Hepatitis B virus: specific binding and internalization of small HBsAg by human hepatocytes

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Previously, we identified human liver endonexin II (EII) present on human hepatocyte plasma membrane as a specific hepatitis B surface antigen (HBsAg) binding protein. We also showed the spontaneous development of anti-idiotypic (anti-HBsAg) antibodies in rabbits immunized with EII and in chicken immunized with the F(ab')2 fragment of rabbit anti-EII IgG. These findings suggest the existence of a receptor–ligand relationship between EII and HBsAg. In the present study, we demonstrate that small HBsAg conjugated to 10 nm colloidal gold also binds specifically to human hepatocytes. Invagination of the coated pit region at the HBsAg binding sites on the human hepatocyte plasma membrane results in the internalization of the HBsAg–gold particles. The binding and consequently the internalization of HBsAg is inhibited by anti-EII or anti-idiotypic (anti-HBsAg) antibodies. These findings indicate that EII is directly involved in the binding and uptake of hepatitis B envelope proteins.

Although considerable information on the molecular biology of hepatitis B virus (HBV) has been gained in the past decades, still little is known about the mechanism of attachment and penetration of HBV into human hepatocytes, the natural host cells of HBV. Binding of virus particles to plasma membranes of human hepatocytes is considered an important initial step in viral entry and infection of HBV. The envelope of HBV is composed of three polypeptides: the small, middle and large hepatitis B surface antigens (HBsAg) (Tiollais et al., 1985). These three proteins might play an important role in recognizing specific molecules on the surface of the human hepatocytes. The three HBsAg proteins have the S sequence in common, but differ in their amino-terminal extensions. Middle HBsAg consists of the S and PreS2 sequences, while the large HBsAg consists of the S, PreS2 and PreS1 sequences. Recently, we have identified endonexin II (EII), isolated from human liver tissue, as a specific HBsAg binding protein (Hertogs et al., 1993). Furthermore, we described the spontaneous development of anti-idiotypic (anti-HBsAg) antibodies in rabbits immunized with human liver EII and in chicken immunized with the F(ab')2 fragment of rabbit anti-EII IgG (Hertogs et al., 1994). These antibodies are able to compete with EII for the binding to HBsAg, indicating that the anti-idiotypic antibodies mimic a region of EII that interacts with HBsAg. These findings strongly suggest the existence of a receptor–ligand relationship between HBsAg and EII. In the present study, we report evidence at the ultrastructural level for the specific binding and internalization of small HBsAg by human hepatocytes. The binding and internalization of small HBsAg are inhibited by anti-EII or anti-idiotypic (anti-HBsAg) IgGs.

Human livers were obtained from organ donors with the permission of the local committee on human experimentations and informed consent of the families. The hepatocytes were isolated by a two-step collagenase perfusion according to the method of Rijntjes et al. (1986). Viability of the cells, as determined by trypan blue exclusion, was 90%. The cells were seeded at a density of 2 × 10^6 cells/cm^2 as described by Rijntjes et al. (1986). Cells were cultured for 3 days prior to use for ultrastructural experiments in six-well dishes (Costar) coated with extracellular matrix obtained from human liver tissue (Rijntjes et al., 1986). Cells that were not used directly for experiments were cryopreserved as described by Rijntjes et al. (1986).

Recombinant yeast derived small HBsAg was kindly provided by Dr Miller (Merck Sharp & Dohme Research Laboratories, Westpoint, Pa., USA) (Emini et al., 1986). HBsAg (2 μg) was labelled with Na^125I (Amersham) using Iodogen (1,3,6,4-tetrachloro-3α,6α-diphenylgly-
couril (Pierce) as described previously (Leenders et al., 1990). Specific activity was 10⁷ c.p.m./μg.

Anti-endonexin II (anti-EII) antibodies were obtained from rabbits immunized with 20 μg recombinant human liver EII in complete Freund’s adjuvant and purified as described previously (Hertogs et al., 1994). F(ab')₂ fragments were isolated from the IgGs and used for immunization of chickens for production of anti-idiotypic (anti-HBsAg) antibodies as described (Hertogs et al., 1994). IgGs isolated from non-immune serum (NIS) as reported (Hertogs et al., 1994) were used in control experiments.

To perform binding studies of radiolabelled HBsAg and human hepatocytes, cryopreserved hepatocytes were thawed as described (Leenders et al., 1990) and resuspended in WEM containing 0.5% BSA. Viability of the hepatocytes after thawing was 50% as determined by trypan blue exclusion. About 100000 cells were incubated for 1 h at 4 °C to reduce non-specific binding. This was followed by incubation for 1 h in WEM–0.5% BSA with serial dilutions of rabbit anti-EII, chicken anti-HBsAg or non-immune serum (NIS) IgGs as control. Radiolabelled HBsAg (100 ng) was added and cells were incubated for an additional 2 h in the presence of the IgGs. Cells were subsequently washed three times with WEM and bound HBsAg was determined in a gamma-counter. Non-specific binding was determined by incubation with radiolabelled ligand in the presence of a 10-fold excess of unlabelled HBsAg. All experiments were performed in duplicate.

HBsAg was coupled to colloidal gold and used as a tracer for binding studies and uptake of HBsAg by primary cultures of human hepatocytes. The preparation of the 10 nm gold solution and the coupling of the gold to the HBsAg were done according to the method described by Slot & Geuze (1985). The gold particles were coupled to 200 μg HBsAg at pH 7.8. Gold conjugated HBsAg was separated from free HBsAg and unbound gold particles by centrifugation on a 10% glycerol cushion in phosphate-buffered saline (PBS) with 0.1% BSA for 1 h at 25000 g at 4 °C. The HBsAg–gold pellet was resuspended in PBS and used directly for binding and internalization studies.

After culture for 3 days, human hepatocytes were incubated for 1 h at 4 °C in WEM containing 1% BSA and subsequently for 1 h with rabbit anti-EII or NIS IgGs diluted 1:10 in WEM–1% BSA (final concentrations were, respectively, 5 μg/ml and 14 μg/ml). The HBsAg–gold pellet was diluted 1:10 in WEM–1% BSA and added to the cells. After incubation for 2 h at 4 °C in the presence of the antibodies, hepatocytes were washed three times with ice-cold WEM and kept for 0, 5, 10 and 30 min respectively at 37 °C. The cells were washed three times with ice-cold PBS and fixed for 0.5 h with 2% glutaraldehyde in PBS at 4 °C. To investigate whether chicken anti-idiotypic (anti-HBsAg) IgGs were able to block the binding and uptake of HBsAg–gold particles by human hepatocytes, the following experiment was performed: HBsAg was preincubated for 0.5 h with a 1:100 dilution of chicken anti-HBsAg (0.8 μg/ml) or NIS (1.4 μg/ml) IgGs at room temperature before HBsAg–gold was added to the cell cultures.

Fixed hepatocytes were harvested, resuspended in 2% agarose at 60 °C and pelleted. The cell pellet was cut into 1 mm³ pieces and postfixed with 1% osmium tetroxide for 20 min at 4 °C. The cell blocks were dehydrated with ethanol and embedded in Epon. Ultrathin sections were counterstained with uranyl acetate and lead citrate and were examined with a Philips EM 301 at 80 kV. Sections were examined ‘blind’ by two independent investigators.

To investigate whether anti-EII or anti-idiotypic (anti-HBsAg) antibodies were also able to inhibit the specific binding of radiolabelled small HBsAg to human hepatocytes, binding studies were performed in the presence of rabbit anti-EII, chicken anti-idiotypic (anti-HBsAg) or NIS IgGs. As shown in Fig. 1, binding of small HBsAg to human hepatocytes was specifically inhibited by both anti-EII and anti-HBsAg antibodies as compared to the incubations with NIS IgGs. Incubation with a 1:10 dilution of the anti-EII (5 μg/ml) did not completely inhibit binding of HBsAg to the hepatocytes. However, when a 1:5 dilution (10 μg/ml) of the rabbit anti-EII IgG preparation was used in the incubation, specific binding of HBsAg was totally abolished (Fig. 1). Chicken and rabbit NIS IgGs gave similar results in the binding studies. The results were compared to those obtained in experiments with rabbit NIS IgGs.

The specific binding and uptake of HBsAg by human hepatocytes was studied using electron microscopy. If EII is involved in the binding/uptake process, incubation of hepatocytes with rabbit anti-EII IgGs prior to
Table 1. Mean number of gold particles attached per cell under different experimental conditions*

<table>
<thead>
<tr>
<th>IgGs used in the incubation</th>
<th>Gold particles bound per cell (±SD)</th>
<th>Percentage of control</th>
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<tbody>
<tr>
<td>NIS</td>
<td>28±6.6</td>
<td>100%</td>
</tr>
<tr>
<td>Anti-EII</td>
<td>12.5±4.95</td>
<td>44%</td>
</tr>
<tr>
<td>Anti-HBsAg</td>
<td>3.5±1.7</td>
<td>12.5%</td>
</tr>
</tbody>
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* Per experimental group 60 cells were analysed by two independent investigators and the average number of gold particles per cell was determined in three experiments (Wilcoxon test, P < 0.005).

HBsAg–gold administration or preincubation of HBsAg–gold conjugate with anti-idiotypic (anti-HBsAg) antibodies before addition to the cells should inhibit this binding. After incubation at 4 °C, cells were washed and fixed for electron microscopic analysis. The number of gold particles per cell was determined (Table 1). Cells incubated with gold conjugated HBsAg in the presence of NIS IgGs displayed the highest labelling density (28±6.6 gold particles/cell). After preincubation of the hepatocytes with anti-EII IgGs (5 μg/ml) the number of gold particles per cell was significantly reduced by 66% (12.5±4.95 gold particles/cell). When the HBsAg–gold was preincubated with anti-idiotypic antibodies (8 μg/ml), binding to the hepatocytes was inhibited by 87% (3.5±1.7 gold particles/cell) as compared to results obtained when experiments were performed using NIS IgGs (14 μg/ml). After incubation at 4 °C the HBsAg–gold particles were found to be attached to the plasma membrane of the hepatocytes (Fig. 2A). In Fig. 2D binding of HBsAg–gold to a typical coated pit region is shown. Warming the cells at 37 °C resulted in invagination of this region (Fig. 2B, E). After incubation for 5 min at 37 °C endocytotic vesicles containing gold particles could be observed (Fig. 2C, F), indicating that internalization took place within a few minutes. After incubation at 4 °C no endocytotic vesicles containing gold particles could be detected. When cells were incubated for 30 min at 37 °C, virtually no gold conjugated HBsAg could be detected on the surface of the plasma membrane, indicating that bound HBsAg was internalized. Rat hepatocytes, which served as negative control, did not bind and internalize HBsAg (result not shown).

Electron microscopy is an elegant method for visualization of the binding and uptake of a ligand by its specific receptor protein. In this study, HBsAg conjugated to 10 nm colloidal gold was used to investigate whether EII is involved in the binding and uptake of HBsAg by human hepatocytes. Binding of HBsAg–gold to human hepatocytes could be inhibited specifically by incubation with rabbit anti-EII or with anti-idiotypic (anti-HBsAg) IgGs. The results obtained from binding studies using radiolabelled ligand, shown in Fig. 1, were consistent with those obtained with HBsAg–gold par-

Fig. 2. Binding and internalization of HBsAg conjugated to 10 nm colloidal gold by human hepatocytes. Cells were incubated with HBsAg–gold for 2 h at 4 °C and subsequently warmed to 37 °C for 0 (panels A and D), 5 (panels B, C and E) or 10 (panel F) min. The bars represent 10⁻³ μm. The bar in panel D is for panels A–D; the bar in panel F is for panels E and F.
articles (Table 1). The use of HBsAg-gold is therefore a suitable method for studying the specific binding and internalization of HBV envelope proteins.

In the present study we showed that invagination of the coated pit region occurred at the HBsAg-gold binding sites, after warming the cells (Fig. 2). Moreover, the coated pit region was pinched off which led to the formation of endosomes containing HBsAg-gold. The process of invagination and formation of endocytotic vesicles takes place within a few minutes after warming the cells. Rabbit anti-EII and anti-idiotypic (anti-HBsAg) IgGs could specifically inhibit the binding of HBsAg-gold to human hepatocytes and subsequent formation of endocytotic vesicles containing HBsAg-gold. Since it is generally accepted that coated pits play an important role in receptor mediated endocytosis of specific ligands (Heuser et al., 1988; Lin et al., 1992), our results strongly suggest that human liver endonexin II is actively involved in receptor mediated endocytosis of small HBsAg. At this moment the effect of anti-EII and anti-HBsAg IgGs on infection of primary cultures of human hepatocytes with hepatitis B virus is under investigation in order to establish the possible role of EII in viral infection.

The authors thank Mrs H. Roelofs and Mrs T. Crabbe for their technical assistance with the hepatocyte isolation, and Mr H. Croes, Mrs M. Weijers, Mrs M. Wagemans and Dr J. Franssen for their help and advice with the electron microscopy. This work was supported in part by a grant of the Nederlands Organisatie van Wetenschappelijk Onderzoek (NWO) and Vlaams Progamma Biotechnologie (VLAB 033).

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(Received 1 September 1994; Accepted 24 November 1994)