The asialoglycoprotein receptor is a potential liver-specific receptor for Marburg virus

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The liver is one of the main target organs of Marburg virus (MBG), a filovirus causing severe haemorrhagic fever with a high fatality rate in humans and non-human primates. MBG grown in certain cells does not contain neuraminic acid, but has terminal galactose on its surface glycoprotein. This observation indicated that the asialoglycoprotein receptor (ASGP-R) of hepatocytes may serve as a receptor for MBG in the liver. Binding studies revealed that the attachment of MBG to ASGP-R-expressing HepG2 cells, but not to ASGP-R-negative E6 Vero cells, has the characteristics of ligand binding to the ASGP-R: binding is dependent on calcium and is inhibited by excess asialofetuin and by anti-ASGP-R antiserum. Asialofetuin and the specific antiserum also inhibited MBG infection of HepG2 cells. In addition, it was shown that expression of ASGP-R cDNA in NIH 3T3 cells enhanced the susceptibility of these cells to MBG infection 4.5-fold. Interaction of MBG with the hepatic ASGP-R could thus explain the marked hepatotropism of the virus.

Introduction

Marburg (MBG), Ebola and Reston viruses are the members of the Filoviridae, the third family of negative-strand RNA viruses with non-segmented genomes (Order Mononegavirales) besides the Paramyxoviridae and Rhabdoviridae. MBG, the prototype filovirus, was isolated during an outbreak of haemorrhagic fever among laboratory workers in 1967 in the city of Marburg, Germany (Peters et al., 1971). The mortality rate of MBG infection was unusually high: 30% of the cases had a fatal outcome. MBG was isolated from almost every organ of the infected patients. However, the predominant targets of the MBG infection seemed to be the liver and the cells of the reticular endothelial system (Bechtelsheimer et al., 1971; Ignatyev et al., 1993). In every case of MBG infection the hepatic functions were severely impaired (Bechtelsheimer et al., 1971).

MBG is a bacillus-shaped virus (680 nm in length and 80 nm in diameter) composed of seven structural proteins. The nucleoprotein, NP (94 kDa) (Sanchez et al., 1992; Becker et al., 1994), two smaller proteins with hitherto unknown function (VP35 and VP30) and the viral polymerase, L (267 kDa) (Mühlberger et al., 1992) are the protein constituents of the nucleocapsid. VP40 and VP24 probably serve as matrix proteins connecting the helical nucleocapsid and the viral membrane. The glycoprotein, GP (170 kDa) (Will et al., 1993) is inserted as a homotrimer in the viral envelope. GP is the only surface protein and is supposed to be responsible for the specific recognition of the host cells by the virus. Geyer et al. (1992) found that GP is highly glycosylated with 50% of its molecular mass consisting of N- and O-linked sugar side chains. The O-linked glycan chains belong to the neutral mucin type. Among the N-linked sugars tri- and tetra-antennary species are predominant. When MBG was grown in E6 cells, a subline of Vero (monkey kidney) cells, GP showed an unusual glycosylation pattern with respect to the terminal sugar residues: neither N- nor O-linked sugar side chains contained neuraminic acid. The lack of neuraminic acid together with the marked hepatotropism of the MBG infection led us to suggest that the asialoglycoprotein receptor (ASGP-R) of hepatocytes may serve as a receptor for MBG in the liver. ASGP-R (for a review see Spiess, 1990) is almost exclusively found in hepatocytes where it is located at the basolateral membrane and therefore faces the bloodstream. The human ASGP-R is a hetero-oligomer which is composed of two homologous subunits (46 and 50 kDa). ASGP-R recognizes with high affinity ($K_D$ in the nanomolar range) tri- and tetra-antennary N-linked sugar side chains with terminal galactose residues. Glycoproteins with such glycosylation patterns are rapidly endocytosed by the ASGP-R via clathrin-coated...
pits and vesicles. The interaction between the receptor and its ligands depends strongly on the presence of calcium ions in the millimolar range. The binding is released at acidic pH below 6-5.

Here we present evidence that MBG lacking sialic acid specifically binds to the ASGP-R. This observation may at least in part explain why the liver is a central target for MBG and may thus contribute to an understanding of the pathogenesis of this infection.

Methods

Biochemicals. All biochemicals were obtained from Merck (Germany) if not otherwise indicated.

Cell lines. For MBG propagation, E6 cells, a cloned line of Vero cells (ATCC CRL 1586), were used (Mühlberger et al., 1992). For binding studies, the HepG2 cell line was employed which was originally isolated from a human liver biopsy (Knowles et al., 1980). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, HEPES modification; Sigma) supplemented with 1% minimal essential medium non-essential amino acids (Gibco BRL), 1% l-glutamine (Gibco BRL) and 10% fetal calf serum (Gibco BRL). Cells were split 1:3 or 1:4 as soon as they reached confluence (at about 4 days). The 171 cell line is a derivative of NIH 3T3 cells expressing the functional human ASGP-R (Shia & Lodish, 1989). Cells (171 and 3T3) were grown in DMEM (Sigma) which was supplemented with 8% newborn calf serum (NCS; Gibco BRL), penicillin, and streptomycin (Gibco BRL).

Virus growth and purification. The Musoke strain of MBG isolated in 1980 in Kenya (Smith et al., 1982) was propagated in E6 cells. The cells were infected with MBG at an m.o.i. of \(10^{-2}\) p.f.u. per cell. Purification of viral particles and preparation of viral antigen were performed as described previously (Mühlberger et al., 1992). The final pellet of purified virus particles was resuspended in TNE (0·01 M-Tris-HCl pH 7·4, 0·15 M-NaCl, 2 mM-EDTA).

Metabolic labelling of MBG. 14×10⁶ E6 cells were infected with MBG at an m.o.i. of \(10^{-3}\) p.f.u./cell. Seven days post-infection the medium was removed, and cells were starved for 2 h in methionine- and cysteine-deficient Dulbecco's medium (ICN). Thereafter new deficient medium was added supplemented with TRANSM-S-LABEL (24 µCi/ml; ICN). After 24 h the supernatant was centrifuged at 4000 r.p.m. for 10 min at 4 °C. The resulting supernatant was collected and re-centrifuged at 27000 r.p.m. at 4 °C through a sucrose cushion (20% sucrose in TNE). The pellet was resuspended in 5 ml Dulbecco's medium without serum and centrifuged at 45000 r.p.m. at 4 °C for 20 min. The pellet was resuspended in 1 ml Dulbecco's medium without FCS. The virus suspension (\([{35S}]\)MBG) had an activity of 1×10⁶ c.p.m./20 µl.

MBG attachment to HepG2 cells. Nearly confluent HepG2 cells (7×10⁶) were used 4-5 days after splitting (1:3). Monolayers were rinsed with DMEM lacking FCS and left for 5 min at room temperature (RT). Cells were then incubated at 4 °C with PBS (supplemented with 1·7 mM-calcium ions) for another 5 min. Thereafter PBS was removed, and cells were incubated with 1 ml PBS containing 15 µl \([{35S}]\)MBG at 4 °C. After 4 h the supernatant was removed, and cells were washed three times with PBS. Optionally cells were treated with 5 mM-EDTA in PBS (without calcium) for 10 min at room temperature. Thereafter cells were lysed with PBS containing 1% SDS. An aliquot of the cell suspension was mixed with 3 ml scintillation cocktail (Ecoscint; Roth) and the radioactivity was counted in a liquid scintillation counter.

Inhibition of MBG binding to HepG2 cells. The experimental procedure was essentially the same as described under MBG attachment. In addition, a preincubation step was carried out. Prior to incubation of the cells with \([{35S}]\)MBG, cells were treated for 1 h at 4 °C with the following agents: (i) varying amounts of asialofetuin (Sigma) (5-250 µg asialofetuin/ml PBS, 1·7 mM-calcium chloride), (ii) a specific rabbit antiserum raised against the isolated human ASGP-R (anti-ASGP-R antiserum, dilution 1:40 in PBS, 1·7 mM-calcium chloride; Geffen et al., 1989) or (iii) a rabbit non-immune serum (dilution 1:40 in PBS, 1·7 mM-calcium chloride). After 3 h incubation at 4 °C with \([{35S}]\)MBG, unbound virus was removed by washing the monolayer with PBS (three 5 min washes). Bound radioactivity was determined by liquid scintillation counting (see above).

MBG internalization into HepG2 cells. Cells were grown in 3cm dishes until they reached confluence. Cells were rinsed twice with DMEM lacking FCS and subsequently incubated for 30 min at 37 °C with different concentrations of asialofetuin and fetuin (Sigma). Thereafter 15 µl of \([{35S}]\)MBG was added, and samples were taken after the indicated times. Unbound virus was removed by washing the cells three times with PBS. Subsequently the monolayers were treated with 1 ml of 5 mM-EDTA in PBS for 10 min at RT. Supernatants were collected (containing virus which was bound to the cell membrane), and cells were lysed with 1 ml 0·2 M-NaOH (these samples contained virus which was internalized into the cells). All samples were probed for radioactivity in a liquid scintillation counter.

MBG infection. Cells grown on glass coverslips to near confluence were incubated with MBG at an m.o.i. of \(10^{-2}\) p.f.u./cell. The inoculum was removed after 35-40 min at 37 °C. Thereafter cells were incubated with the respective medium at 37 °C for 72 h and subsequently analysed by immunofluorescence.

Inhibition of MBG infection. HepG2 cells were grown on glass coverslips until 80% confluence. The medium was removed and cells were incubated at 4 °C with asialofetuin (400 µg/ml medium), the anti-ASGP-R antiserum (1:40 in medium without FCS), fetuin (400 µg/ml) or a rabbit non-immune serum (1:40 in medium without FCS). After 1 h MBG was added to the medium at an m.o.i. of \(10^{-2}\) p.f.u./cell, and cells were further incubated for 1 h at 4 °C. Thereafter the inoculum was removed, DMEM supplemented with the respective inhibitor and 10% FCS was added and cells were incubated at 37 °C for 72 h until they were analysed by immunofluorescence.

Indirect immunofluorescence test. Monolayers of 5×10⁵ HepG2, 171 or 3T3 cells grown on glass coverslips were infected with MBG at an m.o.i. of \(10^{-2}\) p.f.u./cell. After an adsorption period of 40 min, medium was removed and 2 ml DMEM containing 10% FCS or 8% NCS in the case of 3T3 and 171 cells was added. Cells were further incubated for 72 h at 37 °C. Thereafter the monolayer was rinsed twice with PBS, and cells were fixed with 3% paraformaldehyde in PBS (RT for 20 min) followed by acetic acid treatment (–20 °C for 15 min). Subsequently cells were incubated for 5 min in 100 mM-glycine, rinsed with PBS and probed with an anti-MBG guinea-pig serum (1:40; Becker et al., 1994) which had been preadsorbed to HepG2 proteins (200 µl serum) diluted 1:20 in PBS) were incubated for 1 h at 4 °C with a lysate of 1×10⁵ cells in 200 µl PBS. Monolayers were incubated for 1 h in a moisture chamber with the preadsorbed serum. Bound antibody was detected by rabbit anti-guinea-pig immunoglobulins (Ig) which were conjugated to FITC (1:50; DAKO). Samples were analysed by immunofluorescence microscopy.

Immunoblot procedure. Cells were lysed by adding sample buffer under non-reducing conditions (20% v/v glycerol, 8% SDS, 125 mM-Tris-HCl pH 6·8, bromophenol blue). Aliquots of the lysates were separated by 10% SDS-PAGE (PAGE) as described by Laemmli.
(1970). After electrophoresis the proteins were blotted onto PVDF-membranes (Millipore, #P-15552) by the semi-dry blot technique. Immunodetection was performed using the anti-ASGP-R antiserum described above (dilution 1:300). A swine anti-rabbit secondary antibody was used which was coupled to horseradish peroxidase (dilution 1:500; DAKO).

Results

Binding of MBG to HepG2 and E6 cells

To test whether the ASGP-R of hepatocytes is responsible for the efficient infection of the liver by MBG, we compared the binding of MBG to the hepatoma cell line HepG2 and to E6 cells, a Vero cell clone routinely used to propagate MBG. Western blot analysis using specific antibodies raised against the ASGP-R confirmed that E6 cells do not express ASGP-R to any detectable extent (Fig. 1). In HepG2 cells, however, several bands in the molecular mass range between 40 kDa and 50 kDa were observed corresponding to the high-mannose and complex glycosylated forms of ASGP-R subunits (Lodish et al., 1992).

To analyse binding of MBG, HepG2 cells were incubated with [35S]methionine-labelled MBG for 4 h at

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\begin{align*}
\text{MBG binding (\%)} & \quad \text{Control} \quad \text{EDTA (5 mM)} \quad \text{Anti-ASGP-R} \quad \text{Nonimmune serum} \\
\text{HepG2 cells} & \quad 100 \quad 91 \quad 95 \quad 95 \\
\text{E6 cells} & \quad 100 \quad 75 \quad 95 \quad 95
\end{align*}
\]

Fig. 2. Marburg virus binding to HepG2 and E6 cells. (a) Approximately \(7 \times 10^5\) HepG2 cells were preincubated with the indicated substances for 1 h at 4 °C. Then [35S]MBG was added and incubation was continued for 3 h. Subsequently, unbound virus was removed using PBS and optionally cells were treated with 5 mM-EDTA for 10 min at RT. Then cells were lysed and radioactivity was determined. Values represent activity bound to the cells as a percentage of the value obtained with untreated HepG2 cells. (b) MBG binding to E6 cells. Experimental procedure was essentially the same as described in (a). Values represent radioactivity bound to the cells as a percentage of the value obtained with untreated E6 cells.

Fig. 1. Expression of ASGP-R in HepG2 cells versus E6 cells. Cells (7.5 x 10^5) were lysed, subjected to SDS-PAGE, and blotted onto PVDF-membranes. The membranes were probed with the anti-ASGP-R antiserum (dilution 1:300). Bound antibody was detected using a swine anti-rabbit immunoglobulin coupled to horseradish peroxidase. The ASGP-R is marked with an arrow.

4 °C. Between 3 and 10 % of the radioactivity was bound to the cells corresponding to approximately 85 ng viral protein per 10⁶ cells. This value corresponds to approximately 140 virus particles per cell [assuming a protein content of \(6.274 \times 10^{-16}\) g per virion (according to Kiley et al., 1988)]. As shown in Fig. 2(a), binding of MBG to HepG2 cells is affected by the same agents known to influence the interaction between ASGP-R and its ligands: EDTA, excess competitor ligand, and anti-ASGP-R antibodies. Preincubation with the anti-ASGP-R antiserum resulted in the strongest inhibition (almost 90 %) of MBG binding to HepG2 cells. Although a non-specific rabbit serum inhibited MBG binding to the cells by 48 %, the effect of the specific serum was more substantial. The calcium chelator EDTA reduced the attachment of the virus to the cells by 81 %. Increasing concentrations of asialofetuin caused a concomitant
Fig. 3. Effect of asialofetuin on MBG binding to HepG2 cells. Approximately $7 \times 10^6$ HepG2 cells were preincubated with various compounds at the indicated concentrations for 1 h at 4°C. Then [\(^{35}\)S]MBG was added and incubation was continued for 3 h. Subsequently unbound virus was removed by washing with PBS. Cells were lysed and radioactivity was determined. Values represent activity bound to the cells as a percentage of the value obtained with HepG2 cells which were incubated without asialofetuin.

A decrease of radioactive virus bound to HepG2 cells (Fig. 3). A concentration of 250 \(\mu\)g/ml of asialofetuin resulted in an 82% reduction of virus binding, almost the same inhibition of virus binding as was observed with EDTA and by preincubation with the anti-ASGP-R antiserum (Fig. 3 and Fig. 2a).

In contrast, the characteristics of MBG attachment to E6 cells were clearly different. As shown in Fig. 2(b), neither EDTA, asialofetuin, the non-immune serum, nor the anti-ASGP-R antiserum had a significant effect on the binding of the virus to E6 cell membranes. However, the absolute amount of bound virus was nearly identical to that observed with the HepG2 cells.

**Internalization of MBG into HepG2 cells**

To test for internalization of MBG into HepG2 cells, we took advantage of the fact that virus bound to the cell surface via the ASGP-R can be released into the supernatant by EDTA treatment, whereas internalized virus is protected. After addition of [\(^{35}\)S]MBG to the incubation medium the amount of virus that was internalized, i.e. resistant to EDTA treatment, increased with time (Fig. 4a). Internalized viral protein amounted to $88.5 \pm 10^6$ ng/h/10^6 cells (corresponding to approx 150 viral particles per cell: three independent experiments, duplicate determinations). Internalization was inhibited by the addition of excess asialofetuin into the incubation medium (Fig. 4b, c). The presence of 900 \(\mu\)g asialofetuin/ml (Fig. 4c) inhibited internalization by more than 50%. Equal concentrations of fetuin, however, did not influence the uptake of virus into the cells (data not shown).

**Role of ASGP-R during MBG infection**

Since binding and internalization of MBG to HepG2 cells followed the characteristics of other ligands interacting with ASGP-R, the susceptibility of HepG2 cells to MBG infection was analysed. Cells were incubated with MBG and subsequently tested for MBG replication by indirect immunofluorescence. As shown in Fig. 5, incubation of HepG2 cells with MBG resulted in inclusion bodies typical for MBG infection (Becker et al., 1992).

In order to analyse the importance of the ASGP-R for the infection process we tried to inhibit MBG infection by the same agents that were shown to affect the interaction between the receptor and the virus. HepG2 cells were preincubated with anti-ASGP-R antiserum, asialofetuin, fetuin, or non-immune serum and thereafter
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Fig. 5. Susceptibility of HepG2 cells to MBG infection. HepG2 cells grown on glass coverslips were incubated at 37 °C with MBG at an m.o.i. of 0.2 p.f.u./cell. After 45 min the inoculum was removed and cells were further incubated with DMEM supplemented with 10% FCS at 37 °C. After 3 days the monolayer was examined by immunofluorescence. Infected cells show typical inclusion bodies. Bar marker represents 10 μm.

Fig. 6. Inhibition of MBG infection by asialofetuin and anti-ASGP-R antiserum. HepG2 cells grown on glass coverslips were incubated for 1 h with the indicated agents [asialofetuin (400 μg/ml), fetuin (400 μg/ml), anti-ASGP-R antiserum (1:40) and non-immune serum (1:40)]. The cells were then infected with MBG by an m.o.i. of 0.2 p.f.u./cell in the presence of the inhibitors. After the adsorption period unbound virus was removed and the cells were further incubated in the presence of the respective inhibitor. After another 72 h monolayers were analysed by indirect immunofluorescence. Values represent the number of infected cells as a percentage of the control (untreated infected cells).

Discussion

Hitherto no cellular receptor of MBG had been identified. Based on the observation that MBG infection concerns predominantly the liver (Martini et al., 1968; Gedigk et al., 1968) and on the findings of Geyer et al. (1992) that the tri- and tetra-antennary N-glycans of MBG GP propagated in E6 cells are devoid of terminal sialic acid, we postulated that the ASGP-R of hepatocytes serves as a receptor of MBG in the liver. ASGP-R recognizes glycoproteins containing tri- and tetra-antennary N-linked oligosaccharides with terminal galactose...
residues with high affinity \((K_d \approx 10^{-8} \text{ to } 10^{-9})\) (for a review see Spiess, 1990). ASGP-R is located at the basolateral surface of hepatocytes facing the capillaries. Asialoglycoproteins are rapidly endocytosed by the receptor and removed from the circulation (Ashwell & Morell, 1977). The physiological function of the ASGP-R is not completely understood, but it appears to play a role in the homeostasis of serum glycoproteins and in the metabolism of glycoprotein hormones (Drickamer, 1991). The ASGP-R is thus a candidate for a liver-specific receptor for MBG that could mediate virus binding and endocytosis.

To test this model, we studied the binding of MBG to ASGP-R-expressing cells in comparison with receptor-negative cells. Our main model system was the human hepatoma cell line HepG2 which expresses the ASGP-R in almost the same amount as hepatocytes (Schwartz et al., 1981). Incubation of HepG2 cells with MBG resulted in attachment of the virus to the cells. This binding was inhibited by the calcium chelator EDTA and by asialofetuin, a known ligand of the receptor (Fig. 2a and Fig. 3). Viral attachment to HepG2 cells was further inhibited by an antisera against the human ASGP-R. Residual virus binding to HepG2 cells after treatment with a maximal dose of the respective inhibitor was very similar (approximately 20\% ; Fig. 2a and Fig. 3) and is either non-specific or mediated by an alternative receptor. The rabbit non-immune serum used also had an inhibitory effect on the binding of MBG to HepG2 cells, although significantly less than the specific antisera. This may be due to asialoglycoproteins present in the serum (Ashwell & Morell, 1977) and/or non-specific blocking of ASGP-R by serum components. These results indicate that attachment of MBG to HepG2 cells is primarily mediated by the ASGP-R.

Internalization of MBG into HepG2 cells was also shown to depend on ASGP-R (Fig. 4), since it was also inhibited by high concentrations of asialofetuin but not by fetuin, which does not bind to the ASGP-R.

HepG2 cells are susceptible to MBG infection, as was evident from the typical inclusion bodies visualized by indirect immunofluorescence using anti-MBG antibodies (Fig. 5). Both asialofetuin and anti-ASGP-R antisera inhibited MBG infection (Fig. 6). Consistent with a role of the ASGP-R in MBG infection, 171 fibroblast cells expressing the functional receptor were 4-5 times as susceptible to infection as the parental 3T3 cells. Addition of asialofetuin to the culture medium of 171 cells reduced the number of infected cells.

Interestingly, the concentrations of asialofetuin required for inhibition of MBG binding, internalization and infection (400 μg/ml and 900 μg/ml) were considerably higher than in a comparable study (Markwell et al., 1985) where infection of HepG2 cells by a HN-deficient mutant of Sendai virus was inhibited by as little as 20 μg/ml of an asialoglycoprotein. In this special case the ligand for the ASGP-R was the fusion protein of Sendai virus, which the authors supposed to contain terminal galactose residues. However, the carbohydrate structure of the F protein does not conform well to the structural requirements for high-affinity binding to the ASGP-R. Yoshima et al. (1981) showed that the F protein contains several bi-antennary N-linked sugar side chains but no tri- or tetra-antennary species. In contrast, the MBG GP contains up to 19 N-linked glycans: 50 mol\% of these are tri- and tetra-antennary species with terminal galactose residues (Geyer et al., 1992). So the MBG GP could bind many more ASGP-R molecules, which would explain the requirement for higher competitor concentrations.

From our data it cannot be ruled out that a second, minor receptor exists in hepatocytes, a receptor that may not necessarily be tissue-specific. Several cell lines (E6, MA 104 and Vero), which do not express the ASGP-R, support replication of MBG. Furthermore, MBG is able to infect human endothelial cells which also lack the ASGP-R (Schnittler et al., 1993). For this reason it has to be postulated that, in addition to the ASGP-R, other receptors may mediate entry of MBG into cells. Our study supports the view that a pantropic virus like MBG uses different receptors in different organs of the same host as also appears to be the case for human immunodeficiency virus type 1 (Fantini et al., 1991; Yahi et al., 1992) and Semliki Forest virus (Marsh & Helenius, 1989).

In some of the above-mentioned cells the GP of MBG is at least partly sialylated (Feldmann et al., 1994). It has to be investigated if sialylation concerns N-linked oligosaccharides, and if the presence of sialic acids on some of the carbohydrates prevents the interaction between MBG and ASGP-R. The fact that E6 cells, which do not detectably sialylate MBG, do sialylate other proteins, indicates that to be undersialylated is a property of the MBG surface proteins.

Our results suggest that the ASGP-R serves as a receptor for MBG in HepG2 cells, in ASGP-R-positive fibroblasts, and, by analogy, in hepatocytes. We have shown that a wild-type virus with a marked hepatotropism binds to the ASGP-R and that this binding seems to be the prerequisite for an infection of the cells. Because of the clinical course of MBG illness, which shows that the liver is one of the main targets of the MBG infection, the identification of a hepatic receptor is an important step towards a better understanding of the pathology of MBG infections.

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