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Antiviral activity of chondroitin sulphate E targeting dengue virus envelope protein

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ABSTRACT

Sulphated glycosaminoglycans such as heparin inhibit the early step of dengue virus infection through interaction with envelope (E) protein. Here, we found that chondroitin sulphate E (CSE), but not CSD, which contains the same degree of sulphation, inhibited dengue virus (DENV) infection of cells with adsorption. CSE significantly reduced infectivity of all dengue virus serotypes to BHK-21 and Vero cells. DENV preferentially bound to CSE immobilised on plastic plates. Also, virus binding to CSE or heparin was cross-inhibited by soluble CSE or heparin. These findings suggested that common carbohydrate determinants on CSE and heparin could be essential epitopes for interaction of DENV, and may be responsible for inhibition of the early steps of DENV infection. A recombinant E protein directly bound heparin and CSE, but not CSD, meaning that interaction of CSE with E protein contributes to the inhibitory action of this glycosaminoglycan. These observations indicate that a specific carbohydrate structure rather than polysulphation or addition of negative charges of the glycosaminoglycan molecule would be necessary for direct binding to DENV E protein. In conclusion, CSE showed antiviral activity as an entry inhibitor targeting E protein of dengue virus.

1. Introduction

Dengue virus (DENV) belongs to the genus Flavivirus, family Flaviviridae (Kuno et al., 1998; Mukhopadhyay et al., 2005). DENV is transmitted by *Aedes* mosquitoes, and predominantly infects humans. DENV causes human diseases, such as dengue fever, dengue hemorrhagic fever and dengue shock syndrome (Gubler, 2002; Halstead, 2007). There are four dengue virus serotypes, type 1 (DENV-1) to type 4 (DENV-4), which have similar clinical manifestations and epidemiology in tropical and subtropical regions of the world where more than two billion people are at risk of infection (Kuhn et al., 2002; Mackenzie et al., 2004; Weaver and Barrett, 2004; Halstead, 2007).

Flaviviruses are enveloped viruses with an envelope (E) protein on the surface of the lipid bilayer membrane. The Flavivirus genome is a single-stranded, positive-sense RNA approximately 11 kb in length, which contains a single open reading frame encoding a polyprotein (Lindenbach and Rice, 2003; Zhang et al., 2003c). The polyprotein is posttranslationally cleaved into three structural (C, PrM and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) proteins by host- and viral-derived proteases. Among the three structural proteins, E protein consists of 500 amino acids divided into three functional domains: domain I, domain II and domain III. Domains II and III contain a fusion peptide and a cellular receptor binding site(s), respectively (Rey et al., 1995; Wu et al., 2003). E protein, which is the major antigen, is considered to be involved in viral attachment, fusion, neutralisation, host range and tissue tropism (McMinn, 1997; Crill and Roehrig, 2001; Chu et al., 2005; Chin et al., 2007; Stiasny et al., 2007).

Flavivirus infection is initiated by the interaction between E protein and protein, lipid, or carbohydrate host receptor(s) in a complex extracellular matrix structure (Schneider-Schaulies, 2000; Lescar et al., 2001; Aoki et al., 2006). The structures and antibody binding sites of DENV E proteins have been elucidated by X-ray crystallography and NMR (Modis et al., 2003, 2005; Zhang et al., 2003b). These studies provided a structural basis for understanding the molecular mechanisms of immunological protection and virus

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entry. Several lines of evidence regarding host receptors demonstrated that heparan sulphate (HS) or the highly sulphated form of glycosaminoglycan on the host cell surface are essential for the early stages of flavivirus infection (Chen et al., 1997; Lee and Lobigs, 2000; Mandl et al., 2001; Gemi et al., 2002). Virus particles attached to the host cell surface enter the cell by receptor-mediated endocytosis. Acidification of the endosomal vesicle causes conformational changes in E protein, which result in fusion and viral disassembly (McMinn, 1997; Stiasny et al., 2007).

At present, no specific treatments for DENV infection are clinically available. Control of DENV by safe, low-cost and long-lasting vaccination has not been established. Therefore, there is a requirement for effective antiviral agents and therapeutic concepts for DENV infection. Although Japanese encephalitis and yellow fever viruses, which belong to the same family as dengue virus (family *Flaviviridae*), are controlled by specific vaccinations, no licensed dengue vaccines or anti-dengue agents are clinically available. The only available disease treatment is supportive therapy. Several types of antiviral agent have been sought intensively, including inhibitors against viral replication (Zhang et al., 2003a), posttranslational processing of viral proteins (Courageot et al., 2000; Knox et al., 2006; Whitby et al., 2005; Wu et al., 2002) and E protein functions such as membrane fusion (Hrobowski et al., 2005; Poh et al., 2009) and virus attachment (Aoki et al., 2006; Bai et al., 2007; Hidari et al., 2008; Marks et al., 2001; Wang et al., 2009; Yennamalli et al., 2009). Blocking of virus attachment or entry into host cells is an effective strategy to control virus infection (Altmeyer, 2004). This type of inhibitor, termed an entry inhibitor, blocks structural rearrangements of the viral envelope that are essential for viral infection.

Glycosaminoglycans are unbranched sulphated polysaccharides expressed widely on the cell surface or in the extracellular matrix (ECM). There are five different isomers of glycosaminoglycans based on the sugar components, the extent of sulphation and the number of repeating units: heparin, HS, chondroitin sulphate (CS), dermatan sulphate and keratan sulphate. Glycosaminoglycans play important roles in cell adhesion and growth, maintenance of ECM integrity and signal transduction (Gallagher et al., 1986; Kjellen and Lindahl, 1991; Yanagishita and Hascall, 1992; Iozzo, 1998). It has been reported that some viruses utilise glycosaminoglycans as an initial step to enter host cells (Lycke et al., 1991; Trybala et al., 1994; Roderiquez et al., 1995). Some glycosaminoglycans with a high degree of sulphation, such as heparin and highly sulphated chondroitins, show potent inhibition of flavivirus infection (Chen et al., 1997; Su et al., 2001). The degree of sulphation has been suggested to be involved in inhibition of infection (Marks et al., 2001). However, detailed chemical structures responsible for the interaction of DENV with host receptors have yet to be elucidated.

In the present study, we examined the anti-dengue virus activity of sulphated glycosaminoglycans. We also investigated viral infection and molecular interaction with a number of glycosaminoglycans, *i.e.*, chondroitin sulphate A, B, C, D and E, heparin, heparan sulphate and hyaluronic acid.

2. Materials and methods

2.1. Materials

Chondroitin sulphates (CSA, CSB, CSC, CSD and CSE), HS and heparin were purchased from Seikagaku Corp. (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. A sensor chip SA was obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire). Cell proliferation kit (Cat# 11465007001) for MTT assay was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were of the highest quality commercially available.

2.2. Cell culture and virus

BHK-21 and Vero cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 5% FBS. *Aedes albopictus* clone C6/36 cells were grown at 28 °C in Eagle's minimal essential medium supplemented with 10% FBS and 0.2 mM non-essential amino acids. DENV-1 strain, D1/Lao/03, DENV-2 strain, ThNH-7/93, DENV-3 strain, D3/BDH02-01, DENV-4 strain, ThD4-17/97 and JEV strain, JaOArS982, were inoculated into C6/36 cells and the supernatant was harvested on the fourth day of culture (Igarashi, 1978; Thant et al., 1996). The supernatant was centrifuged, followed by addition of polyethylene glycol 6000 and NaCl to final concentrations of 8% and 0.5 M, respectively. This mixture was gently stirred overnight at 4 °C and centrifuged again. The virus pellet was suspended in 2 ml of STE buffer (0.15 M NaCl, 0.1 M Tris and 0.01 M EDTA, pH 7.2). The virus aliquots were stored at −80 °C before use.

2.3. Inhibition of virus infection by glycosaminoglycans

Virus titres were determined by focus-forming assay using BHK-21 and Vero cells as described previously (Aoki et al., 2006). The cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C in DMEM supplemented with 5% FBS. After three washes with serum-free DMEM containing 25 mM HEPES, DENV or JEV was premixed on ice with glycosaminoglycans at the indicated concentrations. The virus–glycosaminoglycan premixtures were immediately inoculated onto the cells for 2 h at 37 °C. After removal of the virus solution, overlay medium (DMEM containing 1% FBS and 0.5% tragacanth gum) was added, and plates were incubated at 37 °C for 43 or 19 h for DENV or JEV, respectively. The cells were fixed and permeabilised with PBS containing 5% paraformaldehyde and 1% NP-40. Infectious foci were detected with human serum from dengue hemorrhagic fever patient (anti-dengue antisera), followed by HRP-conjugated goat anti-human immunoglobulin. Virus infectivity was determined as focus-forming units (FFU). The optimal titre of inoculated virus was predetermined, such that more than 100 foci appeared per well. Anti-dengue antisera used in this study equivalently react with all serotypes of dengue viruses as described previously (Hidari et al., 2008).

2.4. Determination of cellular cytotoxicity of glycosaminoglycans by MTT assay

To evaluate cytotoxicity of glycosaminoglycans against BHK-21 cells, MTT assay was performed according to the manufacturer's instruction. Briefly, the cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C in DMEM supplemented with 5% FBS. After three washes with serum-free DMEM containing 25 mM HEPES, glycosaminoglycans diluted up to 200 μg/ml with serum-free DMEM containing 25 mM HEPES were added onto the cells for 2 h at 37 °C. After removal of the compound solution, the overlay medium was added, and plates were incubated at 37 °C for 43 h. The cells were incubated with the MTT solution for 4 h. After this incubation period, the wells were added the solubilisation solution and incubated overnight at 37 °C under 5% CO_2. The solubilised product was spectrophotmetrically quantified at 550 nm (reference at 690 nm).

2.5. Cell-surface binding of viruses to cultured cell lines

Direct binding of viruses to cultured cells was performed as described previously (Aoki et al., 2006). Briefly, cells were seeded onto 96-well plates and cultured at 37 °C in DMEM supplemented with 10% FBS. After blocking with DMEM containing 2% BSA, the plates were incubated at 4 °C for 2 h in DMEM containing viruses