Original Article

Receptivity of Human Choriocarcinoma JEGIII Cells and Isolated Trophoblast Cells to Hepatitis B Virus Infection and Enhancement by Tumor Necrosis Factor Alpha

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SUMMARY: Intrauterine infection of the fetus is clearly an important mode of vertical transmission of hepatitis B virus (HBV). The trophoblast layer of the human placenta must be traversed by HBV in order to reach underlying cells and fetal capillaries. Although HBV has been detected in the trophoblast layer in situ, the degree of receptivity of primary trophoblast cells to direct HBV infection in vitro remains unknown. To determine the receptivity of trophoblast cells to HBV infection and to discover the cellular basis for the molecular mechanism responsible for the passage of HBV from the maternal to the fetal circulation, we infected choriocarcinoma JEGIII cells and primary trophoblast cells with HBV. Our findings suggest that the cells could be reproducibly infected with HBV and that the infective patterns of the isolated trophoblasts and JEGIII cells were remarkably similar. In vitro infection resulted in an intracellular viral DNA and hepatitis B surface antigen signal and the secretion of hepatitis B surface antigen into culture medium. The results suggest that both isolated trophoblast cells and trophoblast-derived choriocarcinoma cells are sensitive to infection with HBV in vitro. In addition, we have found that infection of trophoblast cells and JEGIII cells by HBV was enhanced in the presence of tumor necrosis factor alpha. This supports an additional role for tumor necrosis factor alpha in the entry of HBV into trophoblast cells during pregnancy.

INTRODUCTION

Hepatitis B is an important public concern in China. However, insufficient knowledge of the mechanisms of trophoblast-viral interactions limits our ability to prevent transplacental hepatitis B virus (HBV) infection. Along with the universal application of efficient vaccines against hepatitis B, studies on HBV intrauterine transmission have been one of the key methods aimed at controlling hepatitis B prevalence in China. The molecular mechanisms responsible for HBV intrauterine transmission are not well understood because the narrow host range of HBV has restricted the availability of suitable experimental systems for these studies. While studies performed on placental explants (1,2) have stated little about the mechanisms through which HBV enters the placenta and proceeds to the fetus. Although the pathogenesis of HBV transmission to the fetus during pregnancy is unknown, congenital HBV infections are commonly associated with infection of the placenta. Thus, passage likely occurs through the placenta, which may also act as a viral reservoir. The trophoblast is a polarized epithelial structure in direct contact with maternal blood, which forms the first cellular barrier between the mother and the fetus. By its location in the tissue, the trophoblast lies on a direct route for HBV entry to the fetal blood vessels, although the role of the trophoblast monolayer in transmission of HBV across the placental barrier is unclear. In vitro studies on the mechanism of HBV intrauterine transmission have been hampered by the lack of appropriate systems for culturing corresponding susceptible cells chronically infected with HBV.

The development of an effective culture model of trophoblast infection by HBV must address two interdependent problems: fibroblast contamination and susceptibility to HBV. Choriocarcinoma cells, which derive from the trophoblast layer, share numerous properties with trophoblast cells and provide an ideal monocellular system with which to study HBV infection of the placental barrier. According to their surface markers, choriocarcinoma cell lines JEGIII, JAR, and BeWo (3) are representative of cytotrophoblasts from early placenta. JEGIII cells form a polarized, tight monolayer that mimics the trophoblastic barrier in vivo (4). Additionally, these cell lines are positively labeled with anti-cytokeratine and with trophoblast-specific antibodies and are human chorionic gonadotropin (hCG)-producing cells. Here we used these culture models to study HBV infection of human placental cells in vitro. This also gave us an interesting opportunity to compare the susceptibility of the two cells to HBV infection.

MATERIALS AND METHODS

Isolation and culture of isolated human trophoblasts: Highly purified cytotrophoblasts were isolated from 6- to 10-week placentas. Briefly, placenta (6-10 weeks gestation) were obtained immediately after artificial abortion. Soft villous material was cut away from connective tissue and vessels. The tissue was washed with 0.9% sodium chloride and coarsely minced with scissors, and then transferred to

The concentration of 10 ng/ml) at 37°C under a 5% CO2 humidified atmosphere to allow for virus uptake. Infectious material was demonstrated by enzyme-linked immunosorbent assay (ELISA): Cell-free supernatants from the different time points were harvested from cultures postinfection and frozen at −20°C until they were analyzed for HBsAg secretion by ELISA. Incubations were performed as indicated by the manufacturer. As a control, supernatants from noninfected cultures were used.

Western blotting analysis of intracellular viral protein: After removal of the supernatant, the cells at different time points postinfection were washed several times with phosphate-buffered saline (PBS) and lysed by the addition of 500 μl of protein sample buffer (200 mM Tris-HCl [pH 8.8], 10% sucrose [glucose], 5 mM EDTA, 0.1% bronopol blue, 3% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol). Ten microfilters (equivalent to 4 × 106 cells) of the cell lysates was separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) and blotted to a polyvinylidene difluoride membrane. Monoclonal antibody (MoAb) to HBsAg (mouse anti-human IgG) was diluted in phosphate-buffer saline and Tween-20 (PBST) and incubated with the transfer membrane for 12 h at room temperature. For this purpose, MoAb was diluted 1:500. After antibody incubation, the membrane was washed three times with PBST. Then the filter was incubated for 2 h at room temperature with rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP). Protein bands were visualized using the enhanced chemiluminescence (ECL) system.

HBsAg particles in the infected cells visualized by transmission electron microscopy: Approximately 5 × 106 HBV-infected cells were harvested for identification of HBsAg particles by transmission electron microscopic examination. The cells were digested with trypsin routinely and centrifuged at 1,000 rpm for 10 min. The cells were resuspended into 4% paraformaldehyde, 0.125% trypsin (Gibco Laboratories, Grand Island, N.Y., USA), pH 7.4. The mixture was incubated in a shaking water bath at 37°C for 20 min. If, after the digestions, the supernatant was viscous, 0.15 U/ml DNase I (Sino-American Biotech, Co., Henan, China) was added, and the mixture was allowed to incubate for an additional 10 min. The mixture went through filtration with a 200-μm screen sieve. The remaining villous material was subjected to the digestion and filtration procedure again. The cell suspensions were pooled and then centrifuged at 1,000 rpm for 10 min, after which the supernatant was discarded and the pellets were retained.

The cells were washed 3 times with serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco) and then resuspended in serum-free medium for culture. One hour later, the attached cells were discarded, and the suspended cells were harvested and subcultured with DMEM containing 25 mM glucose and supplemented with 15% heat-inactivated fetal calf serum (Sijiqing Biotech, Co., Ltd., Hangzhou, China), 20 mM glutamine, 25 mM Heps (pH 7.4), and antibiotics (50 IU of penicillin/ml and 50 μg of streptomycin/ml), and incubated in a CO2 incubator. Cells were cultured continuously for passaging. During the passaging procedure, we performed the same step repeatedly. The cells were identified immunocytochemically with mouse anti-cytokeratin (Dako, Glostrup, Denmark) and mouse anti-vimentin (Dako) and transmission electron microscopically. An avidin-biotin-peroxidase complex (ABC) Immuno Detects kit was purchased from Sino-American Biotech. All solutions were autoclaved or sterilized by filtration before use. The sixth to eighth passages of the isolated cells were used for the following experiments.

Human choriocarcinoma cells: The human choriocarcinoma JEGIII cell line, obtained from the Reproductive Center, Institute of Zoology, the Chinese Academy of Sciences, was maintained in Ham's F12 and DMEM (FD medium) (Gibco) under conditions similar to those described for isolated trophoblast cells. The medium was changed every 2 days. Cultures were observed daily using a phase-contrast microscope. At confluency, cell monolayers were trypsinized and passaged, or frozen in liquid nitrogen until we had gathered sufficient numbers of them for subsequent experimental manipulation.

Human sera: HBV-positive sera were taken from HBV-infected patients after their informed consent to participate in the study. None of them was under antiviral therapy. The sera were snap frozen in aliquots and were stored at −80°C. All virus preparations underwent only one freeze-thaw cycle before initiation of infection studies.

HBV infection protocols: Prior to inoculation, the virus stock was diluted into prewarmed DMEM for infection of isolated trophoblast cells or FD medium for JEGIII cells. When the cells reached a 50–80% confluence, 2 × 106 to 6 × 106 of cells were incubated with 2 × 106 to 6 × 106 of HBV (corresponding to approximately 100 viral genomes equivalent/cell) in the presence or absence of tumor necrosis factor alpha (TNF-α) (+TNF-α or −TNF-α) (at a final concentration of 10 ng/ml) at 37°C under a 5% CO2 humidified atmosphere to allow for virus uptake. Infectious material was removed from the cell monolayers immediately after 12-h interaction, and the cells were extensively washed, including one wash with prewarmed trypsin for 1 min at 37°C to remove any adherent virus particles. The last of the six washes was negative for HBsAg. The cells were then subcultured for 4 additional days to monitor the infection. Cultures were observed daily. At the designed time points, monolayers and supernatants were collected for virus detection.
RESULTS

Characteristics of isolated trophoblasts in culture: Light microscopic examination revealed patchy spreading and irregular polygonal cells, with big and oviform nuclear and transparent cytoplasm. The isolated cells were positive for cytokeratin and negative for vimentin, indicating satisfactory removal of contaminating elements. Transmission electron microscopy revealed that the cells had fine structural features typical of trophoblasts, exhibiting numerous microvilli and containing many organelles, such as mitochondria, smooth membrane vesicles, well-developed Golgi, lipid droplets, occasional coated pits, and so on (Fig. 1).

Secretion of HBsAg in cell-free supernatants: Infectious material was removed from the cell monolayers immediately after 12-h interaction, and the cells were extensively washed, including one wash with prewarmed trypsin for 1 min at 37°C to remove any adherent virus particles. Trypsinization and extensive washings eluted viruses weakly associated with the cells. No HBsAg was detected in the last wash. The cells were then continuously cultured for 4 additional days to monitor the infection. Culture medium was collected at hours 36, 48, 60, 72, 84, and 96 postinfection. Secretion of HBsAg could be observed in similar patterns at hour 36 postinfection in both cell cultures. As shown in Fig. 2, a productive infection was detected in both cell supernatants. In addition, the amount of HBsAg production differed in the absence or presence of TNF-α (P < 0.05). In the absence of TNF-α, a faint HBsAg expression was detected in HBV-infected cell supernatants. In the presence of TNF-α, a similar enhancement was observed in the two cell cultures following infection with HBV. Unexpectedly, the differences observed between the various time points did not reach a statistically significant level (P > 0.05).

HBsAg determination in the infected cell cultures: Western blotting assay demonstrated that HBV-infected iso-
lated trophoblast cells as well as JEGIII cells pretreated with TNF-α repeatedly gave a positive intracellular HBsAg signal (Fig. 3). In addition, electron microscopic examination revealed the presence of band-form HBsAg particles in diluted rough surfaced endoplasmic reticulum cavities of isolated trophoblast cells and JEGIII cells infected with HBV in the presence of TNF-α (Fig. 4). The morphological appearance was consistent with the previous reports (5-7). And in the HBV-noninfected JEGIII cells, no tubular structures of HBsAg were present in the rough-surfaced endoplasmic reticulum cavities of the cells. In contrast, when the cells were infected with HBV in the absence of TNF-α, the intracellular HBsAg expression level was so low that it could hardly be detected by Western blotting analysis or electron microscopic examination (data not shown).

**Detection of HBV PreS cDNA in the infected cells:** The results of the detection of HBV PreS cDNA in infected JEGIII cells pretreated with TNF-α are shown in Fig. 5. At various time points, an approximately 522-bp DNA band was detected. A similar pattern was observed for the isolated trophoblast cells (Fig. 5). In the HBV-infected JEGIII cells and isolated trophoblast cells that were not pretreated with TNF-α, no band or a very light specific band of PreS cDNA was observed (results not shown).

**DISCUSSION**

The isolation of trophoblasts from human placenta is confounded with the presence of other cells released from the tissue during the isolation procedure (8,9). Possible contaminants include placental macrophages (Hofbauer cells), endothelial cells, and fibroblasts. Trophoblasts consist of 40 to 95% of the isolated cells from placenta, depending on the isolation procedure used (9). Cytokeratin is the known marker for epithelial cells. Vimentin was one of a class of intermediate filament proteins known to be present in endothelial cells and fibroblasts. In this study, only trophoblasts were the epithelial cells that are cytokeratin-positive and vimentin-negative, while other types of cells are cytokeratin-negative and vimentin-positive.

In utero infection of the fetus is dependent mostly on placental infection, although transmission during delivery cannot be completely excluded. Indeed, HBV has been detected on both the maternal and fetal portions of the placenta, such
as decidual macrophages, leukocytes, trophoblasts, Hofbauer cells, and villous endothelial cells (2, 10). The question of viral entry is crucial, since the trophoblasts are not the target cell for HBV (tropism). Thus, it remains controversial whether HBV can sustain a productive cycle in trophoblastic cells. The data we reported here indicated that highly enriched human isolated trophoblast cells were indeed sensitive to HBV infection in vitro and that a choriocarcinoma cell line of JEGIII cells, free of any residual contaminant, were also permissive to infection with HBV. Additionally, the infection manners were quite similar, suggesting that the choriocarcinoma cell line, corresponding to the maturation state of villous cytotrophoblasts deriving from human early placenta, can support viral replication in vitro.

Our data support the assertion that isolated trophoblast cells, as well as trophoblast-derived cells, in a well-characterized choriocarcinoma cell line, should be used as in vitro models for continued critical investigation of the molecular mechanism of viral infection of fetuses in pregnancy. Choriocarcinoma cells as the representatives of trophoblast cells were more favorable to work with than were isolated trophoblast cells in the study. First, insufficiency of primarily cultured trophoblast cells isolated from placenta could be circumvented, and second, the repeated primary culturing of trophoblast cells could be avoided.

In the study, we found that the HBV infection of the two cell cultures occurred at a relatively low level in the absence of TNF-α. The reasons behind this limited infection are ill defined but are thought to be due to factors related to HBV receptors in conjunction with particular cellular environments. Numerous extra-cellular soluble factors are present in the vicinity of the placenta during gestation. These agents play pivotal roles during gestation and are mandatory for a successful pregnancy. On the other hand, some of these agents are also known to be up-regulated in vivo in HIV-1 infected patients and to modulate viral expression (11-14). Thus, specific cytokines and/or modulatory factors present in the placental microenvironment while controlling cellular activities may also play a regulatory role in protecting the host or, inversely, in driving viral expression. More specifically, some of these factors could be, in part, the triggering element necessary for viral expression in infected trophoblastic cells. This would translate into productive viral expression and spreading of the virus to the fetus. We propose that exposure of target cells to TNF-α before inoculation with HBV may parallel physiological conditions more closely.

Our results suggest that the presence of cytokine TNF-α in the vicinity of trophoblastic cells would create more favorable conditions leading to vertical transmission of HBV. Considering that expression of these cytokines is highly regulated according to the stage of placentation development, it can be proposed that windows of opportunity are transiently created for the induction of viral expression by extracellular factors in trophoblasts. In line with our results, the relative importance of TNF-α during the vertical transmission of HIV-1 has been indirectly put forward in previous works. First, trophoblast cells from HIV-1-infected placentas were found to express higher levels of TNF-α, and such levels also correlated with the amounts of HIV-1 Gag transcripts found in trophoblastic cells (12). Second, pretreatment of trophoblast cells with TNF-α increased lymphocytic cell adhesion to trophoblastic cells (15). Third, cell contact between macrophages and trophoblasts resulted in up-regulation of HIV-1 expression that was mediated by the release of TNF-α by macrophages (16). Based on these published data together with the present findings, it can be proposed that TNF-α is produced by virus-infected trophoblastic cells and/or macrophages upon cell contact with trophoblasts. As a consequence, these cytokines would in turn up-regulate virus production, which is a potential mechanism of virus replication in trophoblastic cells leading ultimately to vertical transmission.

It is interesting to note that TNF-α exerts its effects primarily at the onset of pregnancy and again during labor (17-25). Interestingly, it coincides with the timing of a higher risk of HIV-1 vertical transmission (26-28). This expression profile suggests that productive HBV infection of trophoblasts may be favored at two different time points during the course of pregnancy, first at the onset of pregnancy, where there are highly proliferative and invasive trophoblastic cells, and later at term. Since there is a limited time frame of TNF-α expression during pregnancy, this may also help to explain in part why HBV transmission may be limited during pregnancy.

We believe that achieving a better understanding of the molecular basis of the interactions between viruses and trophoblast cells may yield novel strategies to prevent the vertical transmission of viruses. Our in vitro models are very useful tools to further study the molecular basis of HBV intrauterine infection and transfer. However, the role of other cells from the placenta should still be investigated, in particular if a step-by-step mechanism is involved in the transport and spreading of the virus through the placental barrier.

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REFERENCES


