Journal of General Virology (2006), **87**, 2269–2278 DOI 10.1099/vir.0.81071-0

Transcytosis of *Human immunodeficiency virus 1* across the placenta is enhanced by treatment with tumour necrosis factor alpha

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The human placenta is relatively resistant to *Human immunodeficiency virus 1* (HIV-1), but obstetric complications associated with inflammatory processes, including chorioamnionitis and spontaneous preterm delivery, are associated with increased rates of vertical transmission. It was hypothesized that the pro-inflammatory mediator tumour necrosis factor alpha (TNF-α), which promotes HIV-1 transmission across endothelial membranes, increases HIV-1 transmission across the placenta. Flow cytometry and immunostaining studies were performed, which demonstrated that the HIV-1 receptors CD4, CCR5 and CXCR4 were not expressed by villous trophoblast cells. Consequently, primary villous trophoblast cells were not infected with cell-free HIV-1 isolates, as measured by *in situ* PCR and quantitative PCR, but villous trophoblast cells were infected by HIV-1-infected peripheral blood mononuclear cells (PBMC). HIV-1 from infected PBMC was rapidly transported across confluent transformed trophoblast cell monolayers by transcytosis, and TNF-α significantly upregulated transcytosis of HIV-1 across the trophoblast layer without disrupting cell viability or confluency. Inhibitors of TNF-α (antibodies against TNF-α and TNF-α receptors) and an anti-inflammatory drug (tenidap) significantly reduced transcytosis rates. It was concluded that the villous trophoblast is resistant to infection by cell-free HIV-1 but susceptible to transcytosis of HIV-1 from infected PBMC, and inflammatory mediators such as TNF-α may play a critical role in promoting maternal–fetal transmission of HIV-1.

Received 30 March 2005
Accepted 16 March 2006

INTRODUCTION

In the USA, highly active anti-retroviral therapy throughout pregnancy and the use of prophylactic caesarean delivery in women with elevated RNA levels of *Human immunodeficiency virus 1* (HIV-1) have reduced the rate of vertical transmission to less than 2 % (Connor *et al.*, 1994; International Perinatal HIV Group, 1999). However, among women who do not register for prenatal care and among women in underdeveloped countries where adequate prenatal care may not be available, the risk of vertical transmission remains quite high. Consequently, it is estimated by the World Health Organization that 1600 neonates are still being infected daily with HIV-1 worldwide, and according to some estimates, up to 25 % of maternal–fetal transmission occurs before labour and delivery (Bertolli *et al.*, 1996; Bryson *et al.*, 1992; Douglas *et al.*, 1998). Unfortunately, we are still largely ignorant of the mechanisms by which HIV-1 is transmitted across the placenta to the fetus.

Development of the placenta is dependent upon the differentiation of trophoblast cells along two pathways (Damsky *et al.*, 1992; Parry *et al.*, 1997). In one pathway, a subset of undifferentiated cytotrophoblast cells in anchoring placental villi invades maternal tissues securing the attachment of the placenta to the maternal uterine wall and the development of an adequate vascular supply. In the other pathway, mononucleated cytotrophoblast cells within the placental villi fuse to form the multinucleated syncytiotrophoblast. As pregnancy progresses, cytotrophoblast cells become more sparse within the villi, and the syncytiotrophoblast forms the only continuous layer separating the maternal intervillous space and the fetal capillary endothelium.

In vivo evidence of transplacental HIV-1 transmission includes identification of the virus in fetuses aborted before labour and delivery, and the detection of HIV-1 DNA in placental tissue sections from cases of HIV-1 vertical transmission (Lagaye *et al.*, 2001; Mano & Chermann, 1991; Sheikh *et al.*, 2000). However, laboratory data regarding the

ability of HIV-1 to infect and cross the placenta are conflicted. For example, trophoblast cells from first trimester placentas were found to be permissive to HIV-1 infection, and latent infection of syncytiotrophoblast in explant cultures of third trimester placentas was detected using *in situ* PCR (Moussa *et al.*, 1999; Sheikh *et al.*, 2000). Conversely, other groups of investigators were unable to infect villous syncytiotrophoblast and cytotrophoblast cells using numerous HIV-1 isolates, including non-syncytium-inducing strains (M- or CCR5-tropic) that are prevalent in infected neonates (Bourinbaiar & Nagorny, 1993; Douglas *et al.*, 1991; Kilani *et al.*, 1997; McGann *et al.*, 1994). One group used plasmids containing HIV-1 DNA sequences to successfully transfect isolated trophoblast cells, indicating that trophoblasts were resistant to viral entry but did not suppress late-stage virus replication and assembly (Kilani *et al.*, 1997). Finally, villous trophoblast cells purified from placentas in HIV-1-infected mothers with children diagnosed as HIV-1-negative were negative for HIV-1 DNA and RNA, suggesting that the placental trophoblast layer served as a barrier to HIV-1 transmission in these cases (Tscherning-Casper *et al.*, 1999).

Clinical factors that appear to increase the rate of maternal–fetal HIV-1 transmission include elevated maternal serum levels of HIV-1 RNA, co-infection with *Hepatitis C virus* or other sexually transmitted diseases, and obstetric complications commonly associated with a pro-inflammatory response, such as premature rupture of fetal membranes, spontaneous preterm delivery and the presence of chorioamnionitis (Garcia *et al.*, 1999; International Perinatal HIV Group, 1999; Hershow *et al.*, 1997; Landesman *et al.*, 1996; Mandelbrot *et al.*, 1996). Evidence of inflammatory changes (i.e. histological chorioamnionitis) can be detected in most placentas from spontaneous preterm deliveries before 30 weeks of gestation, and placental membrane inflammation has been associated with an increased risk of HIV-1 vertical transmission (Goldenberg *et al.*, 2000; Wabwire-Mangen *et al.*, 1999). Hallmarks of the inflammatory response, including elevated levels of cytokines such as tumour necrosis factor alpha (TNF-α) in maternal serum, amniotic fluid and placental samples have been found consistently in association with spontaneous preterm labour and delivery (Gucer *et al.*, 2001; Maymon *et al.*, 1999; Steinborn *et al.*, 1996). TNF-α promotes HIV-1 replication in host cells through its interaction with the transcription factor NF-κB and elevated amniotic fluid TNF-α levels have been associated with increased rates of maternal–fetal HIV-1 transmission (Anderson, 1997; Duh *et al.*, 1989). Based on these observations, other investigators postulated that elevated maternal cytokine levels instigate the migration of HIV-1-infected leukocytes into the placental circulation and amniotic fluid (Goldenberg *et al.*, 1998). We hypothesized that TNF-α promotes HIV-1 transmission across the placental barrier in pregnancies complicated by pro-inflammatory conditions, including co-infection with *Hepatitis C virus* or other sexually transmitted diseases, chorioamnionitis, premature rupture of the fetal membranes and spontaneous preterm delivery.

The villous syncytiotrophoblast appears to be susceptible to endocytosis of HIV-1-infected maternal monocytes (Bourinbaiar & Nagorny, 1993; Douglas *et al.*, 1991, 2001; Phillips & Tan, 1992). After endocytosis, the virus remains in vesicles and can be transported from the apical to the basolateral surface of the trophblast layer by transcytosis, which is a rapid transcellular transport pathway that is specific to polarized epithelial cells (Bomsel, 1997; Lagaye *et al.*, 2001). Cytokines such as TNF-α stimulate receptor-mediated endocytosis and transcytosis of macromolecules across the blood–brain barrier, and TNF-α upregulates transcription of HIV-1 promoters in trophoblast cells (Descamps *et al.*, 1997; Zachar *et al.*, 2002). Therefore, we hypothesized that TNF-α may promote transcytosis of HIV-1-infected monocytes across the placenta and anti-inflammatory agents may abrogate this effect. To test our hypothesis we sought to determine if: (i) placental villous trophoblast cells express HIV-1 receptors; (ii) these cells can be infected by HIV-1 isolates from cases of *in utero* HIV-1 transmission; (iii) trophoblast cells are permissive to transplacental transmission of HIV-1 by non-classical routes, including cell-mediated infection of villous trophoblast cells and transcytosis of HIV-1 across the confluent villous trophoblast barrier; and (iv) the inflammatory mediator TNF-α regulates HIV-1 transmission across the placenta.

METHODS

Cell preparation and culture. We isolated villous cytotrophoblast cells from third trimester placentas using a protocol originally described by Kliman *et al.* (1986) and modified in our laboratory (Parry *et al.*, 1998). The purity of the primary trophoblast preparations was confirmed by immunostaining with monoclonal antibodies (mAbs) that recognize cytokeratin-18 (expressed by trophoblast cells; Sigma) and vimentin (expressed by fibroblasts/monocytes; Sigma). These purification procedures yielded preparations that contained greater than 99·5 % cytotrophoblasts. The isolated villous trophoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % heat-inactivated fetal bovine serum (FBS).

Immortalized trophoblast (BeWo) cells were cultured in DMEM supplemented with 10 % FBS. BeWo cells (ATCC #CCL-98) are transformed human choriocarcinoma cells that have retained the cellular characteristics and endocrine profile of villous cytotrophoblasts (Ringler & Strauss, 1990).

HIV-1 isolates. Cell-free HIV-1 primary isolates were provided by the National Institutes of Health AIDS Research and Reference Reagent Program. Strains of HIV-1 were isolated from neonates in the USA within the first week of life, indicating probable *in utero* infection. Because most cases of *in utero* HIV-1 transmission involved CCR5-tropic strains of HIV-1, we used two CCR5-tropic strains (92US072 and 93US142) and a dual-tropic strain (93US151, which is CCR5-tropic and CXCR4-tropic), to conduct our experiments. HIV-1 isolates were propagated in peripheral blood mononuclear cells (PBMC), which were isolated from healthy volunteers. PBMC were stimulated with phytohaemagglutinin and infected with

HIV-1 isolates, and the concentration of HIV p24 antigen in the supernatant was determined by ELISA (Coulter HIV-1 p24 antigen assay; Coulter Corporation) 5–10 days after infection.

Trophoblast cells were infected with HIV-1 isolates at ratios equivalent to 25–50 ng p24 antigen (cell-free HIV-1) per 1×10^5 cells. Alternatively, when the p24 antigen concentration in the supernatant from HIV-1-infected PBMC exceeded 1000 pg ml^{-1}, the HIV-1-infected PBMC were co-cultured in a 1 : 1 concentration with trophoblast cells. The trophoblast cells were washed 2 h after infection, and incubated for another 3–6 days before assays were performed to measure infection rates.

Treatment with TNF-α. BeWo cells were treated with physiologically relevant amounts of TNF-α (10–100 ng ml^{-1}; R&D Systems) immediately preceding HIV-1 infection (Zachar *et al.*, 2002). After treatment with TNF-α, lactate dehydrogenase (LDH) levels were measured in cell culture medium to ensure that cell viability was maintained (CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega). LDH levels in cell lysates were divided by total LDH levels (culture medium + cell lysates) to determine the percentage of cells that remained viable. To determine if TNF-α-induced transcytosis was specific to activation of TNF-α receptors, we utilized a dual approach in which BeWo cells were treated with: (i) 0·8 μg ml^{-1} of a polyclonal antibody that neutralizes human TNF-α bioactivity (anti-human TNF-a neutralizing antibody; R&D Systems) or (ii) 0·8–20 μg ml^{-1} of mAbs that block the TNF-α receptors I and II (anti-TNFRI and anti-TNFRII; R&D Systems). BeWo cells were also treated with tenidap (compound CP-66248; Pfizer), which is an anti-inflammatory drug that affects diverse biological processes, including cytokine production, arachidonic acid metabolism and intracellular free calcium concentration, which is a critical event leading to inflammatory cell proliferation (Matsumoto & Fujii, 2002; Palacios *et al.*, 1998; Sipe *et al.*, 1992). Tenidap also decreases HIV-1 replication in acutely infected monocytes (Dezube *et al.*, 1997). We treated BeWo cells with 10–100 ng TNF-α ml^{-1} and 12·5–50 μM tenidap immediately preceding HIV-1 infection.

Indirect immunofluorescence assays. Primary cytotrophoblast cells and BeWo cells were treated with normal goat serum to block non-specific binding, after which the cells (at least 4×10^4 cells for each experiment) were incubated with murine mAbs against HIV-1 receptors (CD4, CCR5, CXCR4). Primary antibodies were purchased from R&D Systems (anti-CD4 and anti-CXCR4 antibodies) and Pharmingen (anti-CCR5 antibodies). Human astroglioma (U87) cells stably transduced with the MV7neo-T4 retroviral vector and expressing CD4, CCR5, and/or CXCR4 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and were used as positive controls. After treatment with primary antibody, the cells were incubated with fluorescein-conjugated goat antibody to mouse immunoglobulin (diluted 1 : 20 to 1 : 100), and fluorescence was measured using an Epics XL flow cytometer (Coulter Corporation).

Immunohistochemistry. Permanent histological sections from second trimester human placentas were deparaffinized and stained to detect CD4, CCR5 and CXCR4 according to previously published techniques (Vectastain ABC kit; Vector Laboratories) (Arechavaleta-Velasco *et al.*, 2004).

Indirect *in situ* PCR. We conducted *in situ* PCR experiments to determine if primary villous trophoblast cells could be infected with HIV-1 isolates. Briefly, 1×10^5 cells were plated on to coverslips in 24-well plates and infected with cell-free HIV-1 isolates or HIV-1-infected PBMC. Twenty-four to 48 h after infection, the cells were fixed and counterstained with anti-cytokeratin-18 antibodies to delineate trophoblast cells (i.e. purple staining in the cytoplasm). The fixed cells were treated with pepsin, which permeabilized the cells to permit entry of nucleotides, PCR primers for a 115 bp sequence in the HIV-1 *gag* gene (primers SK38 and SK39; Applied Biosystems) and *Taq* polymerase, during target sequence amplification in an Eppendorf Mastercycler (Applied Biosystems). PCR conditions were 32 cycles of 94 °C for 1 min, 56 °C for 2 min and 72 °C for 2 min. After PCR amplification, the trophoblast cells were hybridized with a 40 nt DNA probe for the *gag* gene (probe SK19; Applied Biosystems) and labelled with digoxigenin. After an overnight incubation with viral probe, antibody against digoxigenin was added and viral DNA was detected by brown staining in the nuclei of infected cells [diaminobenzidine (DAB); Sigma]. Negative controls included the use of target primers with irrelevant probe, irrelevant primers with target probe, DNase digestion before DNA amplification, and the exclusion of *Taq* polymerase or primers. Each experiment was conducted at least in triplicate.

Real-time PCR. The susceptibility of BeWo cells to latent infection by cell-free HIV-1 isolates was determined by quantitative PCR. Two to 48 h after infection, cells were washed and DNA was extracted using High Pure PCR Template Preparation kits (Roche Molecular Biochemicals). Integrated HIV-1 DNA sequences were amplified using a forward primer (5′-GCTAGCTAGGAAACCCAC-TGCTTA-3′) and reverse primer (5′-GCTAGAGATTTTCCACAC-TGACT-3′) that flank a 138 bp segment of the HIV-1 *strong stop* sequence, which is the initial DNA strand produced from viral RNA in target cells. Real-time PCR was performed as described previously (Elovitz *et al.*, 2003). The copy number of a housekeeping gene (*GAPDH*) that was not affected by HIV-1 infection was also quantified. Results of quantitative PCR experiments were calculated by dividing the *strong stop* copy number by the *GAPDH* gene copy number for each sample. Primary trophoblast cells were not used in these experiments because the source of HIV-1 *strong stop* DNA (i.e. trophoblast cells or infected cells contaminating primary trophoblast cultures) could not be determined.

Dual-chamber transcytosis systems. We cultured 1×10^5 BeWo cells in DMEM supplemented with 10 % FBS on semi-permeable inserts (0·45 μm pore size) coated with human fibronectin in 0·33 cm^2 transwells in a dual-chamber system (Becton Dickinson). Confluency of the endothelial monolayers was determined from 4 to 6 days after plating the cells by measuring transmembrane electrical resistance (Millicell Electrical Resistance system) and transport of [^{14}C]inulin across the membrane over a 120 min period (Bomsel, 1997; Hemmings *et al.*, 2001). Results of these assays were compared with 1×10^5 Madin-Darby canine kidney (MDCK) cells (ATCC #CCL-34), which form a tightly confluent monolayer on insert membranes 48–72 h after plating (Hemmings *et al.*, 2001). In order to provide a comparison to the confluent BeWo and MDCK cells, we treated the cells with 5 mM EDTA, which opens the tight junctions and permits paracellular transport of [^{14}C]inulin.

Transcytosis of HIV-1 across confluent trophoblast monolayers was assessed by removing the apical chambers from the transwells 2 h after infection with cell-free HIV-1 isolates or HIV-1-infected PBMC, and measuring productive infection of fresh PBMC (indicator cells) cultured in the basolateral medium. Three to 6 days later, infection of indicator cells was measured by ELISA to detect HIV-1 p24 antigen in the basolateral medium. In order to confirm that the appearance of infectious HIV-1 in the basolateral medium was consistent with the characteristics of transcytosis, we conducted parallel experiments under conditions that inhibited transcytosis: (i) at 4 °C and (ii) after adding colchicine (10 μM; Sigma) to depolymerize trophoblast microtubules (Bomsel, 1997; Hemmings *et al.*, 2001). To determine if HIV-1-infected PBMC yielded productive infection of trophoblast cells, we serially washed the transwells for 5 days before adding indicator cells to the basolateral medium. We were unable to detect HIV-1 p24 antigen by ELISA using medium from the apical chamber

before the final wash, indicating that PBMC did not remain adherent to the trophoblast cells and secrete HIV-1 particles into the apical medium. The viability of trophoblast cells was confirmed in these experiments (CytoTox 96 Non-Radioactive Cytotoxicity assays). Because transcytosis involves rapid transport across the apical cell monolayer, we concluded that successful infection of indicator cells 5 days later would indicate productive infection of trophoblast cells and could not be attributed to transcytosis.

In some experiments, HIV-1-infected PBMC were loaded with 30 ng calcein-AM (Molecular Probes) ml^{-1}, which becomes trapped by esterification in the cell cytoplasm and provides a fluorescent label without affecting PBMC mobility (Bomsel, 1997). PBMC were labelled before co-culture with BeWo cells in transwells, and basolateral medium was directly observed using a fluorescent microscope to determine if transcytosis of the entire PBMC occurred across the trophoblast layer.

Statistical analysis. Quantitative PCR and ELISA experiments were conducted at least in triplicate, and *strong stop/GAPDH* gene copy numbers or HIV-1 p24 antigen levels were compared by two-tailed *t* tests.

RESULTS

Expression of CD4, CCR5 and CXCR4 receptors in trophoblast cells

The CD4, CCR5 and CXCR4 receptors were not detected by indirect immunofluorescence assays on primary villous trophoblast cells (Fig. 1d–f). Expression of the HIV-1 receptors on each cell type was compared to non-specific binding of labelled secondary antibody (IgG2a) alone (blue curves). As expected, U87 cells transduced with retroviral vectors and receptor cDNA produced positive (right) shifts in binding of antibodies against CD4, CCR5 and CXCR4, which were expressed on 65–85 % of the cells (Fig. 1a–c). The CD4, CCR5 and CXCR4 receptors were not detected by indirect immunofluorescence assays on BeWo cells, while the epidermal growth factor receptor (positive control) was detected on approximately 80 % of trophoblast cells (data not shown).

Immunostaining of permanent histological sections from second trimester placentas confirmed that the CD4, CCR5 and CXCR4 receptors were not expressed in the villous trophoblast (Fig. 2). The HIV-1 receptors were detected in mesenchymal cells surrounding villous capillaries, which served as internal positive controls.

HIV-1 infection of trophoblast cells

We performed indirect *in situ* PCR experiments to demonstrate that 5–10 % of primary villous trophoblast cells were infected by HIV-1 after co-culture with HIV-1-infected PBMC, but primary villous cytotrophoblasts were not infected by cell-free HIV-1 isolates (Fig. 3). The HIV *gag* gene sequence in trophoblast cell nuclei was detected by brown staining. Susceptibility or resistance to infection by the three different HIV-1 isolates (92US072, 93US142 and 93US151) did not vary within each cell type. Negative and positive controls yielded anticipated results.

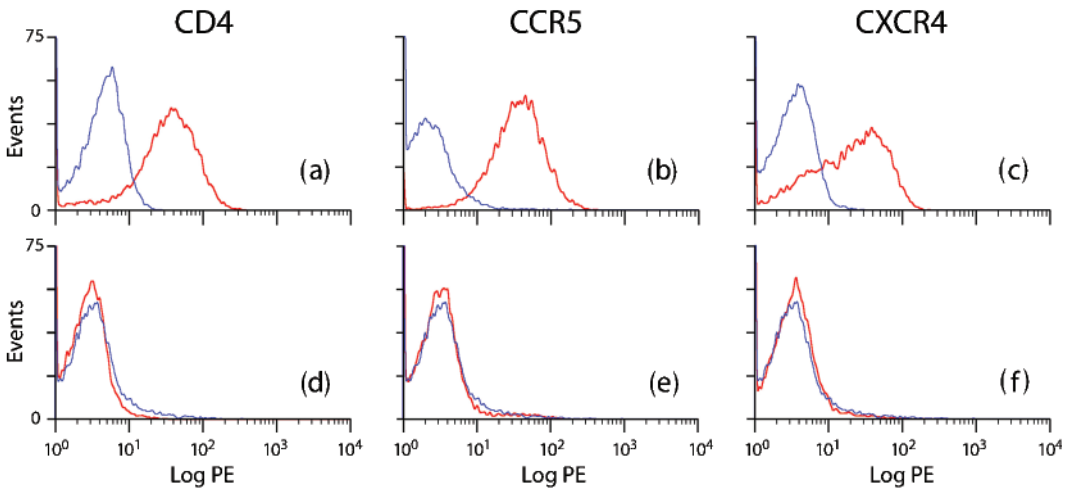

Fig. 1. Indirect immunofluorescence assays of HIV-1 receptors on primary trophoblast cells. Fluorescence intensity was measured in cells that were incubated with antibodies against the HIV-1 receptors (CD4, CCR5 and CXCR4) and fluorescein-conjugated secondary antibody. The *y* axis indicates the cell number (events) detected in one channel; 10 000 events were counted with each experiment. The *x* axis indicates logarithm of fluorescence intensity. The blue curves represent cells that were incubated with fluorescein-conjugated antibody alone (negative controls). Cell populations expressing CD4, CCR5 or CXCR4 (red curves) are located to the right of the non-specific binding region. (a–c) Demonstrates that 65–85 % of human astroglioma (U87) cells stably transduced with a retroviral vector and HIV-1 receptor cDNA expressed CD4, CCR5 and CXCR4 on the cell surface (positive controls). (d–f) Demonstrates negative results from primary villous cytotrophoblast cells.

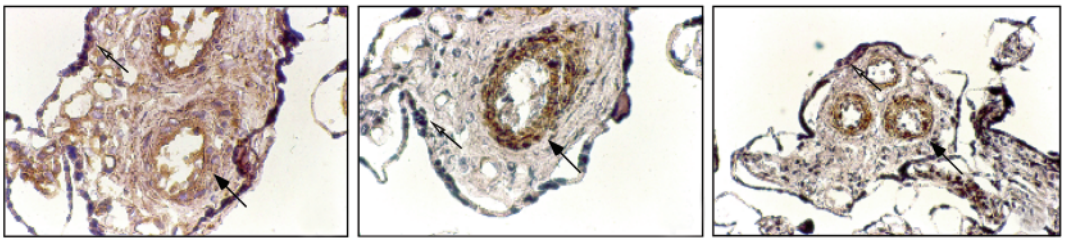

Fig. 2. Immunostaining for the HIV-1 receptors (CD4, CCR5 and CXCR4) within second trimester placental villi. None of the receptors were detected in villous trophoblast cells (open arrowheads), but CD4, CCR5 and CXCR4 protein was detected as brown staining in mesenchymal cells surrounding fetal capillaries within the villous core (closed arrowheads).

In order to detect latent HIV-1 infection with a high degree of sensitivity, real-time PCR experiments were performed to detect the HIV-1 *strong stop* sequence in BeWo cells from 2 h to 4 days after infection with HIV-1 isolates. Infection rates did not vary for the different HIV-1 isolates used, so results were pooled. The mean ± SD *strong stop*/*GAPDH* gene copy number in HIV-1-infected BeWo cells was 0·0005 ± 0·0007 after 2–4 h of infection and 0·0001 ± 0·0002 after 24–48 h of infection. Negative controls (uninfected BeWo cells) yielded similar *strong stop*/*GAPDH* gene copy numbers, while the mean ± SD *strong stop*/*GAPDH* gene copy number of HIV-1-infected PBMC (positive controls) was 0·8535 ± 0·1335. These results indicate that villous trophoblast (BeWo) cells were not susceptible to latent infection by cell-free HIV-1 isolates.

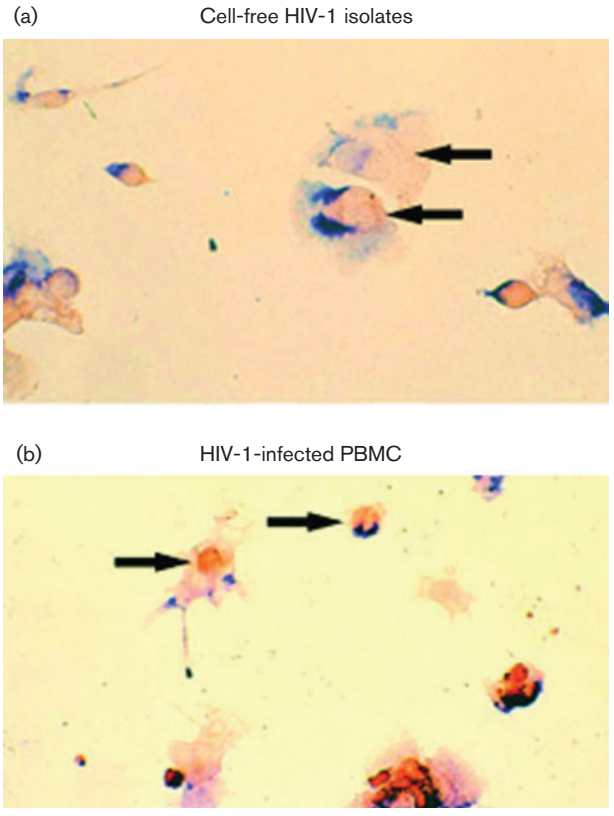

Fig. 3. Indirect *in situ* PCR to detect HIV-1 infection of primary villous trophoblast cells. The HIV-1 *gag* gene sequence was detected by brown staining in the nuclei of infected cells. Arrows point to nuclei of trophoblast cells counterstained with antibodies detecting cytoplasmic cytokeratin-18 (purple staining). (a) Nuclear brown staining was not detected in villous trophoblast cells infected with cell-free HIV-1 isolates. (b) Nuclear brown staining was detected in villous trophoblast cells co-cultured with HIV-1-infected PBMC.

Transcytosis of HIV-1-infected PBMC across the villous trophoblast

Because we were unable to detect infection of primary villous trophoblast cells and BeWo cells by cell-free HIV-1 isolates, we sought to determine if HIV-1-infected PBMC crossed the villous trophoblast layer by transcytosis and if treatment with TNF-α affected HIV-1 transcytosis rates.

Confluency of the BeWo cell monolayers was established by measuring transmembrane electrical resistance and transport of [^{14}C]inulin across the membrane (Table 1). Confluency of the BeWo cell monolayers appeared to be greatest 5 days after plating on the transwell inserts (transmembrane electrical resistance 233 ± 165 ohms × cm^2, [^{14}C]inulin clearance 0·14 ± 0·04 %). Mean [^{14}C]inulin clearance values across MDCK cells ranged from 0·01 % at 30 min to 0·30 % at 120 min incubation. Mean [^{14}C]inulin clearance values across BeWo cells cultured on transwells for 5 days ranged from 0·10 % at 30 min to 0·26 % at 120 min incubation. Mean [^{14}C]inulin clearance values across both cell types increased 15- to 35-fold after EDTA was added to open intercellular tight junctions and allow paracellular transport of [^{14}C]inulin. These findings confirmed the validity of the model as a confluent trophoblast cell monolayer.

Transmission of HIV-1 isolates across the BeWo cell monolayer was observed after co-culture with dual-tropic HIV-1-infected PBMC (36·8 ± 32·9 pg HIV-1 p24 antigen ml^{-1} basolateral medium), but not after infection with cell-free isolates (3·4 ± 1·1 pg ml^{-1}, which was below the range of standard values calibrated for the HIV-1 p24 antigen). These

Table 1. Confluency of BeWo cell monolayers in dual-chamber systems

The confluency of BeWo cells cultured for 3–12 days was compared with MDCK cells cultured for 4 days. Transmembrane electrical resistance was measured as ohms × cm^2, while [^{14}C]inulin clearance was determined by dividing the level of ^{14}C in the basal medium by the level of ^{14}C in the apical medium after a 60 min incubation period. Each value reported below represents the mean value ± SD of at least three transwell experiments. EDTA was added to the monolayers to open the tight junctions and allow paracellular transport of [^{14}C]inulin.

Cells	Transmembrane electrical resistance (ohms × cm^2 ± SD)	[^{14}C]inulin clearance (basal/apical percentage ± SD)
No cells	0	100
MDCK cells × 4 days	395 ± 282	0·13 ± 0·07
BeWo cells × 3 days	168 ± 115	0·23 ± 0·07
BeWo cells × 4 days	183 ± 137	0·17 ± 0·02
BeWo cells × 5 days	233 ± 165	0·14 ± 0·04
BeWo cells × 12 days	432 ± 308	0·30 ± 0·08
MDCK cells + EDTA	–	4·65 ± 2·33
BeWo cells + EDTA	–	2·0 ± 0

results represent the mean values of numerous experiments and are statistically significant ($P = 0·0007$, Table 2). In some experiments, HIV-1-infected PBMC were fluorescent-stained before co-culture with BeWo cells in transwells, but no fluorescent-stained cells were observed in the basolateral medium, indicating that transport of the entire PBMC across the trophoblast layer did not occur. HIV-1 p24 antigen levels in the basolateral medium were not significantly different following co-culture of BeWo cells with PBMC infected with a dual-tropic HIV-1 strain (93US151, which is CXCR4-tropic and CCR5-tropic) or non-syncytium-inducing HIV-1 strains (92US072 and 93US142, which are CCR5-tropic, data not shown). HIV-1 p24 antigen values less than 7·80 pg ml^{-1} corresponded to optical densities below the range of standard values calibrated for the HIV-1 p24 antigen and were considered negative results. Thus, significant levels of HIV-1 transcytosis across the BeWo cell monolayers were not observed when experiments were conducted using cell-free HIV-1 isolates and when cells were co-cultured with HIV-1-infected PBMC at 4 °C or in the presence of 10 μM colchicine, conditions which inhibited transcytosis. Finally, HIV-1 p24 antigen values were less than 7·80 pg ml^{-1} when indicator cells were added to the basolateral medium 5 days after BeWo cells were co-cultured with HIV-1-infected PBMC and serially washed. In cell viability assays, LDH levels remained greater than 95 % (range 93·8–100 %) of total LDH levels in each well (cell lysates plus culture medium), indicating that BeWo cells remained viable but did not secrete infectious HIV-1 particles (e.g. no productive infection) into the basolateral medium 5 days later.

Table 2. Rates of transcytosis of HIV-1 isolates across BeWo cell monolayers

Cell-free HIV-1 isolates or HIV-1-infected PBMC were added to apical medium bathing confluent BeWo cells on semi-permeable membranes in transwell inserts. After 2 h, the inserts were removed and infection of indicator cells (fresh PBMC in basolateral medium) was measured by ELISA 3 days later to detect HIV-1 p24 antigen.

Transcytosis conditions	HIV-1 p24 antigen (pg ml^{-}* ± SD)	*P*-value*
HIV-1† + PBMC	36·8 ± 32·9	–
HIV-1	3·4 ± 1·1	0·0007
HIV-1 + PBMC + Colchicine	5·0 ± 3·4	0·001
HIV-1 + PBMC at 4 °C	2·6 ± 2·5	0·0005

**P*-values are results of two-tailed unpaired *t* tests comparing HIV-1 p24 antigen values for each condition with the HIV-1 p24 antigen value in basolateral medium after BeWo cells were infected with HIV-1-infected PBMC.

†Dual-tropic (CCR5-tropic and CXCR4-tropic) HIV-1 strains (93US151) infect macrophages and CD4-positive T-cells.

TNF-α increases transcytosis of HIV-1 across BeWo cell monolayers

Inflammatory mediators such as TNF-α are produced locally by the placenta and have been reported to augment HIV-1 infection and replication (Coulomb-L'Hermine *et al.*, 2000; Patterson *et al.*, 2001; Zachar *et al.*, 2002). Therefore, we investigated whether TNF-α affected transcytosis of HIV-1 from infected PBMC across BeWo cell monolayers. After treatment with TNF-α, transmembrane electrical resistance was unaffected. In cell viability assays, LDH levels remained greater than 97 % (range 96·93–100 %) of total LDH levels in each well (cell lysates plus culture medium) in BeWo cells treated with TNF-α (10–100 ng ml^{-1}) and untreated BeWo cells. These results indicate that the confluent monolayers were not destroyed and BeWo cells remained viable after treatment with TNF-α.

Treatment with TNF-α yielded significantly increased HIV-1 transcytosis rates when BeWo cells were co-cultured with HIV-1-infected PBMC (Fig. 4). In order to compare the results of numerous experiments, we calculated the ratio of HIV-1 p24 antigen level for TNF-α-treated cells divided by the mean p24 antigen level for untreated cells in each experiment. When maximal levels of TNF-α were used, transcytosis rates were twofold greater than baseline ($P = 0·02$; Fig. 4a). Treatment of BeWo cells with TNF-α did not induce transcytosis of cell-free HIV-1 isolates across the BeWo cell monolayers.

In order to confirm that treatment with TNF-α augmented transcytosis of HIV-1 from infected PBMC across the BeWo cell monolayer, we treated the cells simultaneously with an antibody that neutralizes TNF-α or antibodies that block the

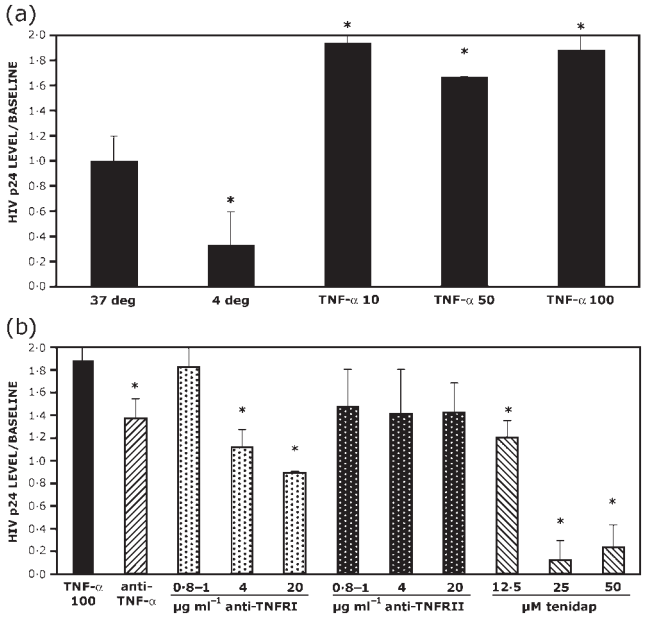

Fig. 4. Bar graphs demonstrating that treatment of BeWo cells with TNF-α significantly increased HIV-1 transcytosis rates (i.e. HIV-1 p24 antigen levels in basolateral medium) across the BeWo cell monolayers. (a) Baseline transcytosis rate (bar labelled 37 deg) was normalized to 1·00, while experiments conducted at 4 °C (bar labelled 4 deg) were performed as negative controls for transcytosis and demonstrated significantly decreased HIV-1 p24 antigen levels in the basolateral medium. Bars labelled TNF-α 10, 50 and 100 indicate cells treated with 10–100 ng TNF-αml^{-1}, and transcytosis rates were increased approximately twofold after treatment with 100 ng TNF-α ml^{-1} ($P=0·02$). (b) In this bar graph, all BeWo cell monolayers were treated with 100 ng TNF-α ml^{-1}. Co-treatment with antibodies that neutralize TNF-α (bar labelled anti-TNF-α) or block TNF-α receptors (bars labelled anti-TNFRI and anti-TNFRII) reduced transcytosis of HIV-1-infected PBMC across trophoblast cell monolayers. Co-treatment with tenidap also reduced transcytosis rates. Transcytosis rates in cells treated with antibodies or tenidap were compared to transcytosis rates in cells treated with TNF-α alone, and significantly reduced rates ($P<0·05$) are indicated by asterisks.

TNF-α receptors (TNFRI and TNFRII). In these experiments, treatment with 100 ng TNF-α ml^{-1} increased transcytosis of HIV-1 from infected PBMC to $1·88\pm0·11$ times baseline, while treatment with antibodies neutralizing TNF-α or blocking TNFR1 significantly reduced this effect toward baseline transcytosis rates ($P<0·05$; Fig. 4b). A dose-response relationship was observed using 0·8–20·0 mg anti-TNFRI antibody ml^{-1}. Treatment with 10 ng TNF-α ml^{-1} and antibodies that abrogate TNF-α activity also demonstrated significantly decreased transcystosis rates ($P<0·05$) after co-treatment with anti-TNF-α antibody and anti-TNFR1.

Because HIV-1-infected PBMC may induce an inflammatory response that promotes transcytosis of HIV-1 across the trophoblast layer, we treated BeWo cells simultaneously with 12·5–50 μM tenidap and 10–100 ng TNF-α ml^{-1}. Treatment with 100 ng TNF-α ml^{-1} and 12·5 μM tenidap significantly reduced the TNF-α effect toward baseline transcytosis rates ($P=0·006$; Fig. 4b), while treatment with higher doses of tenidap reduced transcytosis below baseline rates ($P<0·0001$; Fig. 4b). Similar results were observed when lower doses of TNF-α levels were used. These results suggest that an anti-inflammatory agent such as tenidap may prevent the inflammatory response that is triggered by HIV-1-infected PBMC at the trophoblast interface and may reduce HIV-1 transcytosis across the trophoblast layer.

DISCUSSION

We performed a comprehensive series of experiments characterizing the expression of HIV-1 receptors on placental trophoblast cells, the ability of HIV-1 strains to infect primary villous trophoblast cells, factors that influence HIV-1 infection of trophoblast cells and the potential importance of non-classical routes by which cell-bound HIV-1 may be transmitted from the maternal to the fetal circulation.

Previous investigators have reported conflicting data regarding the expression of HIV-1 receptors and chemokine co-receptors on trophoblast cells (Douglas & Thirkill, 2001; Ishii *et al.*, 2000; Moussa *et al.*, 1999). Most investigators agree that trophoblast cells do not express the CD4 antigen, to which HIV-1 envelope protein binds. Consequently, research efforts have focused on the expression of chemokine receptors, which may be involved in CD4-independent infection of trophoblast cells. The chemokine receptor CCR5 is a co-receptor for macrophage-tropic HIV-1 strains, which are usually non-syncytium inducing and are the most prevalent isolates in neonatal infections, while CXCR4 is a co-receptor for T-cell-tropic HIV-1 strains, which are usually syncytium inducing (Doms & Peiper, 1997). In some reports, these chemokine receptors were detected in first trimester trophoblast cells, but according to most reports, term trophoblast cells do not express the HIV-1 co-receptors CCR5 and CXCR4 (Douglas & Thirkill, 2001; Ishii *et al.*, 2000; Moussa *et al.*, 1999). We used sensitive techniques (i.e. flow cytometry and immunostaining) and were unable to detect expression of CD4, CCR5 and CXCR4, on villous trophoblast cells in second and third trimester placentas. Importantly, positive controls yielded expected results with both techniques. Our results are congruent with those reported for other endothelial cells, including brain and lung endothelial cells, which do not express CD4, CCR5 and CXCR4 on their surface (Kanmogne *et al.*, 2000, 2001). Therefore, we conclude from our findings that the absence of these receptors on trophoblast cells provides one mechanism by which the placenta resists transmission of HIV-1 from the maternal to the fetal circulation.

After failing to detect HIV-1 receptors on villous trophoblast cells, we were not surprised to find that villous

cytotrophoblast cells were not infected by cell-free HIV-1 isolates from established cases of *in utero* infection. Again, we used two sensitive methods (i.e. *in situ* PCR and real-time PCR) to detect HIV-1 gene sequences in trophoblast cells, and positive controls yielded expected results with both techniques. Vidricaire *et al.* (2004) reported that cell-free HIV-1 utilized endocytic host cell machinery and crossed JAR choriocarcinoma cells by transcytosis, which would appear to be contradictory to our findings. However, several points should be noted: (i) BeWo, JAR and JEG-3 cells are similar hormone-producing choriocarcinoma cell lines (Ringler & Strauss, 1990), and Vidricaire *et al.* (2004) indicated that they used JAR cells for their experiments because HIV-1 expression was higher in these cells than in BeWo or JEG-3 cells; (ii) Vidricaire *et al.* (2004) did not utilize wild-type virus to conduct their experiments, but instead, they utilized HIV-1 particles pseudotyped with Ada-M envelope; and (iii) Vidricaire *et al.* (2004) added TNF-α (10 ng ml^{-1}) for 24 h to induce HIV-1 gene expression in JAR cells, and the importance of pro-inflammatory mediators in HIV-1 transmission across the placenta was a central finding in our paper. Furthermore, our findings were consistent with those reported by Lagaye *et al.* (2001), who found that cell-free HIV-1 neither crossed the BeWo cell monolayer nor productively infected BeWo cells. Most importantly, our findings using primary trophoblast cells (no infection following exposure to cell-free HIV-1 isolates) may resolve the contradictory observations seen with choriocarcinoma cell lines and reported by Vidricaire *et al.* (2004), Lagaye *et al.* (2001) and our group. Finally, our results support clinical reports that cite a relatively low (25 %) vertical transmission rate in untreated HIV-1 seropositive pregnant women (Connor *et al.*, 1994).

Although the villous trophoblast appears to be resistant to infection by cell-free HIV-1 isolates, we found that HIV-1 from infected PBMC can infect villous trophoblast cells and rapidly crosses a polarized trophoblast cell layer by transcytosis. Electron microscopic observations indicate that HIV-1 buds from lymphocyte donor cells at points of cell-to-cell contact and is taken up by trophoblasts via endocytosis (Phillips & Tan, 1992). After internalization, the virus remains in vesicles and is rapidly transported from the apical to the basolateral surface of the endothelium, from which infectious HIV-1 particles are released (Bomsel, 1997; Lagaye *et al.*, 2001). We found that co-culture of BeWo cells with HIV-1-infected PBMC resulted in transport of CCR5-tropic and dual-tropic HIV-1 across confluent BeWo cell monolayers, and that features of this transport system were consistent with transcytosis: (i) rapidity (BeWo cells were co-cultured with HIV-1-infected PBMC for only 2 h before transwells were removed); (ii) fluorescent-labelled PBMC were not transported across the BeWo cell monolayer; and (iii) transmission rates were dramatically reduced when cells were incubated with colchicine (which inhibits microtubular function that is important in transcytosis) or cultured at 4 °C (a temperature at which viral infections may occur, but transcytosis is inhibited).

Our findings also provide experimental evidence supporting the clinical observation that inflammatory mediators are associated with increased HIV-1 vertical transmission rates (Goldenberg *et al.*, 1998; Wabwire-Mangen *et al.*, 1999). The placenta produces a number of cytokines that may play a critical role in promoting or inhibiting vertical transmission of HIV-1, and inflammation of the placental villous membrane was associated with an increased rate of mother-to-child HIV-1 transmission in a large cohort of women from Uganda (Coulomb-L'Hermine *et al.*, 2000; Patterson *et al.*, 2001; Wabwire-Mangen *et al.*, 1999; Zachar *et al.*, 2002). We found that TNF-α significantly upregulated transcytosis of HIV-1-infected PBMC across BeWo cell monolayers without disrupting the confluent trophoblast barrier, and that treatment with antibodies that neutralize TNF-α or block TNFRI significantly reduced this effect. In human trophoblast cells, TNFRI is expressed constitutively, and TNFRI appears to be the primary receptor responsible for the actions of TNF-α in inflammatory states (Rasmussen *et al.*, 1999; Yang *et al.*, 2002). Hence, we were not surprised to observe significantly decreased transcytosis rates when cells were treated with mAbs that block TNFRI, but not when cells were treated with antibodies against TNFRII. Our findings support the observations of another group of investigators, who previously demonstrated that TNF-α stimulates promoters of HIV-1 in isolated trophoblast cells (Zachar *et al.*, 2002). Thus, we believe that local cytokine status may be correlated with trophoblast infection and the likelihood of prenatally acquired HIV-1 infection.

Tenidap is an anti-inflammatory agent that inhibits production of interleukin (IL) 1, IL-6 and TNF-α from Hep3B hepatoma cells and PBMC (Dezube *et al.*, 1997; Sipe *et al.*, 1992). In addition, tenidap demonstrates other anti-inflammatory properties, including inhibition of cyclooxygenase-mediated arachidonic acid metabolism, decreased intracellular free calcium concentrations that are required for inflammatory cell proliferation, anion-transport inhibition and cytoplasmic acidification (Dezube *et al.*, 1997; Matsumoto & Fujii, 2002; McNiff *et al.*, 1994). In clinical studies, decreased levels of chemoattractant proteins in synovial fluid and acute-phase proteins in serum were observed in animals and humans treated with tenidap (Loose *et al.*, 1993; Palacios *et al.*, 1998). Finally, tenidap reduced HIV-1 replication in PBMC independent of its ability to reduce IL-6 production (Dezube *et al.*, 1997). Because inflammatory changes in the trophoblast and obstetric conditions associated with an inflammatory response in reproductive tissues are associated with HIV-1 transmission across the placenta, we sought to determine if tenidap could reduce transcytosis of HIV-1 from infected PBMC across the confluent trophoblast layer. Our results indicate that tenidap effectively inhibits the increased transcytosis rates observed after treatment with TNF-α. At higher doses (25–50 μM), the reduction of transcytosis rates to approximately five times lower than baseline indicates that the mechanisms of action of tenidap may not be limited to TNF-α blockade. These results suggest that inflammatory

interactions stimulated by co-culture of HIV-1-infected PBMC with trophoblast cells promote HIV-1 transmission across the placenta, and reversal of these inflammatory effects may prevent vertical transmission of HIV-1.

Based on our data, we conclude that: (i) villous trophoblast cells do not express classic HIV-1 receptors and are resistant to infection by cell-free HIV-1 isolates; (ii) transcytosis of HIV-1 from infected PBMC across the villous trophoblast layer has important implications for transmission of HIV-1 from the mother to the fetus; and (iii) HIV-1 seropositive women with conditions that induce production of pro-inflammatory mediators in the placenta may be at a particularly increased risk of HIV-1 transmission to their fetuses. Therefore, interventions to reduce maternal inflammation may yield decreased rates of maternal–fetal HIV-1 transmission.

ACKNOWLEDGEMENTS

This work was supported by a Women's Reproductive Health Research award from the NIH, a generous gift from the Bill and Melinda Gates Research Foundation and a pilot grant from the Center for AIDS Research at the University of Pennsylvania.

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