Short communication

Transfer of human T cell lymphotrophic virus type I to human term trophoblast cells in vitro

Xiangdong Liu,1 Vladimir Zachar,1,2 Henrik Hager,1 Uffe Koppelhus1 and Peter Ebbesen1*

1 Department of Virus and Cancer, Danish Cancer Society, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark and
2 Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic

We studied the susceptibility of term placental trophoblast cells to in vitro infection with human T cell leukaemia/lymphotropic virus type I in order to provide further insight into the role of syncytiotrophoblast in transplacental passage of the virus. Pure villous trophoblast cultures were exposed to cell-free virus and the extent of infection was analysed by semiquantitative PCR assay to detect integrated proviral DNA. Four different primer pairs targeting the gag, pol, env and pX regions invariably revealed that virus sequences were present in amounts 10²–10³ times less than in the reference cell line MT-2. Virus expression was studied at both the transcriptional and translational levels. Whereas doubly spliced mRNAs coding for the Tax and Rex regulatory proteins could be detected by RT–PCR no virus-specific proteins were found in the cells by immunoperoxidase staining. The present data lend support to the notion that the placental trophoblast may represent a barrier effectively protecting the fetal compartment from exposure to the virus.

Human T cell leukaemia/lymphotropic virus type I (HTLV-I) has been recognized as an aetiological agent of adult T cell lymphoma-leukaemia which is an acute neoplastic disease characterized by mono- or oligoclonal expansion of HTLV-I-transformed T cells (Hinuma et al., 1981) and of the neurological degenerative condition described as HTLV-I-associated myelopathy/tropical spastic paraparesis (Gessain et al., 1985; Osame et al., 1986). Based on sero-epidemiological data, breastfeeding appears to be the most significant route by which HTLV-I is vertically transmitted (Kinoshita et al., 1987). Nevertheless, even among children who were not breast-fed HTLV-I-specific antibodies could still be detected, with the prevalence ranging from 2.6–12.8%, thus suggesting that transmission of the virus may also take place during pregnancy or at birth (Ando et al., 1987; Tsuji et al., 1990). Several other groups have provided additional data in line with the hypothesis that virus passage may occur prenatally by finding HTLV-I-specific proviral sequences and antigens in cord-blood lymphocytes from the offspring of carrier mothers (Saito et al., 1991; Satow et al., 1991; Katamine et al., 1994). In an attempt to highlight the role of the placental barrier in vertical transmission of HTLV-I Fujino et al. (1992) used an in vitro culture system to analyse the placentas from seropositive mothers. The frequency of infection was about 22%. Most importantly, however, by demonstrating that the cytotrophoblast cells may become infected with HTLV-I this study substantiated the assumption that trophoblast is directly involved in virus spread from mother to fetus. As an extension to the above studies a nonprimate (rat) model was developed to explore vertical transmission of HTLV-I (Hori et al., 1995). Although transplacental passage of the virus could be detected in this system, the remote similarity of the materno-placental unit to that of humans precluded further generalization. We have previously described in vitro HTLV-I infection of human transformed trophoblastic cells (Liu et al., 1995). Here we demonstrate that primary human placental trophoblast cells are susceptible to HTLV-I and that cell-free virus provides a sufficient inoculum.

Cytotrophoblasts were isolated and purified from term placentas according to the standard procedure used in our laboratory (Zachar et al., 1991). After trypsinization and Percoll (Pharmacia) gradient centrifugation the yield of trophoblast cells was assessed by flow cytometric analysis for human cytokeratins (DAKO, clone MNF116; Dakopatts). Crude cell suspensions with a cytotrophoblast fraction exceeding 95% were further purified by a negative immunoselection. In this procedure contaminating cells were first reacted with monoclonal antibodies (MAbs) against the MHC-I antigens (DAKO-HLA-ABC, clone W6/32; Dakopatts) and then the labelled cells were removed using superparamagnetic...
Fig. 1. (a) Flow cytometry analysis of immunomagnetically purified human term cytotrophoblast cells. The cells were stained with an FITC-labelled cytokeratins-specific MAb (Dakopatts, clone MNF116) (□) and a conjugate of corresponding isotype was used as a control (■). (b) Isolated human vilous cytotrophoblast cells in culture. Cells were plated in supplemented KGM medium and cultured for 6 h (A), 48 h (B) and 4 days (C). Phase contrast; magnification in all panels is × 200.

...polystyrene microbeads coated with sheep anti-mouse Ig antibody (Dynabeads M-450; Dynal). Finally, the purity of the resulting cell population was determined by flow cytometry. The trophoblast cells were seeded in keratinocyte growth medium (KGM; Clonetics Corp.) supplemented with 10% fetal calf serum and antibiotics at a density of $3 \times 10^5$ cells/cm$^2$. A typical result from the flow cytometry analysis of the purified cytotrophoblasts is shown in Fig. 1(a). The analysis based on detection of cytokeratin as a marker for trophoblast demonstrated that the isolation procedure yielded a homogeneous cell population with practically undetectable contamination. Such results were reproducibly obtained with different placentas and could be corroborated by in situ immunostaining of purified cells and microscopic examination. Fig. 1(b) shows the dynamic changes in the cytotrophoblast cultures leading to the formation of multinucleated syncyti...
Unconcentrated virus was prepared from cell-free supernatant by low-speed centrifugation and filtration (0-45 μm). The stock of concentrated virus was prepared according to the procedure described by Fan et al. (1992). Briefly, after concentration of virus from filtered MT-2 supernatant by ultracentrifugation in a fixed angle rotor (RP-70T) for 3 h at 25000 r.p.m. and 4 °C, the pellet was treated with 80 U/ml DNaseI (Boehringer) at 37 °C for 1 h and adjusted to 1/100 of the original volume with 10% KGM. When the cytotrophoblast cells fused into syncytia after 24–48 h in culture, they were infected by incubation with 1 ml of virus inoculum per 10 cm² culture. The inoculum was unconcentrated MT-2 supernatant or 100 × concentrated virus, incubated with cells for 4 or 24 h at 37 °C, respectively. In control experiments the virus was heat-inactivated at 60 °C for 30 min. After infection was completed, cultures were thoroughly washed with RPMI 1640 medium and further incubated in complete KGM medium for 3 days. The cells were then processed for high molecular weight (HMW) DNA, mRNA and immunostaining. Each of the infection experiments was repeated at least twice with different trophoblast preparations.

Since chromosomal integration of HTLV-I is essential for virus replication the evidence for infection was based on PCR-mediated detection of provirus in the trophoblast nuclei. After infection the trophoblast cells were washed 3 times with PBS, dislodged by scraping and lysed in a lysis buffer (10 mM-Tris–HCl, pH 7.5, 140 mM-NaCl, 5 mM-KCl and 1% NP40) on ice for 1 min. The nuclei were then briefly pelleted and processed further for HMW DNA by proteinase K digestion and phenol–chloroform extraction. The PCR analysis was set up in a semiquantitative fashion and 200 ng of purified DNA was used in each reaction. For reference purposes a dilution series of HMW DNA from MT-2 cells was prepared in carrier DNA extracted from JAR choriocarcinoma cells. Four distinct HTLV-I genes were targeted by a PCR procedure using primer pairs SG166–SG296 (gag), SK110–SK111 (pol), SG219–SG294 (env) and SK43–SK44 (pX) as described previously (Liu et al., 1995). Quantification of the amplified signals was based on direct readings obtained from the blot filters after hybridization, using InstantImager (Packard). The proviral load in infected cell cultures was determined relative to that in MT-2 cells on the basis of a dilution factor which yielded an MT-2 signal comparable to that of the trophoblast. The semiquantitative format of PCR employed enabled us to approximate the HTLV-I proviral load in infected trophoblast cells (Fig. 2). From the series of amplification reactions consistent estimates could be extrapolated across the four genomic regions analysed. Infection experiments were repeated at least twice and provided concordant results. The data
demonstrate comprehensively that in infected trophoblast cultures HTLV-I provirus load, characterized relative to the total genomic DNA, appeared to be $10^2$-$10^3$ times less than that found in MT-2 cells. Interestingly, the amount of virus in the inoculum and/or the duration of incubation with the inoculum did not modify the infection.

Further, we set out to specifically analyse the expression of doubly spliced mRNA which codes for the regulatory proteins Tax and Rex, since this provides a comprehensive assessment of virus expression at the transcriptional level and at the same time acts as a control for amplification of virus RNA originating from the inoculum. This procedure was described in detail by Liu et al. (1995). Briefly, poly(A)$^+$ RNA was directly isolated from the cytosolic fraction of cells which were lysed after infection as described above using Dynabeads Oligo(dT)$_{25}$ (Dynal). mRNA isolated from trophoblast cultures infected with unconcentrated virus and the dilution series of mRNA isolated from $10^6$ MT-2 cells were reverse transcribed using M-MLV reverse transcriptase as specified by the manufacturer (Gibco) and one-tenth of the product was used for PCR. Doubly spliced viral mRNA encoding p40$^{tax}$/p27$^{rex}$ was amplified by targeting the adjacent splicing donor and splicing acceptor sites of the second and third exons, respectively, using RPX3 and RPX4 primers. The $\beta$-actin gene was targeted in a separate set of PCR reactions to assess the quality of samples. Amplification was accomplished using commercially available primers (Stratagene) and the product was visualized by agarose gel electrophoresis with ethidium bromide staining. RT–PCR was carried out in a semiquantitative fashion which enabled us to determine the proportion of virus-specific transcripts in the bulk of the trophoblast mRNA which equalled the yield from $3 \times 10^6$ originally seeded cells. The range in which estimates for the p40$^{tax}$/p27$^{rex}$ mRNA load could be determined was $0.1$-$10^5$ MT-2 cell equivalents (Fig. 3). A set of control reactions targeting $\beta$-actin mRNA confirmed the suitability of individual cDNA samples for amplification. Although expression of p40$^{tax}$/p27$^{rex}$ mRNA in the infected trophoblast cultures could be detected consistently, quantitative analysis showed that the amounts of viral transcripts corresponded to less than $0.1$ MT-2 cell equivalents.

The infected trophoblast syncytia were stained by an immunoperoxidase method in an attempt to detect the viral Tax and p24 proteins. Three to six days after infection the trophoblast cultures were thoroughly washed with PBS and fixed with $80\%$ acetone. MAbs specific for HTLV-I Tax (Lt-4; Tanaka et al., 1990) and HTLV-I p24 (Biosoft) were used as primary antibodies and biotinylated rabbit anti-mouse antibody was used as a secondary reagent. Visualization and subsequent enhancement of the signal were accomplished with streptABComplex/HRP (Dakopatts) and the Immuno-
Pure metal enhanced DAB substrate kit (Pierce), respectively. MT-2 cells which were used as a positive control were subjected to the identical staining procedure. Although the cultures were scrutinized regularly for 5 days post-infection, all efforts to detect the target proteins failed.

In line with a previous report dealing with in vitro infection of choriocarcinoma cells (Liu et al., 1995) the present study provides evidence that primary human placental trophoblasts are susceptible to HTLV-I infection and that virus expression appears to be restricted to the transcriptional level. Although the trophoblast cells could be readily infected with cell-free virus, additional experiments (not shown) demonstrated that cell-mediated infection may be more efficient, even to the point that the p24 expression could be sporadically detected. Taking into account the cell-associated nature of HTLV-I together with its propensity for cell-mediated transmission, as well as the capacity of peripheral blood lymphocytes to adhere to trophoblast cells in vitro (Douglas et al., 1993), it is plausible that in vivo cell-to-cell contact may also be involved in the infection of syncytiotrophoblast. At the maternal–fetal interface spread of the virus may be facilitated by the additional factors that are likely to play a role during pregnancy. Most notably, the duration of syncytiotrophoblast exposure to virus becomes significantly increased. In vivo contact with the infected cells in maternal blood takes place in an uninterrupted manner from as early as day 8 or 9 of pregnancy. Furthermore, throughout pregnancy the placental micro-environment provides an array of cytokines and growth factors with pleiotropic effects which can modulate interaction of viruses and also other pathogenic agents with the trophoblast (Mitchell et al., 1993).

Primary trophoblast cells thereby extend the spectrum of nonlymphoid target cells which have been shown to become infected by HTLV-I. The prominent common feature of these host cells, including among others epithelial (Zacharopoulos et al., 1992), fibroblast (Yoshikura et al., 1984) and endothelial cells (Hoxie et al., 1984), is their capacity to proliferate. Cell division is considered to be crucial for replication of animal oncoretroviruses (Varmus & Swanstrom, 1982). In contrast, term villous trophoblast cells are distinctive in that they do not proliferate in vitro, but differentiate into syncytiotrophoblast cells and HTLV-I (Yoshikura et al., 1993; Fujino et al., 1995) appears to resemble that of lentiviruses.

Reports demonstrating the presence of virus-specific antigenic or genetic components in the placenta and cord blood (Satow et al., 1991; Satow et al., 1991; Fujino et al., 1992; Katamine et al., 1994), and the antiviral humoral response in bottle-fed children (Ando et al., 1987; Tsuji et al., 1990) collectively suggest that the transplacental passage of HTLV-I infection may not be a frequent event. Given the experimental data, it is conceivable that the syncytiotrophoblast is the most important barrier against HTLV-I transmission during pregnancy.

MT-2 cells were obtained from Dr D. Richman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, Md., USA. We thank Dr R. Gallo, NCI, NIH, Bethesda, Md., USA, for providing plasmid PHX3 and Dr Y. Tanaka, University of Kitasato, Japan, for providing anti-Tax monoclonal antibody Li-4.

K. Naito is a member of HTLV European Research Network which is the Concerted Action program of ECC.

References


(Received 23 June 1995; Accepted 2 October 1995)