INTRODUCTION

Successful implantation and embryonic development depend on a series of complex, coordinated interactions between maternal and fetal tissues that is facilitated by a unique cell population, the trophoblast (Cross et al., 1994; Lala and Hamilton, 1996). Based on their distinctive morphology, immunophenotype, and biological functions, several trophoblastic subpopulations have been recognized and defined in the human placenta (Shih and Kurman, 1998). Among them, the implantation site intermediate (extravillous) trophoblast plays a key role in establishing the maternal/fetal circulation by infiltrating the implantation site where it invades and replaces the spiral arteries resulting in unhindered blood flow via the spiral arteries to the intervillous space in the placenta (Kurman et al., 1984). The mechanisms of trophoblastic invasion are similar to those involved in tumor cell invasion (Yagel et al., 1988). For example, implantation site intermediate trophoblastic cells are highly motile, elaborate protease enzymes, and are capable of escaping the host immune surveillance (Lala and Hamilton, 1996; Autio-Harma et al., 1992; Hurskainen et al., 1998; Qin et al., 1997; Fisher and Damsky, 1993; Muhlhauser et al., 1993; Shih and Kurman, 1996; McMaster et al., 1995). However, unlike malignant tumor cells, the migration and invasion of implantation site intermediate trophoblastic cells are spatiotemporally regulated. For example, invasion is relatively superficial never extending beyond approximately one third of the full myometrial thickness at 12 weeks of gestation (unpublished data). Extensive myometrial invasion occurs following the malignant transformation of implantation site intermediate trophoblast as in the case of placental site trophoblastic tumor, a rare gestational trophoblastic neoplasm (Kurman, 1991). The evidence that smooth muscle cells limit the migration/invasion of trophoblastic cells is supported by histologic observation of abnormal pregnancy sites such as tubal (ectopic) pregnancy. The high frequency of ruptured

SUMMARY

An immortalized implantation site intermediate trophoblastic cell line, IST-1, was established from a human placenta of 7 weeks gestation. IST-1 cells phenotypically resembled the implantation site intermediate trophoblastic cells in situ and expressed Mel-CAM (MUC 18 or CD146). Mel-CAM is a cell adhesion molecule belonging to the immunoglobulin gene superfamily. It is involved in heterophilic cell-cell adhesion and plays a role in several biological processes including tumor progression. We have previously shown that Mel-CAM was highly expressed in the intermediate (evallous) trophoblast in the human implantation site. In this study we determined the function of Mel-CAM in the interaction of trophoblast and uterine smooth muscle in the implantation site. IST-1 cells failed to adhere to immobilized recombinant Mel-CAM in solid phase whereas the uterine smooth muscle cells did. The presence of the putative Mel-CAM ligand in smooth muscle cells was further supported by the finding that Mel-CAM-transfected but not the mock-transfected U937 leukemia cells bind to the confluent monolayer of uterine smooth muscle cells. IST-1 cells attached efficiently to the monolayer of the uterine smooth muscle cells and acquired a spindle-shaped morphology simulating smooth muscle cells. The cell binding was only marginally affected by Mel-CAM blocking antibodies. However, Mel-CAM blocking antibodies and recombinant Mel-CAM promoted cell migration from IST-1 cell spheroids on the smooth muscle monolayer. Taken together, our results suggest that IST-1 cells express Mel-CAM but not the putative Mel-CAM ligand. In contrast, the uterine smooth muscle cells express the putative Mel-CAM ligand which binds to Mel-CAM on the surface of the IST-1 cells. The interaction between Mel-CAM and its putative ligand confers a stationary phenotype for trophoblastic cells. These observations are consistent with an important role for Mel-CAM in limiting trophoblastic migration within the myometrium in the implantation site.

Key words: Mel-CAM, Implantation, Myometrium, IST-1

INTRODUCTION

Successful implantation and embryonic development depend on a series of complex, coordinated interactions between maternal and fetal tissues that is facilitated by a unique cell population, the trophoblast (Cross et al., 1994; Lala and Hamilton, 1996). Based on their distinctive morphology, immunophenotype, and biological functions, several trophoblastic subpopulations have been recognized and defined in the human placenta (Shih and Kurman, 1998). Among them, the implantation site intermediate (extravillous) trophoblast plays a key role in establishing the maternal/fetal circulation by infiltrating the implantation site where it invades and replaces the spiral arteries resulting in unhindered blood flow via the spiral arteries to the intervillous space in the placenta (Kurman et al., 1984). The mechanisms of trophoblastic invasion are similar to those involved in tumor cell invasion (Yagel et al., 1988). For example, implantation site intermediate trophoblastic cells

Expression of Mel-CAM in implantation site intermediate trophoblastic cell line, IST-1, limits its migration on uterine smooth muscle cells

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fall the relatively thin layers of smooth muscle and lack of decidua in the wall of fallopian tubes which are not sufficient to restrain the trophoblastic invasion.

Melanoma cell adhesion molecule (Mel-CAM, MUC 18, or CD 146) is a membrane glycoprotein and functions as a Ca^2+-independent cell-cell adhesion molecule involved in heterophilic cell-cell interactions (Lehmann et al., 1989; Sers et al., 1993; Shih et al., 1994, 1997a). Mel-CAM is a member of the immunoglobulin gene superfamily containing the characteristic V-V-C2-C2-C2 immunoglobulin domain structure. The ligand by which Mel-CAM binds one cell to another is as yet unidentified. Mel-CAM expression has been demonstrated to promote tumor progression in human melanoma but it may act as a tumor suppressor in breast carcinoma (Shih et al., 1994, 1997a; Xie et al., 1997). We have previously shown that the differentiation of implantation site intermediate trophoblast is accompanied by Mel-CAM expression (Shih and Kurman, 1996). Mel-CAM immunoreactivity increases in intensity and distribution in intermediate trophoblastic cells from their proximal origin in the trophoblastic columns to the distal tip of the trophoblastic columns that anchor the placenta to the implantation site (Shih and Kurman, 1996). All implantation site intermediate trophoblastic cells are positive for Mel-CAM. Among all the normal tissues examined, they are the cells with the highest staining intensity (Fig. 1) (Shih et al., 1998). In light of the important role of cell adhesion molecules in regulating migration and invasion of trophoblast (Cross et al., 1994), we established a HPV-16 E6E7 transduced immortal implantation site intermediate trophoblastic cell line. We used this cell line as a model to study the role of Mel-CAM in the interaction between implantation site intermediate trophoblastic cells and uterine smooth muscle cells.

![SM](image)

**Fig. 1.** Immunoperoxidase staining for an anti-Mel-CAM antibody, MN-4, in a human implantation site of early gestation. All implantation site intermediate trophoblastic cells exhibit intense membrane staining for Mel-CAM. They infiltrate the endometrium but the invasion is confined within the smooth muscle cells (SM) of superficial myometrium. ×112.

### Materials and Methods

#### Establishment of IST-1 Cell Line

Fresh human placental tissue was collected from a patient undergoing elective abortion at 7 weeks gestation. The request for collection of placental tissue was approved by the Joint Committee for Clinical Investigation and Maternal-Fetal Tissue Research Committee of the Johns Hopkins Hospital. The specimen was washed in cold phosphate-buffered saline (PBS) and was carefully microdissected to remove fetal parts and endometrium. A representative frozen section of the microdissected placental tissue was stained with hematoxylin and eosin to ensure that it contained chorionic villi exclusively. The chorionic villi were transferred to a 10 cm tissue culture dish and were minced into approximately 1 mm³ villous explants as previously described (Yagel et al., 1989; Irving et al., 1995). The explants were plated on dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY) and penicillin/streptomycin (50 U/ml). After 5 days of incubation at 37°C and 5% CO₂ atmosphere the trophoblastic cells grew out from the chorionic villous explants. They were then harvested using trypsin-EDTA and were replated in tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ). The trophoblastic cells were immortalized by transducing the primary culture with a recombinant LXSN16E6E7 retrovirus encoding the HPV16 E6 and E7 transforming proteins with G418 selection marker as previously described (Halbert et al., 1991). Following transduction, the retroviral supernatant was removed and the cells were grown in RPMI 1640 medium containing G418 (400 µg/ml) for an additional 3 days to allow for integration and expression of the recombinant retroviral genes. The IST-1 cell line was obtained from a single clone of HPV-16 E6E7 transduced trophoblastic cells.

#### Cell Lines and Cell Culture

The IST-1 cells were maintained in RPMI 1640 containing 10% FBS and 400 µg/ml G418. The uterine smooth muscle cells (USSMC) which were shown to stain positively with α-smooth muscle actin and desmin were obtained from Clonetics (San Diego, CA). They were cultured in RPMI 1640 supplemented with 10% FBS. Human monocytic leukemia cells, U937, were maintained in RPMI 1640 with 10% FBS. Transfection of U937 cells with PCB6+/Mel-CAM vector was performed using electroporation as previously described (Shih et al., 1994). More than 99% of Mel-CAM transfected U937 cells (U937T cells) were positive for Mel-CAM by flow cytometry (Shih et al., 1994). U937T cells were cultured in RPMI 1640 with 10% FBS and 400 µg/ml. A primary fibroblast culture of chorionic villi was obtained from the fibroblastic outgrowth of villous explants and were maintained in DMEM supplemented with 10% FBS. These fibroblasts were positive for vimentin but negative for cytokeratin. A metastatic melanoma cell line, WM1205Lu, was kindly provided by Dr M. Herlyn (Wistar Institute, Philadelphia, PA) and was maintained in RPMI 1640 supplemented with 2% FBS.

#### Experimental Animals

Five pathogen-free female C.B-17 scid/scid (SCID) mice (Charles River, Cambridge, MA), 6-8 weeks old were injected with 5×10⁶ IST-1 cells in the subcutaneous tissue in the left lower flank and the uterine horns. The mice were sacrificed and were examined for the tumor formation 4 months after injection.

#### Reverse Transcription-Polymerase Chain Reaction

The expression of Mel-CAM, HPV-16 E6 and E7 was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). The reaction was performed following the protocol previously described (Saiki et al., 1985). Total RNA of IST-1 cells was extracted by acid-guanidium and phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The reverse transcription (RT) reaction was carried out with 1-5 µg total RNA in 20 µl reaction buffer and was incubated at 42°C for 50 minutes. Thirty cycles (94°C 1 minute, 52°C 1 minute,
were as follows: HPV-16 E6, 5'-GGGAGATCTTGACCAAGAGAACCTG-3'; HPV-16 E7, 5'-GGGAGAATTCCTGACGCCAGCTGGTTTCTTACGT-3'; HPV-16 E7, 5'-CCCCAGATCTACTACGATG-3' and 5'-TATGGACTCTGGAAACAGAT-3'; Mel-CAM (MUC 18), 5'-CCAAGGCAACCTCAGCATGTC-3' and 5'-CTCGAATCCACCUGTTCGCGACT-3'. The PCR cDNA products of E6, E7 and Mel-CAM were 384, 308 and 437 base pairs, respectively.

**Antibodies**

Anti-Mel-CAM mouse monoclonal antibody, MN-4, which recognizes the epitope in the second extracellular C2 domain and an anti-Mel-CAM rabbit polyclonal antibody have been previously described (Shih et al., 1998). Dr L. Zardi (Genova, Italy) kindly provided the BC-1 antibody which reacted to the ED-B sequence within oncofetal fibronectin (Carnemolla et al., 1989). Dr M. Herlyn (Wistar Institute, Philadelphia, PA) provided anti-integrin β3 antibody and anti-Mel-CAM antiseraum, and Dr S. Fisher (San Francisco, CA) provided the 4H84 antibody against HLA-G. Antibodies that reacted with the specific antigens listed below were purchased commercially: anti-high molecular mass cytoskeleton (903) monoclonal antibody from Enzo Diagnostics (Sarmingdale, NY), anti-HNK-1 (CD57) monoclonal antibody from Becton Dickinson (San Jose, CA), anti-MMP-2 (72 kDa collagenase IV/gelatinase A) monoclonal antibody from Neomarkers (Fremont, CA), anti-inhibin-α monoclonal antibody from Serotec (Kidlington, Oxford, UK), anti-epidermal growth factor receptor (EGF-R) monoclonal antibody, anti-E-cadherin monoclonal antibody, and anti-NCAM monoclonal antibody from Zymed (San Francisco, CA), anti-α-catenine and β-catenine monoclonal antibodies from Sigma (St Louis, MO), anti-cytokeratins 7 and 18 monoclonal antibodies, anti-human placental lactogen (hPL) polyclonal antibody, anti-integrin α5 monoclonal antibody, anti-epithelial membrane antigen (EMA) monoclonal antibody, anti-CD30 monoclonal antibody, anti-α-smooth muscle actin monoclonal antibody, anti-PECAM-1 (CD31) monoclonal antibody, Ber-Ep4 monoclonal antibody (Babury and Moscovic, 1993), anti-HAM 56 monoclonal antibody from DAKO (Carpinteria, CA). The dilution of antibodies and pretreatment of the tissue section were performed according to the manufacturers’ protocols.

**Immunohistochemistry**

For immunoperoxidase staining on IST-1 cells, IST-1 cells derived after 30 or more passages cultured on 8-well chamber CultureSlides (Becton Dickinson) were fixed in 3% paraformaldehyde at room temperature for 10 minutes with or without permeabilization using 0.3% NP-40 for another 10 minutes. After washes in PBS, the cells were incubated with the primary antibodies at 4°C overnight and the immunoreactivity was revealed using the avidin-biotin-complex peroxidase method with an immunostaining kit (Biogenex, San Ramon, CA) and the 3-amino-9-ethylcarbazole chromagen. The slides were counterstained with 0.1% hematoxylin (Sigma). Control samples were treated with normal mouse serum or preimmune rabbit serum.

For immunoperoxidase staining of formalin-fixed, paraffin-embedded tissue sections, five normal human placentas with implantation sites ranging from 7 to 12 weeks of gestation were evaluated for the expression of these antigens. 5 µm tissue sections were deparaffinized and incubated with primary antibodies overnight at 4°C followed by the avidin-biotin complex peroxidase method. Immunoreactivity was detected using 3-amino-9-ethylcarbazole chromagen and sections were counterstained with 0.1% hematoxylin.

**In vitro invasion assay**

Samples (500 µl, 1×10⁵ cells) of each cell suspension of IST-1 cells, the negative control HPV-16 transduced NIH3T3 cells, and the positive control WM1205Lu melanoma cells were plated on 24-well BioCoat Matrigel Invasion Chambers with 6.4 mm transwell inserts. The polycarbonate filters of the inserts contained 8 µm pores which were precoated with the traditional Matrigel containing laminin-rich extracellular matrix substrate and growth factors (Becton Dickinson). After 24 hours of incubation at 37°C, the transwell inserts were washed with culture medium and the cells were fixed with 3% paraformaldehyde for 10 minutes. Cells not entering the pores were removed by wiping the upper side of the membrane and the total number of invaded cells from 5 different fields on the filters underside were counted under a light microscope at ×200 magnification. To quantify invasion, the invasiveness index was defined as the ratio of the number of invaded IST-1 or NIH3T3 cells to that of WM1205Lu cells.

**Western blot analysis**

Subconfluent IST-1 cells were scraped from culture flasks, washed three times with PBS, and then lysed by vortexing vigorously in the RIPA lysis buffer containing 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, 2 µM phenylmethylsulfonyl fluoride, 5 µM EDTA, 1% deoxycholic acid, and 1% Triton X-100 for 10 minutes. After centrifugation, the supernatants were subject to western blot analysis on a 8% SDS-polyacrylamide gel and the membrane was probed with the MN-4 antibody as previously described (Shih et al., 1998). Immunoreactive bands were detected by chemiluminescence (Amersham, Arlington Heights, IL).

**Solid phase cell adhesion assay**

Solid phase cell adhesion assay and the preparation of nitrocellulose-coated plastic Petri dishes have been previously described (Shih et al., 1994). Briefly, 4 µl of purified recombinant Mel-CAM protein (200 µg/ml) and the control sample, bovine serum albumin (200 µg/ml) were applied to the nitrocellulose-coated dishes. Adherent cells were detached using versene containing EDTA (Gibco) and were washed and resuspended in serum-free RPMI 1640 medium. 2 ml of cell suspensions (1×10⁵ cells/ml) of IST-1 cells, UtsMC, WM1205Lu melanoma cells (positive controls), and U937 cells (negative control) were added to the dishes and incubated for 10 minutes at 37°C. Nonadherent cells were removed by three gentle washes with PBS and attached cells were fixed with 3% paraformaldehyde and the cell numbers were determined under a phase-contrast light microscope. Results were expressed as the total number of adherent cells from five randomly chosen fields within protein-coated areas at ×400 magnification from 5 samples.

**Adhesion and migration of cells on uterine smooth muscle cells**

IST-1 cells and U937(T) cells were labeled with 2 µm of fluorescent PKH26-GL vital dye (Sigma) for 2 minutes. For the adhesion assay, single cell suspensions (2×10⁵ cells) of fluorescence-labeled IST-1 cells and U937(T) cells were applied onto the lightly precoated Matrigel Invasion Chambers in 8-well chamber slides and incubated at 37°C for 6 hours for U937(T) cells or 20 minutes for IST-1 cells. The chambers were then gently washed with RPMI 1640 and examined under fluorescence microscope. Micrographs were photographed at a magnification of ×400. Five representative micrographs from each chamber were chosen and the total number of fluorescence-labeled cells was counted. Results were expressed as the average ± standard deviation from 5 chambers. For the competition assay, IST-1 cells were incubated for 15 minutes with Mel-CAM polyclonal antibody (1:500), MN-4 antibody (10 µg/ml), GRGDSP competitor peptide (1.5 mg/ml), or GRGESP control peptide (1.5 mg/ml) (Life Technologies, Grand Island, NY). All reagents were diluted in RPMI 1640 medium.
For the migration assay, 40 μl of the fluorescence-labeled IST-1 cell suspension (5×10⁴ cells) were hung over the lid of a 10 cm tissue culture dish (Becton Dickinson Labware, Franklin Lakes, NJ) at 37°C for 4 hours to allow aggregation into cell spheroids. The cell spheroids were co-cultured onto the confluent monolayer of UtSMC or fibronectin-coated (20 μg/ml) slides on 8-well chamber CultureSlides in RPMI 1640 medium-10% FBS with or without blocking reagents. After 24 hours, the chambers were gently washed, fixed in 3% paraformaldehyde, and examined under a Nikon fluorescence microscope (Melville, NY). Migration was quantitated as the differences between the radii of the region occupied by emigrating cells at the final time and at the initial time (Friedlander et al., 1996). Ten spheroids were analyzed in each group of treatment. For blocking assays, a Mel-CAM specific polyclonal antibody (1:500), MN-4 monoclonal antibody (10 μg/ml), and recombinant Mel-CAM (100 μg/ml) were used. Medium containing these blocking reagents was replaced with new antibodies and recombinant protein at the same concentration after 24 hours of co-culture.

RESULTS

Characterization of IST-1 cells

A cell line, IST-1, of implantation site intermediate trophoblast was established from a human placenta of 7 weeks gestation. Under phase-contrast microscopy, the majority of IST-1 cells were mononucleate and pleomorphic, varying from triangular to fusiform in shape (Fig. 2). Some of the cells exhibited, in part, extremely long cytoplasmic processes. Giant multinucleated cells comprised approximately 5-8% of the cell population. These cells had tightly clustered nuclei arranged in their centers. IST-1 cells formed a monolayer and showed cell-cell contact inhibition. The growth of IST-1 cells was serum-dependent with a doubling time of approximately 4 days in RPMI 1640 medium containing 10% FBS. A chromosomal analysis revealed that more than 90% of the IST-1 cells derived from 40 passages had a diploid karyotype of 46, XX with a normal G-banding pattern. In vitro invasiveness analysis showed that IST-1 cells were invasive through Matrigel with an invasiveness index of 0.91, close to that of highly metastatic melanoma cells, WM1205Lu (index = 1.00). In contrast, the negative control HPV-16 transduced NIH 3T3 cells were not invasive (index = 0). The invasion of IST-1 cells could be inhibited in the presence of the synthetic peptide containing an arginine-glycine-aspartic acid (RGD) sequence (index = 0). An immunohistochemical analysis using a panel of antibodies demonstrated that the immunophenotype of IST-1 cells was consistent with that of the implantation site intermediate trophoblastic cells in vivo (Table 1). IST-1 cells were immunoreactive to cytokeratins 7 and 18 antibodies but not to the high-molecular-mass cytokeratins. Among implantation site intermediate trophoblast-associated markers, IST-1 cells expressed hPL, Mel-CAM, oncofetal fibronectin, integrins α5 and β3, MMP-2, and prolyl 4-hydroxylase but not HLA-G. IST-1 cells failed to express HNK-1 and NCAM which are specifically expressed in villous intermediate trophoblast in the trophoblastic column and in intravascular intermediate trophoblast, respectively (Shih et al., 1997c; Burrows et al., 1994). Furthermore, IST-1 cells were negative for a variety of markers that are expressed by other cell types in the implantation site including decidua, fibroblast, endothelial cells, smooth muscle cells, inflammatory cells and epithelial cells. Representative staining of Mel-CAM and cytokeratin is shown in Fig. 3. To assess the tumorigenicity of IST-1 cells, 5×10⁴ cells were inoculated into the subcutaneous tissue and the uterine horns of SCID mice. No evidence of tumor formation was observed in either site after 4 months of injection. IST-1 cells have been continuously propagated for more than 65 passages in culture. Expression of E7 mRNA in IST-1 cells was demonstrated by RT-PCR (Fig. 4).

Expression of Mel-CAM in IST-1 cells

Immunohistochemically, all IST-1 cells examined including the multinucleated cells in culture were immunoreactive to a Mel-CAM specific monoclonal antibody, MN-4 (Fig. 3). The staining was predominantly confined to the cell membrane and distributed along the lamillapodia extending from the cell surface by immunofluorescence (data not shown). Flow cytometry demonstrated that the staining intensity and percentage of positive cells in early versus late passages were similar (>99% positive). Western blot analysis using MN-4 antibody revealed a single 113 kDa protein band of the expected molecular mass from the IST-1 cell membrane extract but not from the villous fibroblast membrane extract (Fig. 4A). RT-PCR demonstrated the amplified DNA products with the expected size of 435 base pairs from IST-1 cell mRNA (Fig. 4B). The Mel-CAM nucleotide sequence was proved by direct DNA sequencing (data not shown). The expression of Mel-CAM in IST-1 cells appeared not to be affected by several culture conditions including hypoxia (1% oxygen tension), low serum concentration (1% FBS), addition of Forskolin (20 μM), leukocyte inhibiting factor (2,000 U/ml) or hCG (10,000 mIU/ml), and in co-culture with UtSMC (data not shown).

Expression of the putative Mel-CAM ligand in IST-1 cells and UtSMC

The expression of the putative Mel-CAM ligand was evaluated by the ability of cells to bind the immobilized recombinant...
Table 1. Immunophenotype of IST-1 cells

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Abbreviations: IT, intermediate trophoblast; CT, cytotrophoblast; ST, syncytiotrophoblast; FB, villous fibroblast; DC, decidua; ET, endothelial cells; SM, smooth muscle; MC, macrophages; EE, endometrial epithelium; HMM, high-molecular-mass; hPL, human placental lactogen; hCG, human chorionic gonadotropin; FN, fibronectin; pro-4-OHase, prolyl-4-hydroxylase; EGF-R, epidermal growth factor receptor; EMA, epithelial membrane antigen. nt, not tested.

*Indicates the relative immunointensity: ++, strong; +, moderate; +/−, weak; −, negative.

‡The immunointensity is weak or absent in the extravillous intermediate trophoblast.

§The immunoreactivity for intermediate trophoblast is confined to the intravascular trophoblast.

¶The immunostaining is distributed exclusively in the villous intermediate trophoblast but not in the extravillous intermediate trophoblast.

Mel-CAM on a nitrocellulose membrane. As shown in Fig. 5, IST-1 cells, as compared with the negative controls coated with BSA, did not adhere to the areas coated with recombinant Mel-CAM whereas the positive control, WM1205Lu melanoma cells, strongly adhered to recombinant Mel-CAM. In contrast to IST-1 cells, a significant number of UtSMC attached to the

Fig. 3. Immunostaining of Mel-CAM and cytokeratin 7 in IST-1 cells. All the IST-1 cells are positive for both markers. Mel-CAM immunoreactivity is predominantly distributed in the cell membrane. ×144.
immobilized recombinant Mel-CAM. The binding activity was partially inhibited to 60% for UtSMC and to 40% for WM1205Lu by Mel-CAM specific polyclonal antibody.

**Binding of IST-1 cells and U937 cells on UtSMC monolayer**

To further confirm that UtSMC exhibited Mel-CAM ligand binding activity, we co-incubated U937 leukemia cells on the confluent monolayer of UtSMC. Fifty percent of U937T cells which were transfected with an expression vector containing Mel-CAM cDNA attached well onto the UtSMC monolayer (Fig. 6). In contrast, the U937 cells transfected with the vector alone demonstrated negligible binding activity to UtSMC (Fig. 6). The attachment of U937T cells on the UtSMC monolayer was time-dependent and the adherence was not evident until 6 hours of co-incubation. Unlike U937T cells, the adherence of IST-1 cells to the UtSMC monolayer occurred a few minutes after co-incubation. As shown in Table 2, the binding activity appeared to be only slightly inhibited by Mel-CAM polyclonal and monoclonal MN-4 antibodies. Under phase-contrast microscopy, the IST-1 cells which spread over the UtSMC monolayer were exhibiting a fusiform or spindle-shaped morphology that was indistinguishable from the underlying smooth muscle cells (Fig. 7).

Since UtSMC produced abundant extracellular matrix including fibronectin (Kobayashi et al., 1996; and our unpublished result) and trophoblast expressed a variety of integrins (Loke and King, 1995; Damsky et al., 1992, 1994), we tested whether attachment of IST-1 cells on UtSMC monolayer was mediated by the RGD sequence which was recognized by integrins on the cell surface of IST-1 cells. Under phase-contrast microscopy, the IST-1 cells which spread over the UtSMC monolayer were exhibiting a fusiform or spindle-shaped morphology that was indistinguishable from the underlying smooth muscle cells (Fig. 7).

Migration of IST-1 cells on UtSMC monolayer

To assess the functional role of Mel-CAM in the cell-cell interaction of IST-1 cells and UtSMC, we performed a cell migration assay of IST-1 cells on the confluent UtSMC monolayer and on the control fibronectin-coated slides. As shown in Fig. 8, very few fluorescence-labeled IST-cells detached from the cell spheroid and migrated on the UtSMC monolayer after 24 hours of co-culture. For comparison, IST-1 cells were highly migratory from the cell spheroids cultured on the fibronectin-coated slides and the migration ability was not affected by the blocking antibodies or recombinant Mel-CAM. In the presence of Mel-CAM specific polyclonal antibody, Mel-CAM monoclonal antibody, MN-4, and the recombinant Mel-CAM, an increased number of IST-1 cells migrated out from the spheroid. The migration index of IST-1 cells with different treatments was shown in Fig. 9. There was a statistically significant difference in migration index on UtSMC monolayer between controls (without antibody

### Table 2. Binding of IST-1 cells to the UtSMC monolayer and fibronectin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells bound UtSMC</th>
<th>No. of cells bound fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>105±19 (1.00)</td>
<td>125±17 (1.00)</td>
</tr>
<tr>
<td>+Mel-CAM polyclonal Ab</td>
<td>88±15 (0.84)</td>
<td>199±12 (0.95)</td>
</tr>
<tr>
<td>+MN-4 Ab</td>
<td>98±17 (0.93)</td>
<td>130±20 (1.04)</td>
</tr>
<tr>
<td>+GRGESP control peptide</td>
<td>95±21 (0.90)</td>
<td>121±18 (0.97)</td>
</tr>
<tr>
<td>+GRGDSP competitor peptide</td>
<td>106±11 (1.01)</td>
<td>7±51 (0.06)</td>
</tr>
</tbody>
</table>

*Ab, antibody.
†Assays were performed as described in Materials and methods. Averages and standard deviations were shown for five determinants. The values in the parentheses reflect the ratio of the average experimental value to the average control (no treatment) value.
treatment or with preimmune serum) and those with polyclonal antibody, MN-4 antibody, and recombinant Mel-CAM ($P<0.001$).

**DISCUSSION**

Study of the biology of intermediate (extravillous) trophoblast in the implantation site has been limited by the lack of immortalized cell lines that represent this unique cell population in vitro. In the past, HTR-8/SVneo trophoblastic cell line transfected with simian virus 40 large T antigen was the only immortalized cell line available (Graham et al., 1993). In the current study, we established an implantation site intermediate trophoblastic cell line, IST-1, which was immortalized by HPV-16 E6E7. We demonstrated that IST-1 cells were phenotypically similar to the implantation site intermediate trophoblast in vivo.

Morphologically, IST-1 cells resemble other implantation site intermediate trophoblastic cells established previously (Yagel et al., 1989; Graham et al., 1993) and are capable of fusing into multinucleated intermediate trophoblastic cells, a characteristic feature of implantation site intermediate trophoblast in vivo. Immunohistochemically, IST-1 cells expressed the simple epithelium-type cytokeratins including cytokeratins 7 and 18 but not the high-molecular-mass cytokeratins, an immunostaining finding characteristic of trophoblast in vivo (unpublished data). Of all the implantation site intermediate trophoblast-associated markers tested, only HLA-G was negative in the IST-1 cell line. The mechanism underlying the negative HLA-G staining in IST-1 cells is not clear. One of the possibilities is that HLA-G expression in trophoblast is inducible and requires a specific microenvironmental milieu which is missing in the culture conditions. Alternatively, the HLA-G gene in IST-1 cells may harbor mutations that results in a lack of expression since homozygosity for the 1597delC ‘null’ allele in the HLA-G gene has been previously reported (Ober et al., 1998). The negative staining pattern for a variety of markers expressed in other cell types in the implantation site was consistent with the trophoblastic origin of the IST-1 cells. Functionally, IST-1 cells were highly motile and invasive, and they produced hPL. Since immortalization of the intermediate trophoblast may confer a ‘premalignant’ phenotype to these cells in culture (Graham et al., 1993), it is possible that HPV immortalized IST-1 cells may also exhibit a ‘premalignant’ feature. However, many of the normal phenotypic features examined are fully retained by these cells including a relatively prolonged doubling time, serum and anchorage-dependent growth, cell-cell contact inhibition, a normal karyotype and lack of tumorigenicity in SCID mice. These features support our interpretation that IST-1 cells may well represent the in vitro counterpart of the
implantation site intermediate trophoblast of early gestation. Accordingly, this cell line appears as an appropriate model to study the biology of implantation site intermediate trophoblast in vitro although future studies to compare IST-1 cells with non-immortalized trophoblastic cells are required to support this view. In this study, we used IST-1 cells to explore the functional role of Mel-CAM in the interaction of implantation site intermediate trophoblastic cells and smooth muscle cells.

IST-1 cells expressed Mel-CAM as demonstrated by immunostaining, western blot analysis, and RT-PCR. However, these cells did not show the binding activity to Mel-CAM since IST-1 cells failed to bind the immobilized recombinant Mel-CAM in solid phase. To further explore this observation, we performed a heterotypic cell-cell aggregation assay by incubating fluorescence-labeled IST-1 cells and unlabeled U937 cells in mixed culture suspension. IST-1 cells did not aggregate with Mel-CAM transfected U937T cells (data not shown). In contrast to IST-1 cells, UtSMC demonstrated substantial binding activity to the recombinant Mel-CAM in solid phase. In addition, Mel-CAM transfected U937T cells but not the mock-transfected U937 cells adhered to the UtSMC monolayer in cell suspension. These findings suggest that UtSMC expressed the putative Mel-CAM ligand. The binding activity of UtSMC to recombinant Mel-CAM is similar to that of the smooth muscle cells of vascular origin (Shih et al., 1994).

Adhesion of IST-1 cells on the UtSMC monolayer was only marginally inhibited by the blocking antibodies. This binding activity is neither affected by the presence of competitor peptides containing the RGD sequence which is the integrin binding motif present in a variety of extracellular matrix proteins and cell adhesion molecules (Hynes, 1992). In contrast, adhesion of IST-1 cells to fibronectin was competitively inhibited by the RGD-containing peptide. This finding indicates that the RGD sequence is involved in the interaction of IST-1 cells and extracellular matrix and is important for the migration of IST-1 cells on fibronectin substrate which is produced by IST-1 cells (Irving and Lala, 1995). However, the RGD sequence is not involved in cell-cell interaction between IST-1 cells and UtSMC.

Although Mel-CAM expression is not sufficient to mediate cell-cell adhesion between IST-1 cells and UtSMC, Mel-CAM-ligand interaction may impart a signal for a stationary phenotype in implantation site intermediate trophoblast. This is supported by our finding that the blocking antibodies specific for Mel-CAM and recombinant Mel-CAM induced an extensive outgrowth from the IST-1 cell spheroids on UtSMC monolayer. In accordance with our findings, previous study has also demonstrated that integrin subunit β1 did not affect the attachment of the blastocysts to human decidual cells but it was involved in the migration of embryos on the decidual cells (Shiokawa et al., 1996). These results imply that cell attachment and migration are mediated by different mechanisms. Indeed, cell migration is intricately coordinated by a multiplicity of signals and the dynamic interaction between the cell, its substrate, and the cytoskeleton-associated motile apparatus that results in sequential adhesion and dysadhesion of cells (Irving and Lala, 1995; Huttenlocher et al., 1995).

Besides their role in cell adhesion, cell adhesion molecules...
can modulate gene expression and cellular phenotype through a cascade of an ‘outside-in’ signaling system (Weaver et al., 1997). For example, gene expression can be modulated by E-cadherin through the β-catenin/Tcf (Lef-1) pathway (Behrens et al., 1996) and by integrin through the tyrosine phosphorylation of several cytoplasmic/cytoskeletal proteins (Yamada and Miyamoto, 1995; Càry et al., 1998). It has been shown that expression of α5 integrin in myoblasts upregulates N-cadherin expression which in synergy with integrin regulates contact inhibition of migration and motility of myoblasts (Huttenlocher et al., 1998). The cytoplasmic domain of Mel-CAM contains several putative phosphorylation sites which may be responsible for the homodimerization of Mel-CAM (unpublished data). It is possible that ligand binding activates Mel-CAM, leading to a sequential activation of putative downstream effectors which in turn modulate the expression and binding activity of other cell adhesion molecules (Ginsberg et al., 1992).

The direct cell-cell contact between IST-1 cells and smooth muscle cells also modulates the morphology of trophoblastic cells. As compared with the culture of IST-1 cells alone on fibronectin substrate, the IST-1 cells assumed a homogenous spindle shape, similar to UtSMC in which IST-1 cells spread over. This finding is reminiscent of the morphologic plasticity of implantation site intermediate trophoblastic cells observed in vivo as they resemble smooth muscle cells when they infiltrate the myometrium. This finding is in accordance with the previous observations showing that trophoblast behavior during implantation can be influenced by the extracellular matrix in the endometrium (Burrows et al., 1993; Irving and Lala, 1995) and suggests that the phenotype of trophoblastic cells is controlled not only by its developmental lineage but also by the microenvironment.

In conclusion, we established the IST-1 cell line and used it as an in vitro model to dissect the biological mechanisms underlying the interaction between implantation site intermediate trophoblast and its microenvironment. Our study suggests that the regulation of migration/invasion of the implantation site intermediate trophoblast in the myometrium is conferred, at least in part, by the interaction of Mel-CAM expressed by the trophoblastic cells and by the putative Mel-CAM ligand on the juxtaposed smooth muscle cells. Identification of the Mel-CAM ligand and its downstream pathways of signal transduction is important to gain further insight into the role of this novel cell adhesion molecule in normal human implantation and in various pathologic conditions.

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