Trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype

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Summary

At the human feto-maternal interface, trophoblasts differentiate towards extravillous trophoblasts (EVTs) and form the cell column. EVT invasive activity in the distal part of the cell column and begin to migrate into the maternal tissue. We previously reported that dipeptidyl peptidase IV (DPPIV) is expressed on EVTs in the proximal part of cell column and is involved in the inhibition of their migration. Because DPPIV has been shown to degrade several chemokines, we examined possible roles of chemokines in EVT migration.

Immunohistochemistry demonstrated that C-C chemokine receptor 1 (CCR1) was hardly detected on cytotrophoblasts and syncytiotrophoblast but was expressed on EVTs in the cell column. In vitro, CCR1 protein was also present on the surface of EVTs that grew out from chorionic villous explants cultured under 20% O2. Chemokines that can bind to CCR1 (CCR1 ligands), such as regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein-1α (MIP-1α), were confirmed in the decidual tissues by RT-PCR and immunohistochemistry. These CCR1 ligands promoted the migration of the EVTs that were isolated from the explant cultures in vitro. These results indicate that CCR1 is expressed on trophoblasts as they differentiate to EVTs and that CCR1 ligands produced from the decidua tissue induce EVT migration.

By contrast, CCR1 was scarcely expressed on EVTs that grew out from villous explants cultured in 1% O2, indicating that a relatively high oxygenic environment is needed to induce CCR1 expression. Moreover, CCR1 expression on the isolated EVTs was significantly reduced in the presence of decidual-conditioned medium. Such regulation of CCR1 by surrounding oxygenic and decidual environments supports a close correlation between EVT invasion and their expression of CCR1.

This study demonstrates that trophoblasts acquire CCR1 as they differentiate to an invasive phenotype at the villus-anchoring sites and indicates a novel role for the chemokine-CCR1 system in the initial step of trophoblastic invasion towards the maternal tissue.

Key words: CCR1, Chemokine, Extravillous trophoblast, Migration, Cell column, Endovascular trophoblast

Introduction

In human placenta, cytotrophoblasts show two distinct patterns of differentiation. In floating villi, cytotrophoblasts differentiate into syncytiotrophoblast and form the syncytial layer. By contrast, at villus-anchoring sites, cytotrophoblasts differentiate into EVTs and form the stratified structure that is called the cell column (Aplin, 1991). EVTs acquire invasive activity in the distal part of the cell column (Damsky et al., 1992) and begin to migrate towards maternal tissue. EVTs that migrate into the decidual tissue are called interstitial trophoblasts and those that migrate along the maternal vessels are called endovascular trophoblasts. Several molecules have been proposed to regulate EVT invasion. Matrix metalloproteinases and serine proteinases, which degrade the extracellular matrix, are secreted by EVTs to make the decidua preferable for their migration (Bischof et al., 1995). The importance of cell adhesion molecules, such as integrins, in EVT migratory processes has also been suggested (Burrows et al., 1996). At the villus-anchoring sites, EVTs gain integrin α5β1 in the proximal part of the cell column and integrin α1β1 at the distal end (Damsky et al., 1992). Because functional antibodies against these integrins affected trophoblastic migration in vitro, this so-called integrin switching was considered to be important for trophoblasts to become invasive (Damsky et al., 1994). However, the precise mechanism(s) involved in the acquisition of invasive activity has not been clarified.

We previously reported a characteristic expression profile of DPPIV (EC.3.4.14.5) on EVTs (Sato et al., 2002). DPPIV, a membrane-bound peptidase, can metabolize its substrates and regulate their bioactivity on the cell surface. DPPIV was expressed intensely on EVTs in the proximal part of the cell column, but it was downregulated in the distal part where EVTs acquired invasive activity. This suggests that DPPIV modulated the bioactivity of some molecule(s) that affect trophoblastic migration in situ. Supporting this idea, in JEG-3 cells (a DPPIV-positive choriocarcinoma cell line), inhibition of cell-surface DPPIV activity enhanced invasive activity without affecting proliferation. Recently, DPPIV was demonstrated to metabolize several chemokines in vitro (Van et al., 1999).
RANTES, a member of the chemokine family, is a representative substrate of DPPIV and the chemotactic activity of RANTES is abrogated by digestion with DPPIV (Oravecz et al., 1997). Because RANTES is produced in human placental tissues (Denison et al., 1998) and can induce the migration of leukocytes and other cell types (Youngs et al., 1997), we speculated that this chemokine is a candidate for a DPPIV substrate that can affect trophoblastic migration.

To substantiate this possibility, we initially examined the expression of CCR1 receptors on trophoblasts. C-C chemokine receptor 1 (CCR1), CCR3 and CCR5 are reported to be receptors for RANTES (Murphy et al., 2000), and immunohistochemical experiments revealed that EVTs expressed CCR1.

Several chemokines, including CCR1 ligands, have been demonstrated in human placental tissue (Denison et al., 1998; Drake et al., 2001; Red-Horse et al., 2001). They are considered to be involved in either recruiting specific leukocyte populations to the feto-maternal interface (Drake et al., 2001) or modulating trophoblastic function (Ishii et al., 2000). The latter function is supported by the fact that human choriocarcinoma cell lines as well as human placental syncytiotrophoblast express functional chemokine receptors (Ishii et al., 2000; Douglas et al., 2001). To date, however, no chemokine receptor has been demonstrated on migrating trophoblasts (i.e. EVTs). Accordingly, few reports have referred to the effect of chemokines on trophoblastic migration.

Therefore, in this study, we investigated the possible involvement of a chemokine receptor, CCR1, and its ligands in trophoblastic migration. First, we confirmed the CCR1 expression on EVTs in early human placental tissues. Second, using EVTs isolated from chorionic villous explant cultures (Yagel et al., 1989), we examined the expression of several chemokine receptors, including CCR1. Third, the expression

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**Fig. 1.** Expression of CCR1 protein and mRNA on human EVTs at 9-weeks gestation. (A-E) Serial sections of placental tissue from therapeutic hysterectomy at 9-weeks gestation were double-immunostained with anti-CCR1 mAb followed by rhodamine-conjugated secondary antibody plus either FITC-conjugated anti-cytokeratin 7 mAb or FITC-conjugated anti-von Willebrand factor pAb to visualize trophoblasts (A-D) and blood vessels (E), respectively. (C) is a higher magnification of the area indicated in (B). (D,E) The maternal arterial wall is traced with a dashed line. EVTs locating from the cell column (Column) through the trophoblastic shell (Shell) (A,B) and from the trophoblastic shell into the maternal artery (D,E) are shown. CCR1 expression is detected clearly on EVTs in the cell column (B,C) and trophoblastic shell (B), but is scarcely detected on cytotrophoblasts (CT) and syncytiotrophoblasts (ST) (C). CCR1 expression is diminished on interstitial trophoblasts (Int TB) (B,C), whereas it is maintained on endovascular trophoblasts (Endov TB) that migrate from the trophoblastic shell into the maternal artery (E). (F) 35-cycle PCR detects a specific band that corresponds to CCR1 in cDNA derived from the microdissected cell columns at 9 weeks of gestation. No band is observed in the negative control in which total RNA from the cell columns was not reverse-transcribed. AV, anchoring villus; IVS, intervillous space; Gl, decidual gland. Scale bars: 200 μm.
of chemokines that bind to CCR1 (CCR1 ligands) was confirmed in human placental tissues. Fourth, the effects of recombinant CCR1 ligands on proliferation and migration of isolated EVTs were examined to understand the function of trophoblastic CCR1. Last, to clarify the possible mechanism(s) of the regulation of trophoblastic CCR1, we examined the effect of hypoxia and decidua-conditioned medium on CCR1 expression of EVTs in vitro.

Materials and methods

Tissue samples

Placental tissues for immunohistochemistry and laser-capture microdissection were obtained from five therapeutic hysterectomies for either cervical neoplasia or uterine myoma during normal human pregnancies (9-weeks gestation n=3 and 10-weeks gestation n=2). Fresh tissues that either had or had not been fixed with 2% paraformaldehyde (Nakalai, Kyoto, Japan) at 4°C for 24 hours were embedded in OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen and stored at –80°C until use.

Placental tissues for villous and decidual explant cultures were aseptically obtained from legal abortions of normal pregnancies (6-9 weeks of gestation, n=15). Fresh tissues were placed in ice-cold RPMI (Gibco, Grand Island, NY) and used for cultures within 2 hours. In some cases, parts of the chorionic and the decidual tissues were taken separately, washed extensively with PBS and snap-frozen in liquid nitrogen for subsequent RNA extraction.

The gestational age was calculated from the date of the last menstrual period and, if necessary, was adjusted according to ultrasonic measurements of the gestational sac and the fetal crown-rump length. Informed consent for use of these tissues was obtained from all donors. The use of the materials was also approved by the Ethics Committee of Kyoto University Hospital.

Reagents and antibodies

Recombinant human RANTES, MIP-1α, monocyte chemoattractant protein 2 (MCP-2) and hemofiltrate C-C chemokine 1 (HCC-1) were purchased from PeproTech (London, UK). Goat anti-human RANTES, MIP-1α, MCP-2 and HCC-1 polyclonal antibodies (pAbs, IgG fraction) were obtained from Santa Cruz (Santa Cruz, CA). Two mouse anti-human CCR1 monoclonal antibodies (mAbs), clone 53504.111 (IgG1) and clone 141-2 (IgG1), were purchased from Genzyme (Cambridge, MA) and MBL (Nagoya, Japan), respectively.
Mouse anti-human cytokeratin 7 mAb (clone OV-TL12/30, IgG1) and FITC-conjugated mouse anti-human cytokeratin 7 mAb (clone LPSK, IgG2b) were obtained from Dako (Glostrup, Denmark) and Cymbus Biotechnology (Hants, UK), respectively. Mouse anti-human von Willebrand factor mAb (clone F8/86, IgG1) and FITC-conjugated sheep anti-human von Willebrand factor mAb (clone F8/86, IgG1) were obtained from Dako (Glostrup, Denmark) and Binding Site (Birmingham, UK), respectively. Mouse anti-human vimentin mAb (clone V9, IgG1) and FITC-conjugated mouse anti-human vimentin mAb (clone 3B4, IgG2a) were obtained from Dako and Progen Biotechnik (Heidelberg, Germany), respectively. Mouse anti-human melanoma cell adhesion molecule (MCAM) mAb (clone F4-35H7, IgG1) was purchased from Alexis (Lausen, Switzerland). Mouse anti-CCR1 mAb (clone 53504.111, IgG1) and FITC-conjugated mouse anti-CCR1 mAb (clone 141-2, IgG1) as a negative control for 1 hour. After washing in PBS, the sections were incubated with FITC-conjugated rabbit anti-goat Ig pAb (diluted 1:100) for 30 minutes. After the sections were thoroughly washed in PBS, they were mounted with Immunon (PermaFluor, Pittsburgh, PA) and examined under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Some serial sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

### Double immunohistochemistry

The 7-μm-thick sections of frozen placental tissues were fixed with acetone and incubated with mouse anti-human CCR1 mAb (clone 53504.111, 10 μg ml⁻¹ and clone 141-2, 10 μg ml⁻¹) or isotype-matched control mAb (10 μg ml⁻¹) for 1 hour. After washing in PBS, the sections were incubated with rhodamine-conjugated goat anti-mouse Ig pAb (diluted 1:100) for 30 minutes, then washed in PBS and blocked with anti-TNP mAb (20 μg ml⁻¹) for 30 minutes. After washing in PBS, they were incubated with FITC-conjugated mouse anti-human cytokeratin 7 mAb (diluted 1:10), FITC-conjugated sheep anti-human von Willebrand factor pAb (diluted 1:100) or FITC-conjugated negative control antibodies for 1 hour. After the sections were thoroughly washed in PBS, they were mounted with Immunon (PermaFluor, Pittsburgh, PA) and examined under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Some serial sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

### Table 1. PCR primers

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*Primer pairs were taken from published articles (Kojima et al., 1994; Jordan et al., 1999; Ishii et al., 2000; Middel et al., 2001; Huber et al., 2002; Kawaguchi et al., 2002).

### Single immunohistochemistry

The paraformaldehyde-treated placental tissues were cut into 7-μm-thick sections, air-dried on Neoprene (Nissin, Tokyo, Japan)-coated slide glasses and fixed with acetone (Nakalai, Kyoto, Japan) at –20°C for 5 minutes. The serial sections were incubated with goat anti-human RANTES, MIP-1α, MCP-2 and HCC-1 pAb (IgG, 10 μg ml⁻¹) or normal goat IgG (10 μg ml⁻¹) as a negative control for 1 hour. After washing in PBS, the sections were incubated with FITC-conjugated rabbit anti-goat Ig pAb (diluted 1:100) for 30 minutes. After the sections were thoroughly washed in PBS, they were mounted with Immunon (PermaFluor, Pittsburgh, PA) and examined under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Some serial sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

**Human chorionic villous explant culture and isolation of EVTs**

EVTs were isolated from human villous explant cultures as described (Yagel et al., 1989). Placental tissues (6-9 weeks of gestation, n=15)
chemokine receptor in trophoblast

Development and disease

were washed with sterile RPMI and dissected aseptically to remove decidual tissues and fetal membrane. Small fragments of chorionic villi (~2 mm in diameter) were teased apart and soaked in culture medium [RPMI containing 10% FCS (Gibco), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gibco)]. About 20 pieces of the villous fragments were put on 10-cm dishes coated with collagen type I (Iwaki, Chiba, Japan). After incubation for 4 hours under standard conditions (37°C, 20% O₂, 5% CO₂ and 75% N₂) to allow the explants to adhere, 10 ml of culture medium was gently added and the explants incubated under standard conditions for an additional 48 hours. The formation of cell sheets and migration of spindle-shaped cells were observed from the adherent villous tips (a representative example is shown in Fig. 9A). After they were washed gently with PBS, the outgrown cells were dispersed with 0.05% trypsin (Difco, Detroit, MI) and 0.05% EDTA (Nakalai) solution, passed through a 40 μm pore Nylon Cell Strainer (Becton Dickinson, Bedford, MA) to remove the chorionic villous parts, and replated in collagen type I-coated 6-well plates (Iwaki). After 4-hour incubation, nonadherent cells and debris were removed by washing with RPMI. The cells that remained attached were defined as ‘isolated EVTs’ and used for further experiments as described below.

To examine the effect of hypoxia on trophoblastic expression of CCR1, five pieces of ~2-mm villous fragments (6-9 weeks of gestation, n=5) were placed on each of two 3.5-cm dishes coated with collagen type I (Iwaki). After incubation for 4 hours under standard conditions, 2 ml of culture medium was added and the explants incubated for an additional 48 hours under either standard or hypoxic conditions (37°C, 1% O₂, 5% CO₂ and 94% N₂). The explant cultures were then washed gently with PBS and fixed with 0.5% paraformaldehyde at 4°C for 15 minutes. They were immunostained using either mouse anti-human CCR1 mAb (clone 53504.111, 10 μg ml⁻¹) or isotype-matched control mAb (10 μg ml⁻¹), followed by FITC-conjugated rabbit anti-mouse Ig pAb (diluted 1:40), and examined under a confocal laser scanning microscope without mounting.

Preparation of decidua-conditioned medium

To obtain a mixture of soluble factors derived from the decidua, 10 pieces of ~2 mm diameter decidual fragments (6-9 weeks of gestation, n=10) were soaked in culture medium and put on a 3.5-cm dish coated with collagen type I. After incubation for 4 hours to allow adherence of the explants, they were washed gently with PBS and then cultured in 1 ml of RPMI for an additional 24 hours under standard conditions. After this incubation, the supernatant was collected as decidua-conditioned medium, passed through a 0.45 μm-pore filter (Millipore, Bedford, MA), and stored at –80°C until use.

Fig. 3. Expression of CCR1 protein on human endovascular trophoblasts at 10-weeks gestation. Serial sections of placental tissue from therapeutic hysterectomy at 10-weeks gestation were double-immunostained with anti-CCR1 mAb followed by rhodamine-conjugated secondary antibody plus either FITC-conjugated anti-cytokeratin 7 mAb or FITC-conjugated anti-von Willebrand factor pAb to visualize trophoblasts (B,E) and blood vessels (C,F), respectively. Endovascular trophoblasts (Endov TB) near the intervillous space (IVS) (A-C) and those in the deep portion of the decidual tissue (D-F) are shown. Expression of CCR1 is detected on endovascular trophoblasts that have just entered the maternal artery from the trophoblastic shell (Shell) (C), whereas it is hardly detected on the endovascular trophoblasts that reside in the maternal arteries located in the deep portion (F). Gl, decidual gland. Scale bars: 200 μm.
RT-PCR analysis
Total RNA was extracted from the isolated EVTs (6-9 weeks of gestation, n=5) and human peripheral blood mononuclear cells (PBMC; used as a positive control for chemokine receptors) using Trizol (Gibco) as recommended by the manufacturer. Total RNA was also extracted from chorionic and decidual tissues (both 6-9 weeks of gestation, n=5). Two micrograms of total RNA were reverse-transcribed with random primers using a First Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, UK) and diluted with distilled water to a final volume of 200 μl. In some experiments, reverse transcriptase was omitted as a control for the amplification of contaminating genomic DNA. PCR was performed in 20 μl of Taq buffer (+Mg2+), containing 1 μl of the indicated cDNA solution, 2 mM dNTPs and 0.5 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) with 0.5 mM each of sense and antisense primers for chemokines, chemokine receptors and S26 as a housekeeping probe (Table 1) (Kojima et al., 1994; Jordan et al., 1999; Ishii et al., 2000; Middel et al., 2001; Huber et al., 2002; Kawaguchi et al., 2002). The conditions for amplification were: 5 minutes at 94°C, 30 cycles of 1 minute at 94°C, annealing for 1 minute at 56-64°C and incubation for 1 minute at 75°C, followed by a final extension for 5 minutes at 75°C. PCR products were resolved by electrophoresis on 1-1.5% agarose gels and viewed by ethidium bromide staining. Because the genes for chemokine receptors do not contain intron sequences that can be used to control for genomic contamination, an identical, a parallel PCR was performed containing starting material that had not been reverse-transcribed.

Immunocytochemistry
The isolated EVTs (6-9 weeks of gestation, n=5) were trypsinized and replated in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) that had been precoated with collagen type I (Koken, Tokyo, Japan). After incubation for 4 hours under standard conditions to allow the cells to adhere to the slides, they were washed with PBS and fixed with 0.5% paraformaldehyde at 4°C for 15 minutes followed by acetone at −20°C for 2 minutes.

To evaluate the purity of the isolated EVTs, they were double-immunostained using mouse anti-human vimentin mAb (diluted 1:40), anti-human CD45 mAb (diluted 1:50), anti-human von Willebrand factor mAb (diluted 1:40) and anti-human MCAM mAb (diluted 1:20), followed by rhodamine-conjugated goat anti-mouse Ig pAb (diluted 1:100). After blocking with anti-TNP mAb (20 μg ml−1), the EVTs were additionally stained with FITC-conjugated mouse anti-human cytokeratin 7 mAb (diluted 1:10).

To examine the expression of CCR1 on the isolated EVTs, they were double-immunostained with anti-human CCR1 mAb (clone 53504.111, 10 μg ml−1) or isotype-matched control antibody (10 μg ml−1) followed by rhodamine-conjugated goat anti-mouse Ig pAb (diluted 1:100). After blocking with anti-TNP mAb (20 μg ml−1), the cells were additionally stained with FITC-conjugated mouse anti-human cytokeratin 7 mAb (diluted 1:10), FITC-conjugated mouse anti-human vimentin mAb (diluted 1:10) or FITC-conjugated negative control antibodies.

Samples were examined under a confocal laser scanning microscope.

Flow cytometry
The isolated EVTs (6-9 weeks of gestation, n=5) were trypsinized and washed in Hanks’ Balanced Salt Solution (HBSS, Gibco) containing 0.1% bovine serum albumin (Nakalai) and 0.1% NaN3 (Nakalai). The precipitated cells (2 × 10^4 cells tube−1) were incubated with anti-human CCR1 mAb (clone 141-2, 100 μg ml−1, 10 μl) or isotype-matched control mAb (100 μg ml−1, 10 μl) for 30 minutes at 4°C. After the cells were washed twice with HBSS, they were incubated with FITC-conjugated rabbit anti-mouse Ig pAb (diluted 1:40, 20 μl) at 4°C for 30 minutes in the dark. The cells were then washed twice and resuspended in 300 μl of HBSS. Cell surface labeling was analyzed by FITC fluorescence detection using a FACS caliber (Becton Dickinson). Flow cytometric data were obtained from the analysis of 5 × 10^4 cells per sample.

To examine the effect of decidua-derived soluble factors on trophoblastic CCR1 expression, the isolated EVTs (6-9 weeks of gestation, n=5) were incubated in medium containing 10% FCS and 10% human pooled decidua supernatant. The supernatant was obtained from decidua cultures cultured for 24 hours in medium containing 10% FCS. The cells were then incubated with CCR1 mAb (clone 53504.111, 10 μg ml−1) or isotype-matched control mAb (10 μg ml−1) for 30 minutes at 4°C. After the cells were washed twice with HBSS, they were incubated with FITC-conjugated rabbit anti-mouse Ig pAb (diluted 1:40, 20 μl) at 4°C for 30 minutes in the dark. The cells were then washed twice and resuspended in 300 μl of HBSS. Cell surface labeling was analyzed by FITC fluorescence detection using a FACS caliber (Becton Dickinson). Flow cytometric data were obtained from the analysis of 5 × 10^4 cells per sample.
Chemokine receptor in trophoblast

Development and disease

gestation, n=5) were trypsinized and 1 x 10^5 cells ml^{-1} of RPMI with 1% FCS were plated in each well of a collagen type I-coated 24-well plate (Iwaki). Decidua-conditioned medium (100 μl), prepared as above, was added to the wells. Heat-inactivated decidua-conditioned medium was used as control. In each experiment, we used the decidua-conditioned medium derived from the patient from whom the EVTs were isolated. After incubation for 24 hours under standard conditions, the cells were trypsinized and treated for flow cytometric analysis as described above. Differences between percent positivity for CCR1 were analyzed by the two-tailed paired t test.

**Laser-capture microdissection**

The frozen placental tissues were cut into 8-μm-thick sections, mounted on foil-covered glass slides (Digital Micro Systems, Kyoto, Japan) and fixed immediately in 100% methanol (Nakalai) for 3 minutes at room temperature. The sections were counterstained with 1% toluidine blue (Nakalai) in diethylpyrocarbonate-treated (DEPC, Nakalai) water for 10 seconds and then washed in DEPC-treated water at 4°C. Using a Leica AS LMD (Leica Microsystems, Tokyo, Japan), ~30 cell columns were cut out from the sections, taking care not to contaminate the sample with immune cells, decidual cells and syncytiotrophoblast. These cell columns were collected and total RNA was extracted as described above. Half of the sample was reverse-transcribed to synthesize cDNA and the remainder incubated similarly but without reverse transcriptase to control for the amplification of contaminating genomic DNA. The resulting mixtures were subjected to 35 cycles of PCR amplification with either human CCR1 primers or S26 primers (Table 1) as described above.

**Invasion assay**

Invasion assays were carried out as previously described (Sato et al., 2002) with slight modifications. A cell-culture insert (6.4 mm diameter) that contained a polyethylene terephthalate membrane filter with 8 μm-diameter pores (Becton Dickinson) was placed in each well of a 24-well companion plate (Becton Dickinson). The lower surface of the filter was precoated with Matrigel (200 μg ml^{-1}, Becton Dickinson) according to the manufacturer’s instructions. The lower well was filled with 800 μl of serum-reduced medium (RPMI with 1% FCS) containing either intact or heat-inactivated chemokine (50 ng ml^{-1} of either RANTES or MIP-1α, 100 ng ml^{-1} of MCP-2 and 500 ng ml^{-1} of HCC-1). The well containing serum-reduced medium without additive was used for control. The isolated EVTs were trypsinized and 2 x 10^4 cells 200 μl^{-1} of serum-reduced medium were plated in the upper well. The cells were allowed to migrate through the pores for 4-6 hours and those that remained on the upper surface of the filter were thoroughly removed with a cotton swab. The cells that reached the lower surface were fixed with 100% methanol at −20°C for 5 minutes and immunostained with mouse anti-human cytokeratin 7 mAb (diluted 1:40) followed by FITC-conjugated rabbit anti-mouse Ig pAb (diluted 1:40) to visualize trophoblasts. The filters were mounted with Immunon and examined under a confocal laser scanning microscope. The numbers of cytokeratin 7-positive cells were counted using NIH Image 1.61.

This experiment was performed in duplicate to determine the average number of cells that migrated under each experimental condition. The result was expressed as a percentage of number of cells that migrated in the control (without additive). Five independent
5526 experiments were performed using the EVTs isolated from different chorionic samples (6-9 weeks of gestation, n=5). The differences were analyzed by one-way analysis of variance followed by Scheffe’s F-test for multiple comparisons.

Cell-proliferation assay

The isolated EVTs were trypsinized and 1 x 10^4 cells 100 µl^-1 of RPMI plus 1% FCS were plated in each well of a collagen type I coated 96-well plate (Iwaki). Intact or heat-inactivated chemokine (50 ng ml^-1 of either RANTES or MIP-1α, 100 ng ml^-1 of MCP-2 and 500 ng ml^-1 of HCC-1) was added to the well. The well without additive was used for control. After 24 hours incubation under standard conditions, the number of viable cells in each well was assessed using Premix WST-1 Cell Proliferation Assay System (Takara, Kusatsu, Japan) according to the manufacturer’s instructions. Briefly, 10 µl of Premix WST-1 were added to each well and the cells were incubated under standard conditions for 1 hour. WST reduction was determined with automated enzyme-linked immunosorbent assay plate reader (Molecular Device, Menlo Park, CA) at an optical density of 450-650 nm.

This experiment was performed in duplicate and the average was defined as the WST reduction value under each experimental condition. The result was expressed as the percentage of the value in the control (without additive). Five independent experiments were performed using the EVTs isolated from different chorionic samples (6-9 weeks of gestation, n=5). The differences were analyzed by one-way analysis of variance followed by Scheffe’s F-test for multiple comparisons.

Results

Expression of CCR1 protein and mRNA at the human feto-maternal interface

To assess the expression of CCR1 protein at the feto-maternal interface, sections of human placental samples obtained from therapeutic hysterectomies (9-10 weeks of gestation, n=5) were stained with two specific monoclonal antibodies against human CCR1 (clone 53504.111 and clone 141-2). CCR1 was scarcely expressed on cytotrophoblasts and syncytiotrophoblast but was expressed on EVTs in the cell column (Fig. 1B,C). Although expression of CCR1 was hardly detected on EVTs migrating into the decidual tissue (Fig. 1B,C), it was clearly maintained on EVTs migrating from the trophoblastic shell into the maternal arteries (Fig. 1E, Fig. 3C). CCR1 expression was reduced on the endovascular trophoblasts that reside in the maternal arteries located in the deep portion of the decidual tissue (Fig. 3F). Similar staining profiles were observed in all five samples with two anti-CCR1 monoclonal antibodies and no staining was detected with isotype-matched control antibody (data not shown). The specific band for CCR1 mRNA was detected in the 35-cycle PCR amplification of the cDNA derived from the microdissected cell columns (Fig. 1F).

Fig. 6. mRNA expression of CCR1 ligands in human placental tissues. Chorionic and decidual samples were obtained separately from the same cases (6-9 weeks of gestation, n=5) and used for 30-cycle RT-PCR with primers against either CCR1 ligands or S26 as a housekeeping probe. PCR products from the chorionic samples and the decidual counterparts were electrophoresed (lanes C1-C5 and D1-D5, respectively). Specific bands for RANTES, MIP-1α, MCP-2 and HCC-1 are detected in all five decidual samples. These chemokines are hardly detected in their chorionic counterparts.

Differential expression of chemokine receptor mRNAs in isolated EVTs

EVTs were isolated from cultures of human chorionic villi (6-9 weeks of gestation, n=15) as described in Materials and methods. Over 95% of the isolated cells were positive for both cytokeratin 7 and MCAM, a marker of EVTs (Shih and Kurman, 1996). The rest were vimentin-positive (villous stromal cells) and cells positive for CD45 (leukocytes) or von Willebrand factor (endothelial cells) were hardly detected (data not shown). Fig. 4 shows representative results of five independent RT-PCR experiments using primers specific for CCR1-11, CXCR1-5, XCR1 and CX3CR1. Primer pairs and
PCR conditions were validated by examining their ability to detect gene expression in the positive control, PBMC (Fig. 4, top panel). The receptors that were expressed constantly by isolated EVTs after 30 cycles of PCR amplification were CCR1, CCR10 and XCR1 (Fig. 4, middle panel). The negative control (total RNA of the isolated cells that was not reverse-transcribed) was consistently negative (Fig. 4, bottom panel). The sequences of the PCR products were determined and shown to be identical to CCR1, CCR10 and XCR1 sequences.

Expression of CCR1 protein on the surface of isolated EVTs

Expression of CCR1 protein on the isolated EVTs (6-9 weeks of gestation, n=5) was evaluated by immunocytochemistry and flow cytometry using two specific monoclonal antibodies against human CCR1 (clone 53504.111 and clone 141-2). CCR1 was clearly expressed on the surface of the cytokeratin 7-positive cells (Fig. 5A,B) but not on vimentin-positive cells (Fig. 5C). Isotype-matched control antibody produced no staining on the surface of the cytokeratin 7-positive cells (Fig. 5D). The cell-surface expression of CCR1 was also confirmed by flow cytometric analysis; a shift in the fluorescence intensity of cells treated with anti-CCR1 antibody was observed compared to an isotype-matched control antibody (Fig. 5E). In five independent experiments, CCR1 was expressed by 48.2±13.7% (mean±s.d.) of the isolated cells.

mRNA expression of CCR1 ligands in human placental tissues

To examine the mRNA expression of CCR1 ligands at the feto-maternal interface, we performed 30-cycle RT-PCR analysis using chorionic and decidual samples obtained from the same cases (6-9 weeks of gestation, n=5). As shown in Fig. 6, specific bands were detected for RANTES, MIP-1α, MCP-2 and HCC-1. Expression of these chemokines was detected clearly in all of the five decidual samples used (Fig. 6D1-D5) but hardly detected in their chorionic counterparts (Fig. 6C1-C5). Other ligands for CCR1, MCP-3, leukotactin 1 (Lkn-1) and myeloid progenitor inhibitory factor (MPIF-1) were hardly detectable in this RT-PCR analysis.

Fig. 7. Expression of CCR1 ligands at the human feto-maternal interface at 9-weeks gestation. Serial sections of placental tissue from therapeutic hysterectomy at 9-weeks gestation were immunostained with goat anti-RANTES (B,G), anti-MCP-2 (C,H) and anti-MIP-1α pAb (E,I). (A), (D) and (F) shows HE staining of (B,C), (E) and (G,H,I), respectively. In decidual tissue, RANTES is expressed in some of the resident leukocytes (DL; B) and MCP-2 is diffusely expressed in the stromal cells (DS) (C). MIP-1α is expressed in interstitial trophoblasts (Int TB) and endovascular trophoblasts (Endov TB) (E). The expression of RANTES and MCP-2 is hardly detectable in chorionic villi (G,H). There is patchy expression of MIP-1α in syncytiotrophoblast (I). FV, floating villi. Scale bars: 100 µm.
Immunolocalization of CCR1 ligands at the human feto-maternal interface

To determine the immunolocalization of CCR1 ligands in the feto-maternal interface, the sections of human placental samples obtained from therapeutic hysterectomies (9-10 weeks of gestation, \( n = 5 \)) were stained with a goat polyclonal antibody against RANTES, MIP-1\( \alpha \), MCP-2 or HCC-1. Normal goat IgG was used as control. In decidual tissue, RANTES was expressed focally in resident leukocytes (Fig. 7B) and MCP-2 was expressed diffusely in decidual stromal cells (Fig. 7C). As reported recently, MIP-1\( \alpha \) was expressed in EVTs in the maternal tissue (Drake et al., 2000) (Fig. 7E). In chorionic villi, expression of either RANTES or MCP-2 was hardly detectable (Fig. 7G,H). Patchy expression of MIP-1\( \alpha \) was detected in syncytiotrophoblast (Fig. 7I). No staining was detected with anti-HCC-1 antibody or normal goat IgG (data not shown).

Effects of CCR1 ligands on migratory activity and proliferation of isolated EVTs

To understand the function of CCR1 expressed on EVTs, the effects of CCR1 ligands on the migratory activity of the isolated EVTs (6-9 weeks of gestation, \( n = 5 \)) were examined in an invasion assay. In the presence of intact RANTES, the number of cytokeratin 7-positive cells (EVTs) that reached the lower surface increased significantly compared to cells in either the presence of heat-inactivated RANTES or without additive (control) (Fig. 8A-C). Similar migration-promoting effects were observed with other CCR1 ligands, MIP-1\( \alpha \), MCP-2 and HCC-1 (Fig. 8D-F). None of these four chemokines affected the proliferation of isolated EVTs (data not shown).

Effect of hypoxia and decidua-derived factors on trophoblastic expression of CCR1

To determine the possible mechanisms that regulate trophoblastic CCR1 expression, the effect of hypoxia was examined in explant cultures of human chorionic villi (6-9 weeks of gestation, \( n = 5 \)). When the chorionic villous fragments were cultured in 20% O\(_2\), round cells grew out from the explanted villous tips to form a cell sheet from which migration of spindle-shaped cells was observed (Fig. 9A). These outgrown cells were double-positive for cytokeratin 7 and MCAM (data not shown). CCR1 was expressed clearly on these cells (Fig. 9B). When the explants were cultured under 1% O\(_2\), the size of the individual outgrown cells was larger and the intercellular space was wider than at 20% O\(_2\) (Fig. 9C). CCR1 expression was very weak on these cells (Fig. 9D). We confirmed that these cells were also positive for both cytokeratin 7 and MCAM (data not shown).

Next, we examined the effect of decidua-derived soluble
Chemokine receptor in trophoblast

Development and disease

Factors on trophoblastic CCR1 expression. To exclude the influence of chorionic villus-derived factors, EVTs were isolated from the villous explant cultures (6-9 weeks of gestation, n=5) and further incubated in the presence of either intact or heat-inactivated decidua-conditioned medium. As shown in Fig. 9E, flow cytometry revealed that the percentage of CCR1-positive cells in EVTs treated with intact decidua-conditioned medium (26.0±10.9%) was significantly lower than in EVTs treated with heat-inactivated medium (43.1±3.6%).

Discussion

In this study, we demonstrated that a chemokine receptor, CCR1, is expressed on human EVTs. In an immunohistochemical study using early human placental tissues, the expression of CCR1 was hardly detected on cytotrophoblasts and syncytiotrophoblast, whereas it was detected clearly on EVTs in the cell column. The initial expression site of CCR1 was very similar to that of integrin α5. We also detected mRNA encoding CCR1 in the microdissected cell columns by RT-PCR. In maternal tissue, CCR1 expression was diminished on EVTs that were migrating into the decidual tissue, whereas its expression was maintained on those migrating from the trophoblastic shell into maternal arteries.

In primary explant cultures of human chorionic villi obtained during the first trimester of pregnancy, cell sheets and migrating spindle-shaped cells grew out from the explanted villous tips. Because these structures resemble the cell column and the interstitial trophoblasts in vivo, this villous explant culture has been proposed to represent trophoblastic differentiation towards EVTs that occurs at the villus-anchoring sites (Genbacev et al., 1992; Vicovac et al., 1995; Irving et al., 1995). We confirmed that the outgrown cells were positive for cytokeratin 7, an epithelial cell marker that distinguishes trophoblasts and decidual glandular cells from other cell types present at the feto-maternal interface (Haigh et al., 1999). We also observed that MCAM, a specific marker for EVTs and endothelial cells (Shih and Kurman, 1996), was expressed on these cells. These results indicate that the cells that grew out from the explanted villous tips are composed mainly of EVTs, and support the proposal that the cell sheets and migrating spindle-shaped cells correspond to the cell column and interstitial trophoblasts in vivo. In this system, CCR1 protein was expressed clearly on the outgrown EVTs. For further analyses in vitro, we isolated the outgrown EVTs by a previously reported method (Yagel et al., 1989). RT-PCR showed expression of mRNA encoding CCR1 in the isolated EVTs, and immunocytochemistry and flow cytometry confirmed that CCR1 protein is expressed on the surface of these cells. Based on these findings and the above immunohistochemical results, we conclude that trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards EVTs at the villus-anchoring sites.

We then examined whether chemokines that bind to CCR1 are produced at the human fetomaternal interface. Among reported ligands for CCR1, RT-PCR analysis of early placental
samples confirmed the expression of mRNAs encoding RANTES, MIP-1α, MCP-2 and HCC-1, all of which can stimulate CCR1 to induce an increase in cytosolic Ca^{2+} concentration and/or cell migration (Neote et al., 1993; Gao et al., 1993; Gong et al., 1997; Tsou et al., 1998). Interestingly, mRNA for these chemokines was expressed predominantly in the decidual samples and almost undetectable in their chorionic counterparts. Using RT-PCR, Ishii et al. demonstrated mRNAs for RANTES, MIP-1α and MCP-3 in chorionic samples from the first trimester (Ishii et al., 2000). This apparent discrepancy might be due to differences in PCR conditions. In this study, we used a lower amount of cDNA (cDNA reverse-transcribed from 10 ng of total RNA in 20 μl PCR solution) for a fewer amplification cycles (30 cycles) compared to cDNA reverse-transcribed from 1 μg of total RNA in 20 μl PCR solution for 35 PCR amplification cycles (Ishii et al., 2000). In this respect, our PCR data do not indicate the absolute absence of other CCR1 ligands, such as MCP-3, MPIF-1 and Lkn-1, in these chorionic and decidual samples nor do they mean that MIP-1α, MCP-2 and HCC-1 are absolutely absent from chorionic samples. Rather, they indicate the relative abundance of mRNA encoding CCR1 ligands in decidual samples as compared with their chorionic counterparts. Immunohistochemistry of the feto-maternal interface showed the expression of RANTES, MCP-2 and MIP-1α proteins in the decidual tissue. By contrast, the expression of these chemokines was weak or almost absent in the chorionic villi, which supports the above PCR data. In this immunohistochemistry, we failed to determine the localization of HCC-1 protein. However, it was demonstrated by in situ hybridization that mRNA for HCC-1 is expressed diffusely in the decidual stromal cells and invading EVTs (Red-Horse et al., 2001). These findings indicate that CCR1 ligands are produced at the feto-maternal interface. Moreover, it is also implied that these CCR1 ligands are more abundantly produced in the decidual (maternal) compartment than in the chorionic (fetal) compartment.

A study of CCR1-knockout mice showed that CCR1 is not required for normal mouse development (Gao et al., 1997). However, it was recently reported that mouse ectoplacental cone-derived trophoblasts express CCR3, CXCR4 and CCR5 (Athanassakis et al., 2001). This does not agree with our RT-PCR data showing that human EVTs expressed CCR1, CCR10 and XCR1 because it suggests that mouse trophoblasts do not express CCR1. In this respect, the data from mouse knockouts do not negate the importance of CCR1 in human placentogenesis and it might be difficult to perform in vivo experiments using mice to extrapolate the functional relevance of chemokine-CCR1 system in human EVTs. Therefore, in this study, we used the isolated human EVTs to examine the effects of CCR1 ligands in vitro. In the invasion assay, RANTES, MIP-1α, MCP-2 and HCC-1, whose expression was detected in the decidual tissue, promoted the migration of the isolated EVTs. By contrast, the proliferation of isolated EVTs was not affected by these chemokines. Because other chemokine receptors that are known to interact with these chemokines (i.e., CCR3 and CCR5 for RANTES; CCR5 for MIP-1α; CCR2, CCR3 and CCR5 for MCP-2) (Murphy et al., 2000) were hardly detectable in the isolated EVTs by our RT-PCR analysis, these migration-promoting effects are considered to be mainly mediated through CCR1. Taking into consideration that CCR1 ligands were detected predominantly in the maternal side of the feto-maternal interface, the chemokine-CCR1 system might contribute to the initial step of trophoblastic invasion towards maternal tissue.

Trophoblastic invasion and/or differentiation is thought to be influenced by the surrounding oxygen tension (Genbacev et al., 1997). These authors demonstrated that trophoblasts do not become invasive in hypoxic conditions in vitro and proposed that a relatively O_{2}-rich environment, such as in the maternal arteries, stimulates trophoblastic invasion and encourages their arterial infiltration. The factors derived from the decidual tissue are also thought to regulate trophoblastic invasion and differentiation (Bischof et al., 2000). In either ectopic pregnancy or placenta accrete where development of decidual tissue is insufficient, excess trophoblastic invasion has been observed. From these observations, it has been suggested that decidual tissue has a protective effect on trophoblastic invasion. In fact, it was reported that supernatant derived from decidual cells in culture inhibits trophoblastic gelatinolytic activity in vitro (Bischof et al., 1998). We also observed that relatively high O_{2} reduced the expression of DPPV on the outgrown EVTs in the villous explant cultures (Sato et al., 2002), and that decidua-conditioned medium increased the MCAM expression on the isolated EVTs (Higuchi et al., 2003). In this study, therefore, we examined the effects of these two factors, the surrounding O_{2} tension and decidua-derived factors, on the expression of CCR1 on EVTs in vitro. Unlike the findings in villous explant cultures under normoxic conditions, CCR1 expression was hardly induced on the outgrown EVTs under hypoxic conditions. This indicates that a relatively high O_{2} level in the environment is needed to induce CCR1 expression. By contrast, the induction of CCR1 expression on outgrown EVTs decreased significantly after they were isolated from chorionic villous parts and treated with decidua-conditioned medium. This indicates that undefined soluble factor(s) that are derived from decidua reduce the expression of CCR1 on EVTs. Based on the concept that trophoblastic invasion is encouraged by high O_{2} concentrations and inhibited in the presence of decidual tissue, the findings on CCR1 regulation support a close correlation between trophoblastic invasion and expression of CCR1.

After trophoblasts acquired CCR1 in the cell column, CCR1 expression rapidly diminished on the EVTs that were migrating into the decidual tissue. Such downregulation of CCR1 was not observed in villous explant cultures, where the outgrown cell sheets as well as the migrating spindle-shaped cells expressed CCR1 constantly. This might be caused, at least in part, by the absence of decidua-derived factor(s) in explant cultures. By contrast, CCR1 expression was maintained on the EVTs that were migrating from the trophoblastic shell into the maternal arteries, where O_{2} tension is expected to be relatively high. This suggests that high O_{2} tension, which is necessary for the induction of CCR1 expression on trophoblasts in vitro, is also important for maintaining CCR1 expression. Because the expression of chemokine receptors is likely to reflect the responsivity of the cell to chemokine stimulation, it is fascinating to speculate that the chemokine-CCR1 system is involved in the mechanism that leads trophoblasts towards the maternal arteries. To substantiate this speculation, there must be a source of CCR1 ligands in the maternal arteries that provides directional cues that guide EVTs. Unfortunately, our immunohistochemistry failed to show the expression of CCR1
ligands was highest in maternal endothelial cells. However, there is another important cell component that could be the source of CCR1 ligands in the maternal vessels; circulating blood cells. Before 10-weeks gestation, uterine arteries are occluded with aggregates of endovascular trophoblasts, which dramatically slows the arterial blood flow (Drake et al., 2002). This might promote the attachment of blood cells to the vessel wall where they are known to transiently enhance the production of various cytokines and chemokines (Kasahara et al., 1991). RANTES is one of the chemokines produced by cultured human PBMC (Denison et al., 1997). It has also been reported that platelets release RANTES and MIP-1α following activation (Boehlen and Clemetson, 2001). Moreover, we recently demonstrated that PBMC-conditioned medium can promote the invasion of BeWo cells, a trophoblastic cell line (Egawa et al., 2000). In this context, it is possible that some circulating blood cells produce chemokines as they attach to the vessel wall and attract EVTs towards the maternal arteries.

In summary, we have demonstrated that trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype at the villus-anchoring sites. In vivo, CCR1 was not expressed on cytotrophoblasts or syncytiotrophoblast but was expressed on EVTs in the cell column. In vitro, CCR1 was induced on EVTs that grew out from the villous explants cultured under normoxic conditions. Such CCR1 induction was not observed under hypoxic conditions and the induced CCR1 expression was reduced by treatment with decidua-derived factor(s), which supports a close correlation between trophoblastic invasion and CCR1 expression. CCR1 ligands were confirmed in the maternal tissue and could induce the migration of the isolated EVTs in vitro. From these findings, we propose a novel role for chemokine-CCR1 interactions (a well-known regulatory system for leukocyte trafficking) in the initiation of trophoblastic invasion of maternal tissue. In this study, we could not determine the significance of CCR1 expressed on endovascular trophoblasts. The relevance of other chemokine receptors in trophoblastic invasion also remains to be explored. Future work on understanding the possible participation of chemokines and chemokine receptors in trophoblastic arterial infiltration might contribute to clarifying the pathophysiology of preeclampsia and intrauterine fetal growth retardation, condition in which there is insufficient maternal arterial remodeling.

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