ JEG3 cells were suspended in 10 ml of complete medium and plated into each well of a 6-well culture plate (Corning Inc., USA). Decidual tissues were obtained from legally obtained legal tissue, minced into pieces 5 mm in diameter and incubated in the cell culture insert (0.8 µm diameter pore: Becton Dickinson Labware, USA). JEG3 cells were cultured with these decidual tissue preparations for 2 days. As a control, choriocarcinoma tissue obtained from the same conceptus was placed into the cell cultures. JEG3 cells were also co-cultured with decidual tissue for 2 days in the presence of a selective inhibitor of protein kinase A, H89 (20 and 50 mmol/l; Calbiochem-Novabiochem Co., USA) or vehicle alone (culture containing 0.5% dimethylsulphoxide). After the culture, JEG3 cells were subjected to flow cytometry or Northern blot analysis.

JEG3 cells were also cultured for 2 days in the presence of interleukin (IL)-1β, -4, -10, tumour necrosis factor (TNF)-α, interferon (IFN)-γ (10 ng/ml each; Peprotech EC Ltd, UK), transforming growth factor (TGF)-β1 (10 ng/ml; Genzyme/Techne, USA) or forskolin (200 mmol/l; Sigma Chemical Co., USA), and then subjected to Northern blot analysis.

Human chorionic villous explant culture
Villous explant cultures were established using tissues from first-trimester placentae (6–10 weeks of gestation, n = 3) as previously described with some modifications (Aplin et al., 1999). 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 tease apart, soaked in culture medium (RPMI supplemented with 10% fetal calf serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin), and placed into each well of a 6-well culture plate which had been precoated with porcine collagen type I (Koken Co. Ltd, Japan). These placental tissues were cultured for 48 h in the presence of 200 mmol/l forskolin or vehicle alone (culture containing 0.5% ethanol), and then subjected to Northern blot analysis.

Flow cytometry
The dispersed JEG3 cells (2×10⁶) were centrifuged and incubated at 4°C for 30 min with CHL1 mAb (100 µg/ml) or the anti-TNP mAb (negative control, 100 µg/ml). The cells were then washed twice with HBSS, and incubated with 20 µl of FITC-conjugated rabbit anti-mouse immunoglobulins (diluted to 1:40) at 4°C for 30 min in the dark. After washing in HBSS, the cells were resuspended in HBSS and analysed by FACScan.

Statistical analysis
Statistical differences between sample means were calculated by a two-tailed t-test. The results are expressed as means ± SD of six independent experiments, and P < 0.05 was considered significant.

Results
CHL1 antigen expression in human placenta
A murine mAb designated CHL1 (IgG1 isotype) was generated by immunizing mice with human EVT obtained from the chorion laeve of fetal membrane. Using this CHL1 mAb, immunohistochemical analysis was carried out to investigate the localization of CHL1 antigen in human placentae. Simultaneously, each placental section was immunostained with FITC-conjugated anti-human cytokeratin 8/18 mAb to identify trophoblasts and endometrial epithelial cells.

In first trimester placentae, expression of the CHL1 antigen was observed on the majority of cytokeratin-positive EVT which had invaded into the maternal decidua: trophoblasts in the distal portion of cell columns, interstitial trophoblasts in the decidua, and on giant cells in the deep portion (Figure 1A–C). On the other hand, EVT in the proximal portion of cell columns did not express CHL1 antigen (Figure 1B). CHL1 antigen expression was not detected on the villous cytotrophoblasts or syncytiotrophoblasts (Figure 1A). No expression of CHL1 antigen was detected on decidual cells or endometrial epithelial cells, but myometrial cells and endothelial cells in the placental villi and maternal decidua did express CHL1 antigen (Figure 1A–C).

In second and third trimester placentae, the expression profiles of CHL1 antigen were similar to that in first trimester placentae. EVT in placentae of advanced gestation were observed as cytokeratin-positive scattered cells in the placental bed, where the CHL1 antigen was strongly expressed (Figure 2C). In the villi, CHL1 antigen expression was also observed on endothelial cells but not on cytotrophoblasts or syncytiotrophoblasts (Figure 2A and C).

In the fetal membrane at term pregnancy, EVT were observed as a cytokeratin-positive multicellular layer, chorion laeve (Figure 2B and D). CHL1 antigen was strongly expressed on EVT of the maternal portion in the chorion laeve, but not on the fetal portion (Figure 2B and D). Amniotic epithelial cells and decidual cells were negative for CHL1 antigen expression.

In these immunohistochemical analyses of placentae and fetal membranes, no immunostaining was observed when anti-TNP mAb was used as the primary antibody (data not shown).

Identification of CHL1 antigen as MCAM/CD146 antigen
cDNA clones encoding the CHL1 antigen were recovered from a chorion laeve cDNA library by mAb panning of transfected COS7 cells. After four successive rounds of mAb panning and plasmid retrieval, one plasmid clone pCHL1-12 showed cell surface expression of CHL1 antigen on COS7 cells as detected by flow cytometry (data not shown). Immunoprecipitation and electrophoresis showed a prominent band of material precipitated from pCHL1-12 transfected...
COS7 cells by CHL1 mAb (Figure 3, lane 1). The calculated mean molecular mass was 110 kDa.

Plasmid clone pCHL1-12 was subjected to DNA sequencing, and comparison of the nucleotide sequences with the EMBL/GenBank databases showed that clone CHL1 was identical to the human melanoma cell adhesion molecule (MCAM/CD146 antigen) (Lehmann et al., 1989).

Expression of MCAM mRNA in human placentae
MCAM mRNA was detected by Northern blotting as a transcript of 3.3 kb in human placentae as previously described (Lehmann et al., 1989) (Figure 4). Expression of MCAM mRNA in placental tissue was variable from sample to sample, possibly reflecting the amount of MCAM-expressing endothelial cells in floating villi (Figure 4, lanes 1–12). In addition, MCAM mRNA was observed as a single transcript in fetal membrane which contains EVT of chorion laeve (Figure 4, lanes 13–16).

Enhancement of MCAM expression in JEG3 cells by decidual tissue
Under normal culture conditions, weak MCAM expression on the surface of JEG3 cells was detected by flow cytometry (data not shown). When JEG3 cells were cultured for 2 days in the presence of isolated decidual tissues, MCAM expression was enhanced as compared with the cells cultured under the presence of chorionic tissue derived from the same patients (Figure 5A). Northern blot analyses showed that this enhancement of MCAM expression was also observed at the transcriptional level (Figure 5B).

Increased cAMP levels enhance MCAM expression in JEG3 cells and in human chorionic villous explant cultures
To identify the decidua-derived factors which enhance MCAM expression in JEG3 cells, we examined the effects of several cytokines and growth factors which are known to be produced by decidual tissue. JEG3 cells were cultured for 48 h in the presence of IL-1β, IL-4, IL-10, TNF-α, TGFβ3, or IFN-γ, and expression of MCAM mRNA was examined by Northern blot analysis. The expression of MCAM mRNA was faint in JEG3 cells cultured under standard conditions (Figure 6, lane 1), however none of the cytokines or growth factors described above showed any enhancement of MCAM mRNA expression (Figure 6, lanes 2–7).

Next we examined the involvement of cAMP in decidua-induced MCAM expression in JEG3 cells because cAMP is reported to enhance MCAM expression in non-trophoblastic cells (Rummel et al., 1996). In the presence of H89, a selective inhibitor for protein kinase A, decidua-induced MCAM expression in JEG3 cells was attenuated in a dose dependent manner (Figure 7). Furthermore, forskolin, an activator of adenylyl cyclase that leads to increased cAMP levels, significantly enhanced the expression of MCAM mRNA in JEG3 cells.
To examine whether this stimulatory effect of cAMP on MCAM expression also functions in non-neoplastic human placental tissue, a human chorionic villous explant culture was carried out in the presence of forskolin. Northern blot analysis showed a significant enhancement of MCAM mRNA expression in the explant culture with forskolin compared with that of vehicle alone (Figure 9).

Discussion

In the present study, we raised mAb against human EVT in chorion laeve and obtained a mAb, CHL1, which reacted with these cells. To identify a cDNA clone which encoded the CHL1 antigen, we constructed a cDNA library derived from human chorion laeve and subsequent panning analysis revealed that CHL1 antigen was identical to MCAM/CD146. MCAM is an integral membrane glycoprotein of 113 kDa which belongs to the immunoglobulin supergene family and was originally defined as a marker of tumour progression and metastasis formation in human melanoma (Lehmann et al., 1987). MCAM expression has been detected in a variety of tissues (Lehmann et al., 1987; Shih et al., 1994; 1998), and its specific expression on human EVT has already been described (Shih and Kurman, 1996). Our immunohistochemical analyses using the CHL1 mAb showed intense MCAM expression on EVT lineage cells, which was compatible with the previous study (Shih and Kurman, 1996). Comparison of MCAM expression with cytokeratin expression showed that MCAM was not expressed on EVT in the proximal portion of cell columns. In addition, expression of MCAM in the chorion laeve was observed in the maternal portion of EVT but not on the fetal portion. While trophoblasts in chorion laeve are considered as EVT, it has been reported that several stages of EVT differentiation exist in this layer. EVT located nearer the amniotic epithelium are proliferative and undifferentiated, while EVT nearer the decidua are nonproliferative and differentiated (Kaltenbach and Sachs, 1979). From the results of our immunohistochemical analyses, including those with another available anti-MCAM mAb S-Endo1, MCAM appears to be expressed on EVT with relatively differentiated phenotypes. Therefore, we
considered that MCAM is a sensitive marker which is useful to investigate the differentiation mechanism of EVT.

To investigate factors which regulate MCAM expression in human EVT, we used the choriocarcinoma-derived cell line JEG3 which expresses MCAM in addition to the expression of HLA-G, another specific marker of EVT (Kovats et al., 1990; Chumbley et al., 1993; 1994). Using this JEG3 cell line, we examined the effects of isolated decidual tissues on MCAM expression in JEG3 cells because maternal decidua have direct contact with EVT during placentation, and a role for decidual tissues in the differentiation of trophoblasts has been proposed (Ren and Braunstein, 1991; Katsuragawa et al., 1995). When JEG3 cells were co-cultured with decidual tissue, the expression of MCAM was enhanced. In previous reports, soluble factors from decidual tissues or decidualized endometrial stromal cells were shown to suppress trophoblastic production of hCG, which is a marker of villous trophoblastic differentiation (Ren and Braunstein, 1991; Katsuragawa et al., 1995). Since the immunohistochemical analyses showed that MCAM was specifically expressed on EVT, our results support the concept that decidual tissues facilitate trophoblast differentiation into the extravillous lineage. As to the factors causing these effects, some soluble factors secreted from decidual tissues were thought to be important because this stimulatory effect on MCAM expression was observed without direct contact between the decidual tissues and JEG3 cells. To clarify such factors, we examined the expression of MCAM in JEG3 cells in the presence of several cytokines which are present in maternal decidua such as IL-1β, IL-4, IL-10, TNF-α, TGF-β3 and IFN-γ (Saito et al., 1994; Piccinini et al., 1998; Ashkar and Croy, 1999; Bischof et al., 2000). Unfortunately, we could not observe any significant changes in MCAM mRNA expression with these cytokines. In addition, in our preliminary experiments, co-cultures with peripheral blood mononuclear cells also failed to induce MCAM expression on JEG-3 cells (data not shown). From these observations, we speculate that some soluble factors from decidual cells other than cytokines may be responsible for promoting EVT differentiation.

We also examined intracytoplasmic signalling factors which mediate MCAM expression. Several studies have demonstrated that...
cAMP is involved in the regulation of MCAM expression in melanoma and neuroectodermal cells (Rummel et al., 1996). In addition, the cAMP responsive element found in the MCAM promoter was shown to be a major transcriptional activator (Mintz and Johnson, 2000). cAMP has been shown to stimulate hCG production in isolated human trophoblasts (Feinman et al., 1998) and various choriocarcinoma-derived cell lines (Hohn et al., 1998). We have also observed that forskolin enhanced hCG production in JEG3 cells (data not shown). Since increased production of hCG is considered to be one of the characteristics of trophoblastic differentiation into the villous lineage, it is of interest whether cAMP induces or reduces MCAM expression of JEG-3 cells. In our study, the protein kinase A inhibitor H89 attenuated the decidua-induced MCAM expression in JEG3 cells. This suggests that the cAMP-dependent signalling pathway is involved in the regulation of MCAM expression in trophoblastic cancer cells, as observed in melanoma cells. Northern blot analysis clearly indicated that forskolin significantly enhanced the expression of MCAM mRNA in JEG3 cells, supporting the above conclusion. Because MCAM is an excellent differentiation marker for EVT lineage cells, it is speculated that cAMP can induce trophoblast differentiation into the extravillous lineage. To confirm this idea, we examined the effects of forskolin on MCAM expression using a human choriocarcinoma villous explant culture system, which is thought to reproduce trophoblast differentiation occurring in the cell column (Aplin et al., 1999). Northern blot analysis showed that the expression of MCAM mRNA was also enhanced in human choriocarcinoma villous explant culture by forskolin treatment. Considering our results together with the previous reports showing that cAMP induces trophoblast differentiation into the villous lineage, it appears that cAMP is involved in the differentiation of human trophoblasts into both villous and extravillous lineages. Since MCAM expression was observed in cells from a relatively differentiated stage of the EVT lineage, cAMP may be involved not in the initial step when selecting the VT or EVT pathway, but in the following steps after determining the EVT lineage.

In conclusion, this study provides evidence that decidua-derived factors enhance the expression of MCAM in JEG3 cells. As trophoblastic expression of MCAM was confined to the extravillous lineage, a possible role of decidua-derived factors in EVT differen-

Figure 7. Effects of protein kinase A inhibitor H89 on decidua-induced MCAM expression in JEG3 cells. JEG3 cells were co-cultured with or without decidual tissues for 2 days in the presence of 20 mmol/l H89, 50 mmol/l H89 or vehicle alone. MCAM expression on JEG3 cells was analysed by flow cytometry using the CHL1 mAb. MCAM expression was attenuated by H89 in a dose-dependent manner.

Figure 8. Effects of forskolin on MCAM mRNA expression in JEG3 cells. (A) JEG3 cells were cultured for 48 h in the absence of stimulation (lane 1), with vehicle alone (lane 2), or with 200 mmol/l of forskolin (lane 3). Aliquots (10 μg) of total RNA from each culture were subjected to Northern blot analysis using MCAM cDNA (upper panel) and S26 ribosomal protein cDNA probes (lower panel). (B) Levels of MCAM mRNA were determined by densitometric scanning of the autoradiograph from the Northern blot analysis. The MCAM mRNA level for each condition was corrected with S26 mRNA expression.

Figure 9. Effects of forskolin on MCAM mRNA expression in human chorionic villous explant culture. (A) Fragments of placental villi in first trimester of pregnancy were cultured for 48 h in the presence of 200 mmol/l forskolin or vehicle alone. 10 μg of total RNA from each culture were subjected to Northern blot analysis using MCAM cDNA (upper panel) and S26 ribosomal protein cDNA probes (lower panel). (B) Levels of MCAM mRNA were determined by densitometric scanning of the autoradiograph of Northern blot analysis. MCAM mRNA level from each condition was corrected with S26 mRNA expression.


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