

INFECTION OF HUMAN HEPATOCYTE CELL LINES WITH HEPATITIS B AND C VIRUSES IN VITRO

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ABSTRACT

Hepatitis B and C virus (HBV; HCV) infections are of the most prevalent viral diseases in the world, since around 350 and 170 million individuals are infected, respectively. Infection with HBV and HCV can lead to cirrhosis and hepatocellular carcinoma. Despite the use of the chimpanzee, there were no reliable models for the study of the many aspects of HBV and HCV infections. Although several alternative methods have been previously developed for the in vitro studies of these infections, there was still an urgent need for new *in vitro* infection models. Indeed, mostly chimpanzees, but also recombinant mice, marmosets, woodchucks and ducks have been used as animal models for the study of the HBV and HCV infections.

We have shown that the cell-bound lipoproteins are playing a crucial role during the infection process *in vitro*. In order to obtain HBV and HCV infections *in vitro*, the cell-bound lipoproteins have first to be removed from their cellular receptor prior to the addition of viral inocula originating from human sera, the latter being made originally of a virus-lipoprotein complex (Favre, D., Berthillon, P. & Trépo, C. Removal of cell-bound lipoproteins: a crucial step for the efficient infection of liver cells with hepatitis C virus *in vitro*. *C. R. Acad. Sci. III* **324**: pp 1141-1148, 2001; Favre, D., Petit, M.-A., & Trépo, C. Hepatitis B virus (HBV) infection and HBV DNA integration associated with further transformation of hepatoma cells *in vitro*. (In the press in *ALTEX*).

MATERIALS AND METHODS

The cells (7×10^5 to 1×10^6) were grown in 6-well plates (Costar) until confluency. For infection, the cells were first washed with PBS and then treated with high molecular weight dextran sulfate (AppliChem, Darmstadt, Germany) at 10 mg/ml in PBS during an incubation period of 5 to 10 minutes. This incubation with dextran sulfate carried out the removal of the cell-bound lipoproteins. After this incubation step, the cells were extensively washed with PBS to remove traces of the dextran sulfate-lipoprotein complex. The viral inoculum was then added to the cells for an incubation period of 45 min to 1 h. The viral inoculum used consisted of patient's serum titrated to provide up to 10 HCV genome equivalents per cell either undiluted or diluted in a final volume of 500 μ l of PBS. After the infection period, the viral inoculum was removed, and the cells were extensively washed with PBS. The cells were then incubated in complete cell culture medium. Usually, the complete cell culture medium was routinely changed after an overnight incubation in order to remove unbound or loosely bound input virus. Alternatively, a higher efficiency of infection was achieved if a portion of the infection process described above was performed at lower temperatures, for example at 4°C or on ice. Since the binding of lipoproteins to the LDL receptor is a calcium-dependent process [36-39], the calcium chelator ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA; ethylene-bis(oxyethylenitrilo) tetraacetic acid; egtazic acid) at 1 mM was employed concomitantly with dextran sulfate.

RESULTS

Hepatitis C virus

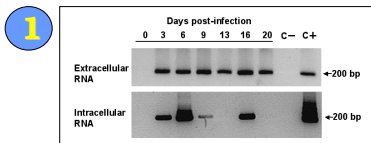


Figure 1. Kinetics of detection of positive-strand HCV RNA in supernatants and cells of HepG2-infected cells. Infection was performed as described in Materials and Methods using human serum positive for HCV. Half of the cell culture medium was changed after 3, 6, 9, 13 and 16 days. At the times indicated, the single-round PCR products were obtained by amplification of the 5' noncoding region and analyzed by agarose gelelectrophoresis followed by staining by ethidium bromide. Negative control (C-) included PCR amplification from cell culture medium and mock-infected cells at the beginning of the infection. RNA isolated from HCV-positive serum and from HCV-positive cells originating from liver biopsies was used as positive controls (C+) for positive-strand RNA detection. PCR amplification of HCV RNA from the cell culture supernatants (higher panel) and from the cells (lower panel).

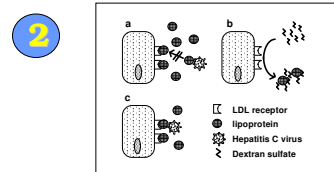


Figure 2. Model for the infection of eukaryotic cells with hepatitis C virus *in vitro*. Removal of cell-bound lipoproteins from the low density lipoprotein (LDL) receptor might be the crucial step for efficient hepatitis C virus infection *in vitro*. a, the binding of the HCV-lipoprotein complex to the LDL receptor is hampered *in vitro* by the cell-bound lipoproteins and by the vast excess of free lipoproteins present in the human blood. b, prior to HCV infection, the cell-bound lipoproteins are removed from the LDL receptor by using dextran sulfate, thus generating free LDL receptors. c, lipoprotein-free LDL receptors can bind the HCV-lipoprotein complex, thus allowing adsorption and penetration of HCV into target cells. A similar involvement of lipoproteins might take place during the infection of cells with HBV.

Hepatitis B virus

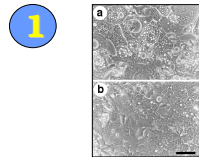


Figure 1. HBV-infected cells in monolayers. a, the HBV-infected HepG2 cells 12 days post-infection are enlarged and contain dense nuclear inclusions of various sizes and cytoplasmic vacuoles. Some cells present long cytoplasmic processes. b, mock infected HepG2 cells. Bar. 60 μ m.

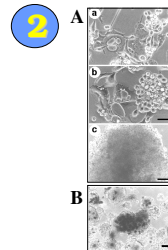


Figure 2. Growth of HBV-infected HepG2 cells in hormone-supplemented cell culture medium (A) and as suspensions in soft agar (B). A, Infected cells were grown for two weeks, trypsinized, washed, and thereafter grown for two more weeks in complete medium supplemented with physiological concentration of insulin (100 nIU/ml). a, b: aberrant morphologies of adherent, HBV-infected cells with appearance of clusters of refringent, rounded cells; c, rounded cells do proliferate and produce large clumps of cells. B, The HepG2 cells were infected with HBV for an incubation period of three weeks, and were thereafter grown for clonogenic assay. Large clumps of cells are growing in the soft agar after three weeks. Dark cells are probably necrotic cells. Noninfected cells and mock-infected cells do not grow in soft agar (not shown). Bars: 40 μ m (A: a, b); 100 μ m (A: c); 120 μ m (B).

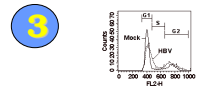


Figure 3. Propidium iodide staining of HepG2 cells. Mock-infected cells (mock) and HBV-infected cells (HBV) were analysed by FACS at 14 days after plating. Cells were in G1, S and G2 phase of the cell cycle. FL2-H: relative fluorescence.

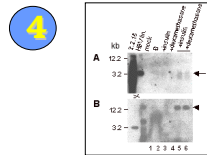


Figure 4. Southern blot analysis of DNA extracted from HuH-7 cells infected with HBV. DNA was electrophoresed on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with a purified HBV DNA probe. A, B: HuH-7 cells four and eight days post-infection, respectively. Lane 1: mock-infected cells. Lane 2: HBV-infected cells, incubated in complete cell culture medium. Lanes 3, 4: HBV-infected cells, incubated with additional insulin or dexamethasone, respectively. Lanes 5 and 6: HBV-infected cells, incubated with both insulin and dexamethasone. Positive controls used were DNA from 2.2.15 cells, and linear HBV DNA (HBV lin.).

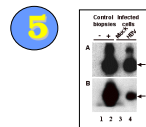


Figure 5. Southern blot detection of the nested-PCR amplification products with primers located in the S gene in HBV-infected HepG2 and HuH-7 cells, respectively, two years post-infection. Negative controls were mock-infected cells (mock; lane 3). Negative (-) and positive (+) liver control biopsies are represented in lanes 1 and 2, respectively.

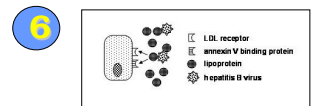


Figure 6. Involvement of the low density lipoproteins during the infection of hepatocytes with HBV *in vitro*. The LDL receptor and the annexin V binding protein have been proposed to mediate HBV adsorption and penetration into the hepatocytes. Both molecules do bind to low density lipoproteins (LDLs). Since HBV does bind to the apolipoprotein H present in the LDLs, HBV might thus enter into the cells via the LDL-receptor and/or the annexin V binding protein. The use of dextran sulfate prior to infection is removing the free lipoproteins from the cell surface and facilitating the subsequent binding and penetration of the LDL-HBV complexes into the hepatocytes.

DISCUSSION

The model of *in vitro* infection reported here may thus serve to look for new pharmacological targets to interfere with HCV replication and HBV DNA integration, and to screen *in vitro* for candidate inhibitors of therapeutic potential, without the use of animals.