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HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION OF CHORIOCARCINOMA-DERIVED TROPHOBLASTS

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Summary. – An *in vitro* model of placental infection by human immunodeficiency virus type 1 (HIV-1) was established using human choriocarcinoma-derived trophoblast lines exposed to free HIV-1 or HIV-1-infected lymphocytic and monocytic cells. Virus infectivity was evaluated by measuring both the levels of p24 HIV-1 antigen and reverse transcriptase activity either from indicator MT-4 lymphocytes after co-cultivation with infected trophoblasts or directly from trophoblast cultures. None of the tested trophoblast lines were permissive, in a detectable manner, to infection by cell-free virus. Furthermore, there were no signs of infection when trophoblasts were exposed to HIV-1-carrying ACH-2 and U1 cells with impaired adhesion capacity. However, the exposure to MOLT-4/IIIB lymphocytes or U937/YH5 monocytes that adhere to substrate cells resulted invariably in productive infection. The ultrastructure of the trophoblasts suggests endocytosis of HIV-1. It appears that the infection of the host cell results from the escape of virions from degradation in lysosomes. Alternatively, HIV-1 may enter by budding directly from the lymphocyte surface into the cytoplasm of trophoblasts. These results confirm previous studies and suggest that CD4-negative placental trophoblasts – the only foetal cells in direct contact with maternal blood – can be susceptible to HIV-1 infection.

Key words: HIV transmission; lymphocyte; monocyte; trophoblast; endocytosis

Introduction

The possibility of intrauterine HIV-1 infection across the trophoblastic placental barrier has been supported by a number of clinical and experimental studies (Amirhessami-Aghili and Spector, 1991; Andimann *et al.*, 1990; Chandwani *et al.*, 1991; European Collaborative Study, 1991; Hill *et al.*, 1987; Lewis *et al.*, 1990; Mattern *et al.*, 1992; Maury *et al.*, 1989; Zachar *et al.*, 1991*a*; Zachar *et*

al., 1991*b*; Zachar *et al.*, 1991*c*). Our investigations (Bourinbaiar and Phillips, 1991; Bourinbaiar *et al.*, 1991*a*; Phillips and Bourinbaiar, 1992) and the recent study by Douglas *et al.* (1991) indicate that CD4-negative epithelial cell-like trophoblasts are more permissive to HIV-1 delivered by contact with infected blood cells than to cell-free HIV-1. Although it appears that HIV-1 enters into placental explants via coated pits (Douglas *et al.*, 1991), little is known about the further cellular sequence of events preceding the establishment of HIV-1 infection in placental tissue. Therefore, it was suggested by Zachar *et al.* (1991*a*; 1991*b*; 1991*c*) that the results from established trophoblast lines which display a close similarity to fresh trophoblasts may provide valuable information for the mechanism of retroviral infection. In this *in vitro* study we characterized the infection of trophoblast cell lines exposed either to free HIV-1 or HIV-infected lymphocytic and monocytic cells.

Materials and Methods

Infection of trophoblast lines by free HIV-1 or by contact with HIV-infected lymphocytic and monocytic cells. Human choriocarcinoma-derived BeWo, JAR, JEG-3 (CCL 98, HTB 144, HTB 36, ATCC, Rockville, MD), and ENAMI (kindly supplied by Dr. N. Matsuzaki, Osaka University Medical School, Osaka, Japan, with permission from Dr. K. Yamashita, National Sapporo Hospital, Sapporo, Japan) trophoblast lines were seeded at concentration of 10^5 cells/cm^2 and grown to near-confluence for three days in a 6-well culture plate. These cells were exposed for 1 hr either to appropriately adjusted concentrations of free HIV-1 (Bourinbaiar *et al.*, 1991*a*) in filtered supernatants from MOLT-4/IIIB, U937/YH5, ACH-2, and U1 cultures or to virus-infected washed MOLT-4/IIIB, U937/YH5 (from Dr. J. Minowada, Fujisaki Cell Center, Okayama, Japan), ACH-2, and U1 (from Dr. T. Folks, AIDS Research and Reference Reagent Program (ARRRP), AIDS Program, NIAID, NIH) cells (10^6/ml) at 37 °C. By the end of incubation suspension-grown donor lymphocytic (MOLT-4/IIIB, ACH-2) and monocytic (U937/YH5, U1) cells were thoroughly washed from the plastic-adherent trophoblasts by repeated rinsing with ice-cold Ca^{--} and Mg^{--} free PBS. Following the removal of non-absorbent HIV-1 and adherent HIV-infected cells, the virus-exposed trophoblasts were trypsinized and replated into new culture dishes. Residual leukocytes which could persist after washing were eliminated by further culture in serum-free medium (Serumless Medium, Neuman and Tytell, GIBCO) for 7 days. Virus production from trophoblasts at days 10, 20, and 30 p.i. was assayed either directly from the culture fluid of HIV-exposed trophoblasts or was examined after amplification by virus culture as follows. Cord blood-derived MT-4 lymphocytes (10^6 cells/ml) were washed twice and added to mock-infected and infected trophoblasts. The MT-4 lymphocytes (from Dr. J. Minowada, Fujisaki Cell Center, Okayama, Japan) remained in contact with inoculated trophoblasts for 1 hr and then were gently removed and washed in PBS three times. MT-4 were resuspended in new wells and incubated at 37 °C in 5 % CO_2 for 7 days. By the third day MT-4 cells, which usually grow in compact clumps, tended to dissociate and some of them formed multinuclear giant cells indicating active viral replication. Instead of relying on manual counting of syncytial cells (a biased and obsolete assay) we determined the signs of infection by standardized and commonly accepted viral assays. In both types of infection, cell-free or cell-mediated, virus production from trophoblasts was assayed by enzyme linked immunosorbent assay (ELISA) for relase of p24 HIV-1 *gag* protein (Viral core p24 antigen ELISA kit, Cellular Products, Buffalo, NY) or by reverse transcriptase (RT) as described by us previously (Bourinbaiar and Phillips, 1991; Phillips and Bourinbaiar, 1992).

Electron microscopy. Individual Millicell-HA (Millipore, Bedford, MA) insert plates containing BeWo monolayers (10^5 cells/cm^2 initial seeding concentration) were removed 20 min, 40 min, 60 min, 2 hr, and 3 hr after the addition of free virus or washed MOLT-4/IIIB (10^6 cells/ml) cells. The

plates were immediately immersed in 3 % glutaraldehyde in 0.2 mol/l phosphate buffer at pH 7.2 and prepared for transmission electron microscopy as described in detail previously (Bourinbaiar and Phillips, 1991; Phillips and Bourinbaiar, 1992).

Results

The inspection of productive infection revealed that all four tested trophoblast cell lines are susceptible to HIV-1 infection as summarized in Table 1. However, the infectivity of HIV-1 was dependent on the mode of virus delivery. None of the trophoblast lines was permissive to cell-free virus in a detectable fashion. Similarly, there were no signs of infection when choriocarcinoma cell lines were exposed to HIV-1 carrying U1 monocytes or ACH-2 lymphocytes which did not adhere to trophoblasts. On the contrary, exposure to adherent MOLT-4/IIIB lymphocytes or U937/YH5 monocytes resulted in infection of all tested trophoblasts. The infection of trophoblasts was characterized by very low RT activity and p24 production. The results of direct viral detection from trophoblast culture fluid 10 and 20 days p.i., revealed that there were barely detectable levels of p24 antigen (under 10 pg/ml of p24 with the detection limit of p24 kit being 7.8 pg/ ml) and RT activity of only 100–300 cpm/10 μl against 100–200 cpm/10 μl in mock-infected trophoblasts. Mock-infected and HIV-infected trophoblasts remained morphologically identical and no cytopathic effect could be observed in trophoblast cells exposed either to free virus or cell-associated virus. One month after inoculation the HIV-1 infection seemed to be established as a latent infection with no detectable p24 release or RT activity. However, coincubation

Table 1. Infection of choriocarcinoma-derived trophoblast lines by cell contact with HIV-carrying lymphocytic and monocytic donor cells or by exposure to free HIV-1 from donor supernatants

Source of HIV	Choriocarcinoma-derived trophoblast lines			
	BeWo	ENAMI	JAR	JEG-3
MOLT-4/IIIB cells	+	+	+	+
MOLT-4/IIIB sup.	-	-	-	-
U937/YH5 cells	ND	ND	+	ND
U937/YH5 sup.	ND	ND	+/-	ND
ACH-2 cells	-	-	-	ND
ACH-2 sup.	-	-	-	ND
U1 cells	-	-	-	ND
U1 sup.	-	-	-	ND

Amount of cell-free viral inoculum in supernatants (sup) was adjucted to the levels of infectious viruses in HIV-producing donor cells according to a procedure described earlier (Bourinbaiar *et al.*, 1991*a*). Positive (+) or negative (-) infection was confirmed by virus culture with MT-4 lymphocytes using both p24 ELISA and RT assay at least in two separate experiments. ND not done: U937/YH5 and JEG-3 cells were discontinued due to persistence of antibiotic-resistant mycoplasma.

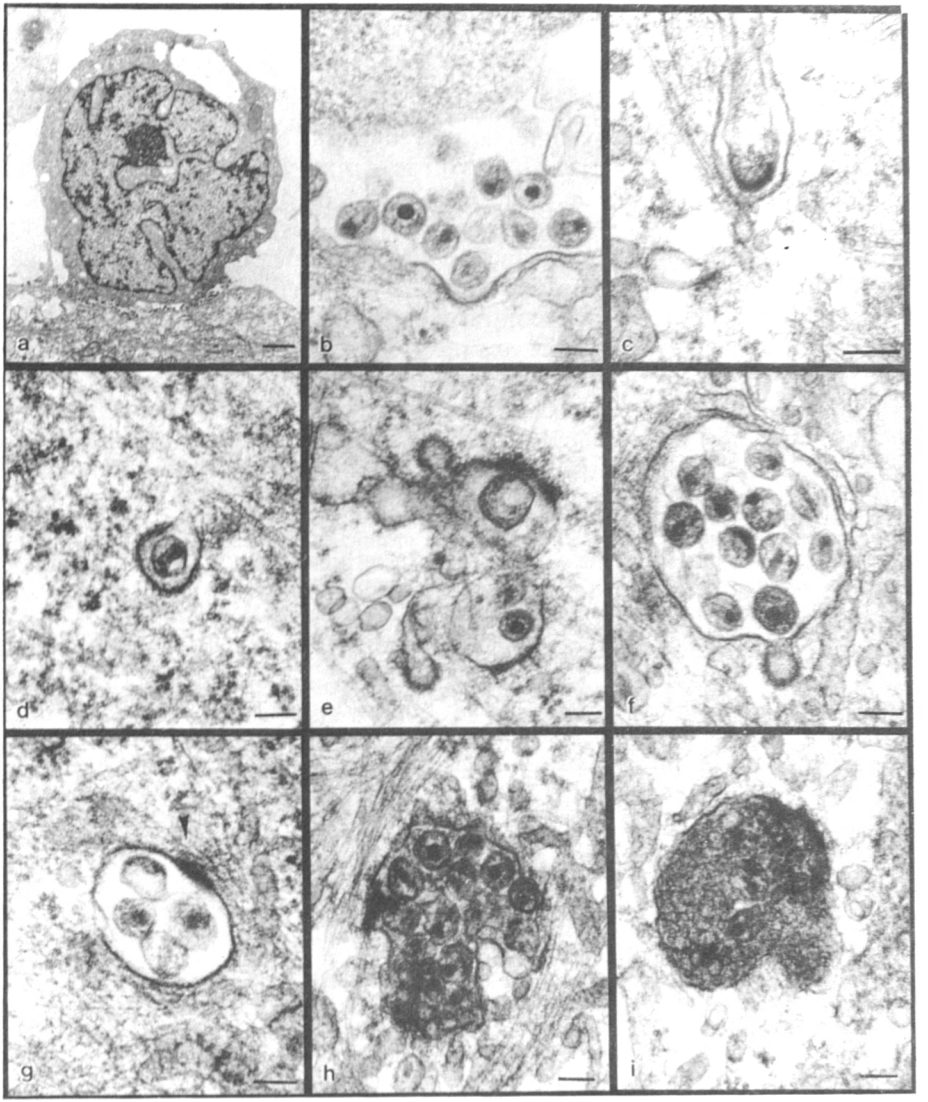

Fig. 1

of all trophoblast lines, exposed beforehand to MOLT-4/IIIB or U937/YH5 cells, with non-infected indicator MT-4 lymphocytes resulted in heavy viral production with typical levels of p24 above 1000 pg/ml and RT activity higher than 3500 cpm/10 μl.

Electron microscopy revealed that within 20 min of cell contact HIV-1 particles, normally invisible on the surface of washed lymphocytes, were found budding from lymphocytes in a highly polarized fashion into the cell-cell contact area and were entering trophoblasts via endocytosis (Fig. 1a-i). Mature and budding viral particles were observed exclusively in trophoblast-lymphocyte contact areas. This indicates that a contact-triggered virus releasing mechanism occurs in a situation similar to that described previously for cell-mediated infection of epithelial cells (Bourinbaiar and Phillips, 1991; Phillips and Bourinbaiar, 1992). This observation support an earlier study by Douglas *et al.* (1991) carried on placental explants. One hour after cell-cell contact most of the viral particles were found in electron dense lysosomes. Morphological evidence suggested that some of the virions may evade the degradation in lysosomes by a classic escape mechanism through fusion of the viral envelope with endosomal wall prior to delivery into lysosomal compartment. As result the viral nucleocapsid may be released into the cytoplasm (Bourinbaiar and Phillips, 1991; Phillips and Bourinbaiar, 1992). In addition, HIV-1 seems to enter target cells by budding from lymphocyte microvilli directly into the cytoplasm of target trophoblast cells (Phillips and Bourinbaiar, 1992). Ultrastructure of trophoblasts exposed to free virus did not show any uptake nor virions in the cytoplasm with few viral particles being immobilized on the surface of the cells.

Discussion

Although it is increasingly clear that in utero transmitted HIV-1 has an important impact on child mortality, the mechanism and factors regulating the

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Fig. 1

Transmission electron microscopy of HIV-1-infected MOLT-4/IIIB lymphocyte in contact with BeWo trophoblast

(a) Bars 1.0 μm for (a) and 0.1 μm for (b-i). Higher magnification reveals that virions are concentrated in the narrow intercellular space between contacting cells (b). Trophoblast membrane investigations resembling coated pits (b) and small coated vesicles containing individual virions (d) suggest the possibility of endocytic uptake by placental cells. At later times single virions were apparently delivered to the tubulo-vesicular compartment resembling early endosomes (c) and then to sphere-shaped late endosomes containing intact viral particles (f and g). The membrane of some endosomes displayed electron dense structures (arrowhead) suggesting the escape of HIV-1 into the host cytoplasm by fusion of the viral envelope with the wall of the limiting vesicle (g). At 1 hr and later remaining virions were found in electron dense lysosomic compartment where they appeared at different stages of degradation (h and i). Alternatively, HIV-1 seems to penetrate target cells by budding from lymphocyte microvilli protruding directly into the cytoplasm of target trophoblast cells (c).

mother-to-child transmission of HIV-1 are still poorly understood (Andimann *et al.*, 1990; Chandwani *et al.*, 1991; European Collaborative Study, 1991; Mattern *et al.*, 1992; Bourinbaiar *et al.*, 1992). The present study was aimed at establishing the intrauterine infection routes of HIV-1 in a situation where no direct blood-blood contact could be implicated as the cause of retroviral infection. An *in vitro* model representing human trophoblasts in contact with cell-free HIV-1 or HIV-carrying lymphocytes and monocytic cells has been established. This model was based on our previous studies where we used the human epithelial cell line exposed to infectious cells or free virus (Bourinbaiar and Phillips 1991; Bourinbaiar *et al.*, 1991*a*; Phillips and Bourinbaiar, 1992).

Viral infection in trophoblasts appeared to result from cell-to-cell contact with HIV-1-carrying MOLT-4/IIIB lymphocytes, or on one tested occasion, with U937/YH5 monocytes. We observed that cellular contact induces rapid release of virions from donor cells and HIV-1 subsequently entered placental cells via coated pits. The mechanism of virus processing within trophoblasts and the process of uncoating at the endosomal membrane is very similar to the infection mechanism described previously for HIV-1 (Bourinbaiar and Phillips, 1991; Douglas *et al.*, 1991; Goto *et al.*, 1988; Grewe *et al.*, 1990; Pauza and Todd, 1988; Phillips and Bourinbaiar, 1992) and for other enveloped viruses (Marsh, 1984). In addition, HIV-1 seems to penetrate target cells by budding from the lymphocyte microvilli protruding directly into the cytoplasm of the target placental cells in a fashion similar to that described previously (Phillips and Bourinbaiar, 1992). All this apparently leads to the establishment of latent infection that was activated by co-cultivation with cord blood derived MT-4 lymphocytes. Infectivity assays with free virus or non-adherent ACH-2 and U1 cells did not reveal productive viral infection. This may reflect the fact the choriocarcinoma lines lack the CD4 viral receptor and thus manifest restricted permissiveness to HIV-1 (Zachar *et al.*, 1991*a*; Zachar *et al.*, 1991*b*; Zachar *et al.*, 1991 *c*). However, the possibility of trophoblast infection by free HIV-1 cannot be excluded, provided that adequate HIV-1 strains and more sensitive virus detection methods will be employed (Zachar *et al.* 1991*a*; Zachar *et al.*, 1991*b*; Zachar *et al.*, 1991*c*) or tested on freshly obtained placental samples that may differ in their properties from choriocarcinoma cells (Amirhessami-Aghili and Spector, 1991; Douglas *et al.*, 1991). All these results are not new on their own. Very similar results were obtained and discussed extensively in our previous studies when we used CD4 negative epithelial cells and in HIV-1 infection studies by a number of other investigators.

The possibility of the transfer of maternal blood cells across the placental barrier has been a subject of considerable controversy (Hunzinker *et al.*, 1984; Olding, 1972; Shroder, 1972), but unfortunately, our model did not provide evidence to support such a mode of HIV-1 spread. The placental cells used in our experiments were human choriocarcinoma cells and thus may not represent exactly the *in vivo* situation. Despite that, the described trophoblast model could serve as a useful tool for the elucidation of viral attachment, entry, and infection

across the trophoblast barrier as proposed earlier by Zachar *et al.* (1991*a*; 1991*b*; 1991*c*). More important is that the knowledge gained from experimental data could be directly applied to the development of preventive measures against HIV-1 transmission in utero (Bourinbaiar *et al.*,1992).

In conclusion, our results support numerous studies by others and indicate a potential route of mother-to-child intrauterine transmission that could be significantly enhanced by contact of the foetal trophoblasts with infected maternal blood cells.

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