Anti-CD9 monoclonal antibody-stimulated invasion of endometrial cancer cell lines in vitro: possible inhibitory effect of CD9 in endometrial cancer invasion

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Cell surface marker CD9 has been reported to play a role in inhibiting trophoblastic cell invasion. Since the invasive properties of cancer cells may resemble those of trophoblasts, we decided to investigate the role of CD9 in the invasion of endometrial cancer cells. In normal human endometrium, CD9 was found to be constitutively expressed on epithelial cells, as reported previously. While epithelial cells of endometrial hyperplasia (n = 5) were also positive for the expression of CD9, endometrial adenocarcinomas (n = 15) showed reduced expression. In order to clarify the significance of this reduced CD9 expression in endometrial cancer, an in-vitro invasion assay system was used to assess the effect of anti-CD9 monoclonal antibody (mAb) on the invasive properties of endometrial cancer cell line. Anti-CD9 mAb significantly enhanced invasion of the RL95-2 and Ishikawa cell lines, without affecting cell proliferation. Since CD9 is associated with the integrin subunits β₁, α₃ and α₆ in human endometrium, we investigated the functional relationship between CD9 and these integrins in the RL95-2 cell line. Monoclonal antibodies against the integrins β₁, α₃ and α₆ inhibited RL95-2 cell invasion. However, anti-CD9 mAb continued to show a stimulatory effect on RL95-2 cell invasion after treatment with anti-integrin α₃ mAb. In contrast, the anti-CD9 mAb had no effect after treatment with the mAb for integrins α₆ and β₁. These findings indicate that CD9 is involved in regulating the invasive properties of endometrial carcinoma cells and that this effect is partially mediated by integrin subunits α₆ and β₁. Thus, CD9 appears to be involved in the prevention of endometrial cancer invasion.

Key words: CD9/endometrium/endometrial cancer/integrin/invasion

Introduction

In a recent study, we found that a cluster of differentiation (CD) antigen 9 molecules was expressed on the human endometrial epithelial cells throughout the menstrual cycle (Park et al., 2000). The CD9 molecule is a 24–27 kDa glycoprotein, originally reported to be expressed on the cell surface of haematopoietic cells, e.g. pre-B-lymphocytes, platelets and activated T-lymphocytes (Kersey et al., 1981; Boucheix et al., 1983). A number of studies using anti-CD9 monoclonal antibodies (mAb) have shown that the CD9 molecule is involved in the functions of haematopoietic cells, e.g. platelet activation, aggregation and adhesion, neutrophil adhesion and pre-B cell adhesion (Jennings et al., 1990; Masselis-Smith and Shaw, 1990; Forsyth, 1991). It has also been reported that the CD9 molecule is involved in Schwann cell migration and adhesion (Hadjiargyrou and Patterson, 1995).

We have found that CD9 is expressed on the extravillous trophoblasts in the cell columns of first trimester human placenta, which then differentiate into invading extravillous trophoblasts (Hirano et al., 1999a). On the other hand, CD9 is not expressed in villous trophoblasts of the human placenta. Moreover, the intensity of CD9 expression in extravillous trophoblast cells differs between early pregnancy and term; i.e. the expression of CD9 in the extravillous trophoblast invading the endometrium in early pregnancy is weak, but that in the placental bed and chorionic laevae of the term placenta is very intense. These results imply that the intense expression of CD9 tends to be associated with the extravillous trophoblasts which have ceased migration or invasion into the endometrium. Furthermore, it has been shown that anti-CD9 mAb significantly enhanced the invasion of BeWo cells, a trophoblast like choriocarcinoma cell line, in vitro (Hirano et al., 1999b). These findings suggest that CD9 may have an important role in inhibiting cell invasion.

Many elements of trophoblast invasion resemble the events that occur during malignant tumour cell invasion (Aplin, 1991; Tabibzadeh and Babaknia, 1995), suggesting that CD9 may also be involved in regulating cancer cell invasion. Therefore, in this study, we examined the expression of CD9 in both endometrial hyperplasia and in endometrial cancer, and observed a reduced expression of CD9 in human endometrial cancer in comparison with normal endometrial epithelium. This observation led us to investigate the effect of anti-CD9 mAb on the cell proliferation and invasive properties of endometrial cancer using endometrial cancer cell lines, e.g. RL95-2 (Way et al., 1983) and Ishikawa (Nishida et al., 1985).
Materials and methods

Tissues
Specimens of endometrial hyperplasia were obtained from five patients aged 44–81 years; specimens of endometrial carcinoma were obtained from 15 patients aged 39–79 years. The endometrial hyperplasias consisted of one simple and four atypical hyperplasias. The 15 cases of endometrial carcinoma consisted of 10 in stage I, two in stage III, two in stage IV and one recurrent according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Histologically, all carcinomas were classified as endometrioid, with six being well differentiated (G1), five moderately differentiated (G2), and four poorly differentiated (G3). None of the patients received any hormonal or drug therapy. This study was approved by the ethical committee of Kyoto University and preoperative informed consent was obtained from all patients.

Antibodies
Two mouse anti-human CD9 monoclonal antibodies (mAbs), TP-82 (immunoglobulin G1 (IgG1) class) and ALB-6 (IgG1 class), were purchased from Nichirei Co (Tokyo, Japan) and Cosmo Bio Co Ltd (Tokyo, Japan) respectively (Boucheix et al., 1983; Higashihara et al., 1985). An anti-human CD9 mAb (SYB-1) a generous gift from Dr C.Boucheix and Dr E.Rubinstein, INSERM U268, Hôpital Paul Brousse, France (Boucheix et al., 1983). An unrelated mouse anti-trinitrophenyl (anti-TNP) mAb (IgG1 class) and an anti-mouse erythroid cell mAb, TER199 (IgG2a class) were used for negative controls (Tsujimura et al., 1990; Ikuta et al., 1997).

Two mouse anti-human integrin α3 mAb, 11G5 (IgG1 class) and PIB5 (IgG1 class), were purchased from Serotec (Oxford, UK) and Gibco-BRL (Gaithersburg, MD, USA) respectively. The mouse anti-human integrin α5 mAb OG-1 (IgG1 class) was developed in our laboratory (Fujiwara et al., 1993). The rat anti-human integrin α6 mAb GoH3 (IgG2a class) was purchased from Serotec. Three mouse anti-human integrin β3 mAb: DF5 (IgG1 class), 3S3 (IgG1 class) and P4C10 (IgG1 class) were purchased from Affinity Research Products Ltd (Nottingham, UK), Serotec and Gibco-BRL. ALB-6, PIB5, GOH3, and 3S3 reportedly have functional blocking effects on their own antigens (Boucheix et al., 1983; Carter et al., 1990; Sonnenberg et al., 1990; Gao et al., 1995). Fluorescein isothiocyanate (FITC)- conjugated rabbit anti-mouse immunoglobulins or FITC-conjugated goat anti-rat immunoglobulins were purchased from Dako (Glostrup, Denmark) and were used as the secondary antibodies for histochemistry. A horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) was used as the secondary antibody for Western blotting.

Indirect immunofluorescence staining
Immunohistochemical analysis was performed as described elsewhere (Fujiwara et al., 1993) with minor modifications. Individual specimens were embedded in OCT compound (Tissue-Tec; Miles Scientific, Naperville, IL, USA), snap-frozen in liquid nitrogen and stored at −80°C. Frozen tissues were sliced to a thickness of 7 µm with a cryostat microtome (Cryocut 1800, Reichert-Jung, Heidelberg, Germany), immediately air-dried on Neoplene (Nisshin EM, Tokyo, Japan)-coated glass slides and fixed in acetone at −20°C for 5 min. The slides were either examined immediately or stored at −20°C until use. The slides were incubated with anti-CD9 monoclonal antibodies, TP-82 (5 mg/ml) and ALB-6 (5 µg/ml) or the anti-TNP mAb (5 µg/ml), for 30 min at room temperature. After washing in phosphate-buffered saline (PBS), they were incubated with FITC-conjugated secondary antibody (diluted 1:40), for 30 min at room temperature in the dark. The slides were then washed, mounted with Perma Fluor Aqueous Mounting Medium (Immunon, Pittsburgh, PA, USA), which reduces fluorescence fading, and examined under a fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometry
The dispersed RL95-2 and Ishikawa cells (2×10⁵) were centrifuged and incubated at 4°C for 30 min with 10 ml of the anti-CD9 mAb (ALB-6, 100 µg/ml), the anti-integrin mAb (PIB5 for α3, OG-1 for α5, or P4C10 for β3 subunit; 100 µg/ml each), or the anti-TNP mAb (negative control, 100 µg/ml). The cells were washed twice with Hanks’ balanced salt solution (HBSS), and incubated with 20 ml of FITC-conjugated rabbit anti-mouse immunoglobulins (diluted to 1:40) at 4°C for 30 min in the dark. The cells were then washed three times and resuspended in HBSS after the second antibody incubation, and the number of viable cells was analysed using flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Three independent experiments were performed.

Invasion assay
The invasion assay was carried out as previously described (Hirano et al., 1999b). The cell lines were cultured on cell culture inserts (6.4 mm in diameter of membrane; Becton Dickinson Labware, NJ, USA) containing polyethylene terephthalate membranes with 8 µm diameter pores, which were placed in each well of a 24-well tissue culture plate (Falcon, Becton Dickinson Labware). Prior to cell culture, the upper surface of the filters was coated with cold Matrigel (100 µg/cm², Collaborative Research Co, Bedford, MA, USA) and air-dried aseptically. The inserts were rehydrated with 100 µl of warm Dulbecco’s modified Eagle’s medium (DMEM) for 2 h. A total of 60 000 viable RL95-2 or Ishikawa cells suspended in 200 ml of DMEM supplemented with 10% fetal calf serum (FCS) were inoculated into the culture inserts (upper chamber), and 700 ml of medium was added to the culture well (lower chamber). The anti-CD9 mAb (ALB-6; 0, 0.05, 0.5, and 5 µg/ml) or the control antibody (anti-TNP, 5 µg/ml) was then added into the culture medium (DMEM with 10% FCS). After 48 h of culture period, the upper surface of the filter was ‘scrubbed’ three times with a cotton swab. Cells remaining on the lower surface of the ‘scrubbed’ filter were fixed in methanol for 10 min at room temperature, and stained with haematoxylin. For quantification, the cells that had migrated to the lower surface were counted under a microscope in five pre-determined fields at ×200 magnification. The assay was performed in triplicate chambers. Five independent experiments of the cell culture lines were performed.

In RL95-2 cells, the effects of the anti-integrin mAb (PIB5 for subunit α3, GoH3 for subunit α5, or 3S3 for subunit β3) (0, 1.0, and 5.0 µg/ml) on invasion were examined. Furthermore, the effect of the anti-CD9 mAb (ALB-6) on RL95-2 cell invasion was also examined in the presence of the anti-integrin α3, α5, or β3 mAb.

Assessment of cellular proliferation
To investigate the effect of ALB6 (anti-CD9) on RL95-2 and Ishikawa cell proliferation, these cells (6×10⁵ cells) were suspended in DMEM with 10% FCS and plated onto Matrigel-coated (100 µg/cm² as described above) or non-coated 96-well polystyrene dishes at 200 ml/well. The anti-CD9 mAb (ALB-6; 0, 0.05, 0.5, and 5 µg/ml) or the control mAb (5 µg/ml) were then added to the culture medium, and the cells were cultured in triplicate. After 2 days of culture, the cultured cells were dispersed with 0.05% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.02% EDTA, and Trypan Blue staining was used to count the number of viable cells.

Affinity chromatography and Western blotting
Affinity chromatography and Western blot analysis were performed as described previously (Hirano et al. 1999a). RL95-2 cells (4×10⁷)
were homogenized in 5 ml of 40 mmol/l phosphate buffer, pH 7.3, containing 150 mmol/l NaCl, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 1% CHAPS and protease inhibitors including p-aminophenylmethanesulphonylfluoride hydrochloride (0.25 mg/ml), 10 mg/ml leupeptin (Peptide Institute Inc, Osaka, Japan), and 10 mg/ml pepstatin (Peptide Institute). After centrifugation (9000 g for 30 min), the concentration of CHAPS in the lysate was reduced by dilution to 0.3%. The lysate was then passed through a column containing 10 ml of anti-TNP-conjugated Affigel 10 (Bio-Rad Laboratories, Hercules, CA, USA, 2 mg IgG/ml gel) at 4°C to remove non-specifically bound compounds. The through-pass fractions were incubated with 0.2 ml of anti-CD9 (TP-82, 100 mg IgG/ml gel), anti-integrin α₃ (11G5, 100 μg IgG/ml gel) or α₉-conjugated Affigel 10 (OG-1, 200 mg IgG/ml gel) at 4°C for 2 h. After thorough washing of the gels, the purified antigens and their associated proteins were eluted with a sample buffer and transferred after 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (non-reduced conditions) onto a polyvinylidine difluoride (PVDF) membrane. The membranes were stained with anti-CD9 mAb (SYB-1), the anti-integrin β₁ mAb (DFS), or the control mAb (anti-TNP) as described earlier.

Statistical analysis
The data are expressed as means ± SEM, and were analysed by the two-tailed paired t test for pairwise comparisons or the one-way analysis of variance, followed by Scheffe’s F test for multiple comparisons.

Results

Immunohistochemical analysis of CD9 expression in human endometrium, endometrial hyperplasia and endometrial cancer
In normal endometrium, a high level of CD9 antigen was detected on the cell surface of luminal and glandular epithelial cells (Figure 1A–C). There were no significant differences in the intensity of expression of CD9 on epithelial cells throughout the menstrual cycle, as reported previously (Park et al., 2000).

In endometrial hyperplasia, CD9 was also expressed on epithelial cells and its expression intensity was similar to that of normal endometrial epithelial cells (Table I). In contrast, the expression of CD9 in endometrial cancer (endometrioid type) appeared to be lower than that in normal endometrium (Figure 1D–I and Table II).

Detection by flow cytometry of CD9 and integrins α₃, α₉ and β₁ in cultured RL95-2 and Ishikawa cells
Flow cytometry was performed in order to confirm the expression of CD9 and integrin subunits α₃, α₉ and β₁ in the endometrial cancer cell lines, RL95-2 (Figure 2) and Ishikawa (data not shown). Flow cytometry revealed that CD9 was expressed in RL95-2 (92 ± 7%) and Ishikawa (95 ± 3%) cells. Integrins α₃, α₉ and β₁ were also expressed in RL95-2 (76 ± 11, 73 ± 10 and 82 ± 12% respectively) and Ishikawa (92 ± 3, 83 ± 3 and 97 ± 2% respectively) cells (mean ± SD, n = 3).

Effect of anti-CD9 on cell invasion and cell proliferation of RL95-2 and Ishikawa cells
In the presence of anti-CD9 mAb (ALB-6), the number of invading RL95-2 and Ishikawa cells increased significantly in a dose-dependent manner (Figure 3). The invasiveness of RL95-2 and Ishikawa cells was enhanced by 230 and 155% respectively by anti-CD9 mAb (ALB-6) at a concentration of 0.5 mg/ml. The control anti-TNP antibody had no effect on invasion by either RL95-2 or Ishikawa cells.

On the other hand, anti-CD9 mAb did not affect cell proliferation of RL95-2 or Ishikawa cells for 2 days in either Matrigel-coated or non-coated dishes (data not shown).

Effect of anti-integrins α₃, α₉ and β₁ mAbs on cell invasion of RL95-2 cells
In order to clarify the functional association of CD9 with integrin α₃, α₉ and β₁, the effect of anti-CD9 mAb on the invasion by RL95-2 cells was examined in the presence of blocking mAbs against these integrins. Anti-integrin α₃ mAb (P1B5), α₉ mAb (GoH3), and β₁ mAb (3S3) reduced the number of invading RL95-2 cells. While the anti-CD9 mAb ALB-6 enhanced RL95-2 cell invasion in the presence of the anti-integrin α₃ mAb, it had no effect on RL95-2 cell invasion following treatment with the anti-integrin α₉ and β₁ mAbs (Figure 4).

Association of CD9 with integrin α₃, α₉ and β₁ in the endometrium and in RL95-2 cells
Western blot analysis of the proteins purified from the RL95-2 cells by anti-CD9 mAb (TP-82) detected integrin β₁ as a protein band at 110 kDa (Figure 5, Lane 4), which is compatible with the findings of the Western blotting analysis using the whole lysate of human endometrial tissue (Park et al., 2000).

In the proteins purified with anti-integrin α₃ (11G5) and α₉ (OG-1) mAbs from the RL95-2 cells, CD9 was clearly detected as a protein band at 26.5 kDa (Figure 5, lanes 2 and 3), indicating that these integrins were associated with CD9. This is also compatible with findings from whole lysate of endometrial tissue (Park et al., 2000).

Discussion
In contrast to that in normal endometrium and endometrial hyperplasia, the expression of CD9 in the endometrial carcinomas appeared to be reduced. This suggests that the production of CD9 might be altered during carcinogenesis in the human endometrium. It is clear that acquisition of the malignant phenotype during carcinogenesis is associated with altered regulation of cellular biological functions, such as cellular proliferation and invasion. In this study, we therefore used endometrial cancer cell lines to investigate the effect of anti-CD9 mAb on cell proliferation and the invasive properties of endometrial cancer. Anti-CD9 mAb significantly promoted the number of invading cells in the endometrial cancer cell lines, e.g. RL95-2 and Ishikawa. Since anti-CD9 did not affect cell proliferation, its stimulatory effect on the number of invading cells must have been due to an enhancement of the invasive property itself, rather than to enhanced cell proliferation. These findings suggest that CD9 may be involved in regulating the invasive properties of these cell lines.

We could not determine whether the binding of anti-CD9 mAb activates or inhibits the function of the CD9 molecule.

Involvement of CD9 in endometrial cancer invasion
Figure 1. Expression of CD9 in endometrium and endometrial cancer as detected by immunofluorescence staining. (A–C) normal human endometrium in the secretory phase (cycle day 21); (D–F) endometrial cancer (endometrioid type G1); (G–I) endometrial cancer (endometrioid type G3). (A) (D) and (G) haematoxylin and eosin staining; (B) (E) and (H) immunostaining with the anti-CD9 monoclonal antibody (mAb). (C) (F) and (I) anti-trinitrophenyl (anti-TNP) mAb (negative controls). Intense expression of CD9 was detected on glandular epithelial cells in the endometrium. CD9 expression was reduced in endometrioid type endometrial cancer. Scale bar = 100 µm.

Table I. Fluorescence intensity scores for CD9 expression in endometrial hyperplasia

<table>
<thead>
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<th>No.</th>
<th>Age of patient (years)</th>
<th>Type of hyperplasia</th>
<th>CD9 intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>Simple</td>
<td>++/++/++</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>Atypical, simple</td>
<td>++/++/++</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>Atypical, complex</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>Atypical, complex</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>77</td>
<td>Atypical, complex</td>
<td>++</td>
</tr>
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</table>

Fluorescence intensity: – = none; + = weak; ++ = moderate; +++ = intense.

Table II. Fluorescence intensity scores for CD9 expression in endometrial cancer

<table>
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<th>FIGO stage</th>
<th>Grade of differentiation</th>
<th>CD9 intensity</th>
</tr>
</thead>
<tbody>
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<td>Ib</td>
<td>G1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>Ib</td>
<td>G1</td>
<td>(+++/++)³</td>
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<tr>
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<td>79</td>
<td>Ib</td>
<td>G1</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>IVb</td>
<td>G1</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>Ib</td>
<td>G1</td>
<td>(++)³</td>
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<tr>
<td>6</td>
<td>42</td>
<td>Ib</td>
<td>G1</td>
<td>(++)³</td>
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<td>7</td>
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<td>G2</td>
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<td>IIIc</td>
<td>G3</td>
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Fluorescence intensity: – = none; + = weak; ++ = moderate; +++ = intense.
FIGO = International Federation of Gynecology and Obstetrics.
³Regional distribution with 30–50% of cancer cells stained.

In this study, however, reduced expression of CD9 was observed in the endometrial carcinomas, which has also been reported in other human solid cancer cells, e.g. breast, lung, pancreas and colon cancers (Miyake et al., 1995; Higashiyama et al., 1997; Mori et al., 1998; Sho et al., 1998). In addition, the level or intensity of CD9 expression has been found to be inversely correlated with prognosis of the patient and metastatic potential of the tumour (Miyake et al., 1995; Higashiyama et al., 1997). The transfection of the CD9 gene into several cell lines reportedly suppresses cell motility and metastasis (Ikeyama et al., 1993). In the human placenta, CD9 was found to be intensely expressed on the extravillous trophoblasts, especially those which have stopped their invasion at the fetomaternal interface (Hirano et al., 1999a). Moreover, anti-CD9 mAb enhanced the invasion of the human trophoblast-like cell line known as BeWo cells, suggesting an inhibitory role for the CD9 molecule in trophoblast invasion (Hirano et al., 1999b). Putting these findings together suggests that CD9 might be an important regulator, inhibiting cell invasion in a...
Involvement of CD9 in endometrial cancer invasion

Figure 2. Flow cytometry histograms of RL95-2 cells. RL95-2 cells were stained with anti-trinitrophenyl (anti-TNP) monoclonal antibody (mAb) (negative control), anti-CD9 mAb (TP82), anti-integrin α3 mAb (PIB5), anti-integrin α6 mAb (OG-1), or anti-integrin β1 (P4C10) mAb. Histograms showed that the majority of RL95-2 expressed CD9, integrin α3, integrin α6, and integrin β1 on their cell surface. x axis = relative fluorescence intensity; y axis = cell numbers.

Figure 3. Effects of the anti-CD9 monoclonal antibody (mAb) on the invasion of (A) RL95-2 and (B) Ishikawa cells. The invasion assay was carried out as described in the text in control medium or in the presence of various doses of the anti-CD9 mAb (ALB6) or a control antibody (anti-trinitrophenyl). The results are expressed as a percentage of the mean number of invaded cells in the control and are expressed as the mean ± SEM from five independent experiments. The data were analysed by a one-way analysis of variance, followed by Scheffe’s F test for multiple comparisons (*P < 0.05, **P < 0.01). y axis = number of invaded cells (% control).

variety of cancers, including endometrial cancer, as well as in trophoblasts of the human placenta.

Changed patterns of integrin expression have been reported in a variety of cancers, which suggests that integrins may be involved in the invasive and metastatic properties of these cancers (Koretz et al., 1990; Zutter et al., 1990; Ruoslahti, 1991). With respect to endometrial cancer, it has been reported that a decline in integrin expression occurs more frequently in poorly differentiated cancers and that the loss of specific integrins may be associated with metastatic nodal spread (Lessey et al., 1995). Moreover, β1 integrins have been suggested to affect the invasive properties of human colorectal carcinoma cells (Chao et al., 1996; Daemi et al., 2000). In this study, using flow cytometry, we confirmed that integrins α3, α6, and β1, which we previously found to be associated with CD9 in human endometrium (Park et al., 2000), are expressed on the cell surface of RL95-2 cells. We have also confirmed that CD9 is associated with integrins α3, α6, and
Effects of the anti-CD9 monoclonal antibody (mAb) (ALB) on RL95-2 cell invasion in the presence of mAb against integrin α3 (PIB5), α6 (GoH3) and β1 (3S3). The results are expressed as a percentage of the number of invaded cells in the control (medium without mAb), and are expressed as the mean ± SEM from five independent experiments. The data were analysed by an one-way analysis of variance, followed by Sheffe’s F test for multiple comparisons (*P < 0.05, **P < 0.01, NS = not significant). x axis = dose of mAb (mg/ml); y axis = number of invaded cells (% control).

Consequently, the effects of blocking mAbs against these integrins on the invasion of RL95-2 cells were examined, and it was found that these mAbs inhibited RL95-2 cell invasion. Since the neutralizing mAbs, which suppress RL95-2 cell invasion, have been reported to block the interaction between integrins and extracellular matrices (Carter et al., 1990; Sonnenberg et al., 1990; Gao et al., 1995), integrins α3, α6, and β1 may be considered to regulate adhesion of RL95-2 cells to extracellular matrices and to regulate their invasion. Next, an investigation of the effects of co-operation between anti-CD9 mAb and anti-integrin mAbs demonstrated that, in the presence of anti-integrin α3 mAb, anti-CD9 mAb enhanced RL95-2 cell invasion compared with that in the presence of anti-integrin α3 mAb alone. This indicates that the CD9 molecule regulates RL95-2 cell invasion independent of integrin α3. On the other hand, anti-CD9 mAb no longer had any effect on RL95-2 cell invasion following treatment with the anti-integrin α6 and with β1 mAbs. These results suggest that the effect of CD9 on RL95-2 cell invasion is directly mediated via integrins α6 and β1. In addition, we have reported that the inhibitory effect of CD9 on the invasive properties of BeWo cells was mediated by integrin α5β1 (Hirano, 1999b). Moreover, CD9 has also been reported to be expressed on the human granulosa cells in association with integrin αβ1, suggesting the involvement of CD9 in the function of human granulosa cells in co-operation with this integrin (Takao et al., 1999). Therefore, CD9 might be an important regulator of these integrins in a variety of cell systems.

In the human endometrium, integrins have also been reported to play important roles in blastocyst implantation (Tabibzadeh and Babaknia, 1995). We have already reported that CD9 was strongly expressed on the epithelial cells in normal human endometrium throughout the menstrual cycle, including the secretory phase, and was associated with integrins α3, α6 and β1, leading us to propose a functional relationship between CD9 and these integrins (Park et al., 2000). In addition, the RL95-2 cell line has been used as an in-vitro model of...
human endometrial epithelial cells for the study of blastocyst implantation mechanisms (Thie et al., 1998). Therefore, the functional association of CD9 with integrins α6 and β1 on RL95-2 cells observed in the present study warrants the need for further investigation of the role of CD9 in the process of blastocyst implantation in the human endometrium.

In conclusion, this study has demonstrated that CD9 is related to cell invasion in the RL95-2 and Ishikawa endometrial cancer cell lines. This finding, together with the observation of a reduced expression of CD9 in endometrial carcinomas compared to that in both normal endometrium and endometrial hyperplasia, suggests that CD9 might be involved in regulating the invasive properties of endometrial cancer by inhibiting cell invasion. In addition, this role of CD9 appears to be associated with the function of integrins. Further clarification of the role of CD9 in association with integrins in human endometrial cancer should contribute to a better understanding of the mechanism of endometrial cancer invasion.

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