Dengue virus type 2 infects human endothelial cells through binding of the viral envelope glycoprotein to cell surface polypeptides

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The endothelial cell line ECV304, derived from human umbilical cord and identified to be susceptible to dengue virus type 2 (DEN-2) infection, was used to study the molecular mechanism of DEN-2 binding to endothelial cells. DEN-2 was found by virus overlay protein-binding assays (VOPBAs) to bind to three ECV304 cell membrane proteins with molecular masses of 29, 34 and 43 kDa. Only a single protein of 29 kDa was observed when VOPBAs were carried out using preparations of trypsin-treated ECV304 cells. Pre-incubation of live ECV304 cells in culture or cell membrane proteins in modified VOPBAs with the recombinant DEN-2 envelope glycoprotein (rEgp) inhibited DEN-2 infection and blocked virus binding to the three proteins identified. These results indicate that DEN-2 rEgp could bind to three proteins on the surface of ECV304 cells. This virus–cell interaction may be associated with the receptor complex specific for DEN-2 infection of endothelial cells.
**Table 1. Production of DEN-2 in supernatants of infected ECV304 cells**

ECV304 cells were infected at various m.o.i. and collected at different times p.i. Virus titres were also assessed after cells were pre-incubated with various concentrations of rEgp. Virus titres were assessed using a modified micro-plaque assay. ECV304 cells infected with heat-inactivated DEN-2 were used as control. ND, Not detected.

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>m.o.i.</th>
<th>DEN-2 (p.f.u. ml(^{-1}))</th>
<th>Pre-incubation with rEgp</th>
<th>DEN-2 (p.f.u. ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td>8.2 × 10(^5)</td>
<td>0-1</td>
<td>1.8 × 10(^2)</td>
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<tr>
<td>48</td>
<td></td>
<td>5.3 × 10(^3)</td>
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<tr>
<td>72</td>
<td></td>
<td>6.6 × 10(^8)</td>
<td>0-1</td>
<td>2.6 × 10(^8)</td>
</tr>
<tr>
<td>96</td>
<td></td>
<td>8.2 × 10(^5)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

100) (Fig. 1a). The most intense fluorescence staining was observed at 72 h p.i. These data showed that DEN-2 could replicate efficiently and mature in ECV304 cells and that this cell line could be used as a model cell line for DEN-binding assays.

ECV304 cells were harvested either by gentle separation from the adherent surface with a cell scraper or by treatment with trypsin. The cell pellets harvested were then used to prepare total cell protein preparations using lysis buffer and membrane protein preparations using 1% glucose pyranoside, according to the methodologies described previously (Marianneau et al., 1996). The preparations of cell lysates and membranes were subjected to SDS-PAGE and protein bands were visualized by staining with Coomassie blue R-250. A single protein band with a molecular mass of approximately 43 kDa was missing from the trypsin-treated membrane preparations, suggesting that this protein on the surface of ECV304 cells is trypsin-labile. A virus overlay protein-binding assay (VOPBA) was performed using a labelled preparation of DEN-2 to compare protein preparations from trypsinized cells and those from scraped cells for their virus-binding capability. DEN-2 labelled with \(^{35}\)S-methionine (Amersham) was purified from DEN-2-infected C6/36 cells, as described previously (Salas-Benito & del Angel, 1997). Labelled virus (4.5 × 10\(^5\) p.f.u. ml\(^{-1}\)) was used in all VOPBAs. Results showed that DEN-2 bound to three proteins, with molecular masses of approximately 29, 34 and 43 kDa, absent from both total cell protein preparations and membrane protein only preparations from scraped ECV304 cells (Fig. 2a, lane 1). When VOPBAs were carried out using total and membrane protein preparations obtained from trypsin-treated ECV304 cells, only a single band of 29 kDa was observed (Fig. 2a, lane 2). No similar bands could be seen in a control VOPBA performed with labelled DEN-1 strain Hainan. As previous studies demonstrated that heparan sulfate (HS) may be involved in the interaction of DEN with cell surfaces (Chen et al., 1997; Shieh et al., 1992), a blocking experiment with HS was carried out in a modified VOPBA to study whether DEN-2 binding to cell surface proteins is mediated by a carbohydrate moiety. ECV304 cell membrane preparations (collected by trypsin treatment or by scraping) were pre-incubated with 10 \(\mu\)G HS (Sigma). Labelled DEN-2 was found to bind to the three proteins identified. The above result suggests a specific interaction of DEN-2 with the three proteins identified on the surface of ECV304 cells.

As a previous report showed that the full-length recombinant DEN-2 envelope glycoprotein (rEgp) expressed in and secreted from *Pichia pastoris* yeast cells retained its antigenicity (Wei et al., 2003), we studied whether pre-incubation of live ECV304 cells with different quantities of purified rEgp could block DEN-2 infection to these cells. Lyophilized rEgp (0-1, 10 \(\mu\)G) was reconstituted and added to the medium of 3 × 10\(^5\) ECV304 cells at 4 °C for 2 h. After the removal of rEgp-containing medium, DEN-2 was added to the cells at an m.o.i. of 10. Virus-binding interactions were allowed to proceed for 2 h at 4 °C in order to avoid virus penetration. ECV304 cells without rEgp were used as control. After unbound virus was removed, cells were incubated at 37 °C for 72 h. Culture supernatants and infected cells were collected every 24 h to assay virus titre and to count the percentage of DEN-2
antigen-positive cells by IFA. The results showed that pre-incubation of ECV304 cells with 10 µg rEgp could inhibit DEN-2 infection completely. Marked inhibition of DEN-2 infection and virus production was found when cells were pre-incubated with 0·1–1·0 µg rEgp. Decreasing yields of DEN-2 in the supernatants of infected cells correlated with increasing concentrations of rEgp (Table 1). The percentages of fluorescence-positive ECV304 cells collected at various times were similar to the results of virus titration (Fig. 1b).

A modified VOPBA was carried out to study whether rEgp could block DEN-2 binding with the three ECV304 cell surface proteins identified. After SDS-PAGE and electrotransfer of proteins, the resultant membranes were incubated for 2 h at 4 °C with 0·1, 1 and 10 µg rEgp. PBS without rEgp was used as control. The membranes were then incubated with 35S-labelled DEN-2 and auto-radiographed under the same conditions as described above. No virus-binding bands could be visualized when ECV304 cell membrane preparations (collected by trypsin treatment or by scraping) were pre-incubated with 10 µg rEgp. When the same membranes were pre-incubated with 1·0 µg rEgp, the single virus-binding band identified (29 kDa) could not be visualized from the trypsin-treated preparations but the three binding bands identified could be visualized, albeit indistinctly, from the scraped cell preparations (Fig. 2b). These results suggest that rEgp could inhibit virus infection by competitively blocking the virus-binding proteins on the surface of ECV304 cells.

Human vascular endothelial cells were found to support DEN replication in vitro but early studies demonstrated (Anderson et al., 1992) that these cells were less susceptible to DEN-4 in vivo. An increasing number of reports have indicated that DEN can infect human endothelial cells efficiently and induce cytokine production, and may play an important role in regulating vascular permeability and
maintaining haemostasis (Huang et al., 2002). Recent studies indicate that vascular endothelial cells can be targets for DEN infection but different serotypes of DEN and different strains of the same serotype might exhibit different infectivity to human endothelial cells (Bielefeldt-Ohmann et al., 2001; Huang et al., 2002). In the present study, an umbilical cord-derived human endothelial cell line (ECV304) was used to analyse susceptibility to DEN-2 infection. Virus growth curves and virus antigen detection (ECV304) was used to analyse susceptibility to DEN-2 infection. Virus growth curves and virus antigen detection (assessed by IFA staining at various times p.i.) indicated that DEN-2 can reproduce efficiently in ECV304 cells and that this cell line could be used as a model for DEN-binding assays.

Although considerable progress has been made in the identification of host cell receptors for viruses (Shieh et al., 1992), little is known about the nature of DEN receptors on the surface of host cells; no host cell receptors for DEN have been identified in human endothelial cells. Previous reports demonstrated that two membrane proteins served as specific receptors for DEN-4 on the surface of C6/36 cells (Salas-Benito & del Angel, 1997). Current studies showed that multiple cell surface molecules, including glycosaminoglycans, HS and lipopolysaccharide-binding CD14-associated molecules, might be involved in DEN binding and subsequent virus infection in target cells (Chen et al., 1997, 1999; Bielefeldt-Ohmann et al., 2001). In this study, the ECV304 cell line was chosen to study how DEN-2 binds to endothelial cells and to identify virus binding-associated proteins on the host cell surface. The results of VOPBAs using labelled DEN-2 as probe show that three virus-binding proteins of 29, 34 and 43 kDa were present in the membrane preparations of this human endothelial cell line. Our results also indicate that the 34 and 43 kDa virus-binding proteins were sensitive to trypsin, as trypsin treatment could prevent recognition of these two proteins by labelled DEN-2.

Anderson et al. (1992) observed that binding of the envelope glycoprotein of DEN-4 to cell surface receptor(s) correlated with cell susceptibility to virus infection. The envelope glycoprotein of *Flaviviruses* presumably plays a role in cellular tropism by being involved in virus binding to host cell receptors and has been identified as the virus attachment protein (Chen et al., 1996, 1997). By preincubating live ECV304 cells or transfected membranes with rEgp in the present study, we demonstrated that DEN-2 infects host cells and virus binding to the three ECV304 cell surface proteins identified (29, 34 and 43 kDa) could be inhibited in a dose-dependent manner. The result indicated that rEgp might compete with DEN-2 for the same receptor on the surface of the target cell through receptor saturation and steric hindrance.

In this paper, we report (i) that DEN-2 bound specifically to three proteins of 29, 34 and 43 kDa on the surface of human endothelial cells and (ii) that rEgp, a full-length recombinant DEN-2 envelope glycoprotein, inhibited infection of ECV304 cells by DEN-2, suggesting the existence of a specific interaction between these cell surface membrane molecules with the viral envelope glycoprotein. Although the nature of the three virus-binding proteins identified remains to be characterized, isolation, purification and characterization of these protein molecules will be of fundamental importance to identify further the host cell receptor for DEN-2 attachment to and penetration of human vascular endothelial cells.

REFERENCES


