Increased levels of serum hyaluronan in patients with dengue infection

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Dengue hemorrhagic fever;
Dengue shock syndrome;
Sinusoidal endothelial cells

Summary
Objective: Serum hyaluronan (HA) is ubiquitously distributed in connective tissues. Circulating HA is degraded by hepatic sinusoidal endothelial cell. The aim of the present study was to evaluate serum HA levels and to determine their importance in dengue fever (DF)/dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) patients.

Methods: Serum HA levels were measured by an ELISA-based method in 16 patients with DF, 14 patients with DHF and 10 patients with DSS. The HA levels were compared with those in 48 acute hepatitis A patients; 8 healthy blood donors serve as controls.

Results: In acute phase, mean serum HA levels significantly increased in patients with DSS (7316.3 ± 10,359.0 ng/ml) in comparison to patients with hepatitis A (93.8 ± 50.4 ng/ml; \( P < 0.0001 \)) and healthy controls (48.3 ± 16.3 ng/ml; \( P < 0.0001 \)). Serum levels of HA in patients with DSS rapidly decreased during the convalescent phase, but were still significantly higher than those in healthy controls (915.2 ± 1294.8, and 48.3 ± 16.3 ng/ml; \( P < 0.0005 \)).

Conclusions: The increased HA in dengue patients could be attributed to sinusoidal endothelial damage rendering the endothelium incapable of HA clearance. The elevation of HA production may involve the pathogenesis of dengue infection. Further studies are needed to determine whether the increase in the HA production or decrease in its clearance is responsible for the elevated serum HA.

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Introduction

Dengue infection is the most common human viral disease caused by an arbovirus in the family of Flaviviridae. Four antigenically distinct subtypes of these RNA-viruses are designated as dengue type 1–4. Their transmission involves ingestion of viremic blood by mosquitoes and subsequent passage to a susceptible human host, the principal vector being Aedes aegypti.1 Dengue virus infection generally causes mild symptoms such as fever, headache, myalgia, arthralgia, nausea, and vomiting, which is called dengue fever (DF). In some cases, however, infection with dengue virus may progress to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF is a severe febrile illness characterized by abnormalities in homeostasis and increased capillary leakage that can progress to DSS.2 Increased vascular permeability is presumed to play a key role in the pathogenesis of DHF/DSS, since it results in the loss of plasma from the vascular compartment, which may give rise to shock in severe cases. However, the mechanism involved in the pathogenesis of dengue viral infection, especially the manifestation of DHF/DSS, remains unclear.

Hyaluronan (hyaluronic acid, HA) is a high molecular weight polysaccharide (10^6 to 10^7 Da) with a ubiquitous distribution in the connective tissues, which is responsible for maintaining extracellular matrix characteristics.3 The molecule of HA is a linear polymer consisting of alternating units of glucuronic acid and N-acetylglucosamine, and can be synthesized by most cell populations.4 HA plays an essential role in the control of tissue hydration through its contribution to osmotic pressure.5 Some portion of the tissue HA can be washed out into the lymph nodes, where a main portion of HA is degraded under normal physiological conditions. The remaining HA enters the circulation through the lymphatic ducts and can be rapidly cleared from the blood by liver sinusoidal endothelial cells via a receptor-mediated endocytosis mechanism.5,6 It has been reported that the maximum binding capacity is approximately 10^9 molecules of HA per liver endothelial cells.6 The HA receptor, which belongs to the polymorphic family of CD44 glycoproteins, mediates the HA uptake into the cell where it can be degraded by acid hydrolase in lysosomes.7,8 Although some of the macromolecules, such as various proteins, can be taken by other cell populations, circulating HA is exclusively cleared by the hepatic sinusoidal endothelial cells.9 Therefore, assessment of the circulating HA levels can be used as a measure of liver endothelial cell function.

It has been demonstrated that circulating HA levels increased significantly during hypodynamic sepsis and hemorrhagic shock.10,11 However, the role of HA toward hemorrhage and hypovolemic shock in dengue infection has never been documented. Therefore, the objective of this study was to investigate the serum levels of HA and to determine their importance in patients with dengue infection. Serum HA levels were also measured on patients with confirmed hepatitis A virus (HAV) infection, one of the most common diseases with fever and acute liver dysfunction, as a viral infectious disease control.

Materials and methods

The protocol of this study was approved by the Ethics Committee on Human Research of the Faculty of Medicine, Chulalongkorn University. All parents or legal guardians of children in this study were informed of the study’s objectives, and written informed consent was obtained from them before the children entered the study.

Patients

Forty Thai children (23 boys and 17 girls) with dengue infection during the period of May 2004 to December 2005 were recruited from Department of Pediatrics of King Chulalongkorn Memorial Hospital. Among them, 16 patients were classified as DF, 14 patients were classified as DHF, and 10 patients were classified as DSS according to the WHO criteria. The study also included 48 Thai children (34 boys and 14 girls) with acute HAV infection during an outbreak occurred in a child care center (n = 37) located in a suburban area of Bangkok between November 2002 and February 200312 and an outbreak among school children at Songkla province, the Southern part of Thailand (n = 11) in early 2005. Eight healthy age-matched controls were obtained from healthy children who were on their routine vaccination visit. The mean age ± SD of patients with DF, DHF, DSS, HAV, and healthy controls were 10.2 ± 3.1, 8.9 ± 1.8, 11.2 ± 2.5, 4.3 ± 2.9, and 9.7 ± 2.6 years, respectively. All dengue viral patients showed typical clinical symptoms and signs of dengue infection and were serological positive for anti-dengue IgG and IgM ELISA (NovaTec Immundiagnostica GmbH, Technologie and Waldpark, Dietzenbach, Germany). Forty-eight stored sera of patients from an outbreak of acute hepatitis A infection were tested using commercially available ELISA kits (Abbott Laboratories, North Chicago, IL) for the presence of anti-HAV IgG and anti-HAV IgM.12

Paired blood samples were collected from each dengue infection patient in this study: one during the acute phase and the other during the convalescent phase. The acute-phase blood samples were obtained at the admission to the hospital (day 3 to day 7 after the onset of fever). Convalescent-phase blood samples were obtained during the convalescent phase of the disease (day 14 to day 21 after the onset of fever). The sera were separated and stored at −70°C until further study.

Hyaluronan measurement

Serum HA level was measured using a competitive inhibition based-ELISA as previously described with modifications.13 Briefly, serum samples (175 μl) containing unknown amounts of HA, as well as a standard containing known concentrations of a highly purified HA preparation (Healon in 6% bovine serum albumin in PBS) were placed in small polypylene tubes with 175 μl of appropriate concentrations of biotinylated-HA binding proteins (B-HABP) and incubated at room temperature (approximately 25°C) for 1 h. Aliquots (100 μl per well, triplicate) of this reaction mixture were applied to umbilical cord HA coated and BSA blocked
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microrotter plates and incubated at 25 °C for 1 h. The wells were then washed with phosphate-buffered saline solution (0.05% Tween-20) and the appropriate dilution of anti-biotin peroxidase conjugate (Zymed Lab Inc., San Francisco, CA, USA) was added to each well, incubated at 25 °C for 1 h and washed, after which peroxidase substrate was added. After incubation at 25 °C for 20 min, the reaction was stopped by the addition of 50 μl 4 M H2SO4. The absorbance ratio at 492/690 nm was measured using a Titertek Multiskan M340 microplate reader. The level of HA in the serum samples was determined by their ability to inhibit color development in the assay relative to a standard curve generated from the purified HA preparation.

Statistical analysis

Statistical analyses were performed using the SPSS version 11.5 software package (SPSS Inc., Chicago, IL, USA). Data are expressed as means ± standard deviation (SD). Comparisons between groups were analyzed by one-way ANOVA and unpaired t-test. The tests were considered statistically significant at P < 0.05.

Results

Clinical characteristics and laboratory findings are shown in Table 1. The mean age of children with DSS was higher than that of children with nonshock DHF and DF, but the difference was not statistically significant. The male-to-female ratio was 12:4 in DF, 6:8 in DHF, and 5:5 in DSS. The peak hematocrit (Hct) for patients with DSS was significantly higher than that for patients with nonshock DHF and DF (43.9 ± 4.8%, 41.5 ± 3.9%, and 39.3 ± 2.6%; P = 0.02). The lowest platelet count for children with DSS was significantly lower than that for children with nonshock DHF and DF (77,900 ± 41,270, 78,428 ± 15,786, and 146,000 ± 21,726 cells/mm³; P < 0.001).

Children with DSS had lower albumin levels than those with DHF and DF (3.5 ± 0.9, 3.9 ± 0.4, and 4.1 ± 0.3 g/dl; P = 0.02). However, the patients with DSS had significantly higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) than those of patients with DHF and DF (226.3 ± 218.5, 80.4 ± 105.3, and 34.9 ± 16.8 U/l, P = 0.002; and 138.6 ± 100.5, 73.6 ± 89.9, 28.0 ± 22.6 U/l, P = 0.003, respectively). As shown in Fig. 1, Dengue patients had significantly greater serum HA levels in acute-phase samples than did acute hepatitis A patients and healthy controls (P < 0.0001). Serum HA levels in convalescent-phase samples from children with dengue infection decreased, compared with levels in acute-phase samples, but were still significantly higher than those in healthy controls (378.6 ± 657.1, 1186.8 ± 1634.8, 915.2 ± 1294.8, and 48.3 ± 16.3 ng/ml; P < 0.0005).

The serum levels of HA in the acute-phase samples from children with DSS were significantly higher than those in samples from children with DF (7316.3 ± 10,359.0 vs. 635.4 ± 1569.9 ng/ml; P = 0.04). The serum HA levels in children with DHF/DSS were found to have rapidly decreased during the convalescent phase. The concentration of HA in convalescent-phase samples from the patients with DSS was somewhat higher than that for the patients with DF, but was not significantly different (915.2 ± 1294.8 vs. 378.6 ± 657.1 ng/ml; P = 0.37).

Discussion

Hyaluronan (hyaluronic acid, HA) is a straight-chain glycosaminoglycan polymer present in the interstitial spaces. HA appears in various forms that are freely distributed in the blood and lymphatic circulation, loosely associate with the extracellular matrix, tightly aggregate proteoglycan, or bind to cell surfaces by specific receptors.14 More than 90% of released HA is specifically metabolized by endothelial cells of hepatic sinusoids and, therefore, damage of hepatic endothelial cells results in increased serum HA levels.15 Elevation in circulating HA has been demonstrated in response to stress, such as massive trauma, major surgical procedures, burns, blood loss, shock, and septicemia.16–18 Furthermore, serum HA levels rise in a number of liver diseases,19 and accumulation of HA in the serum is considered to be mostly due to failure of sinusoidal endothelial cells to degrade HA.20–22 As a result, serum HA levels reflect the functional alterations that occur in sinusoidal endothelial cells in association with various liver diseases. In addition to the sinusoidal endothelial cells integrity, the HA hepatic clearance depends on the sinusoidal level of capillarization and hemodynamic

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics, laboratory findings, and serum HA levels</th>
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<tr>
<td>Parameters</td>
<td>DF</td>
</tr>
<tr>
<td>No.</td>
<td>16</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>12/4</td>
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<tr>
<td>Age (years)</td>
<td>10.2 ± 3.1</td>
</tr>
<tr>
<td>Peak hematocrit (%)</td>
<td>39.3 ± 2.6</td>
</tr>
<tr>
<td>Platelets (cells/mm³)</td>
<td>146000 ± 21726</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>34.9 ± 16.8</td>
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<tr>
<td>ALT (U/l)</td>
<td>28.0 ± 22.6</td>
</tr>
<tr>
<td>Acute-phase HA (ng/ml)</td>
<td>635.4 ± 1569.9</td>
</tr>
<tr>
<td>Convalescent-phase HA (ng/ml)</td>
<td>378.6 ± 657.1</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. NS, not significant; HA, hyaluronan; AST, aspartate aminotransferase; ALT, alanine aminotransferase. a P values are for comparison between DF, DHF, and DSS.
damage. and with acute hepatitis, depending on the severity of liver dengue virus infection with elevation of aminotransferases and disseminated intravascular coagulation. Production resulting in vasculopathy, thrombopathy, shock, complex formation, compliment activation, and chemokine also, infection by dengue virus causes apoptosis, immune therefore disrupt the regulation of the vascular tone. Damage to endothelial cells in dengue virus antigen can be detected in Kupffer cells and sinusoidal endothelial cells in the liver. Endothelial cell damage is caused by either direct virus cytopathy or immune-activated pathology. Damage to endothelial cells in dengue virus infection possibly reduce the effectiveness of the endothelial permeability, induce plasma leakage, and therefore disrupt the regulation of the vascular tone. Also, infection by dengue virus causes apoptosis, immune complex formation, compliment activation, and chemokine production resulting in vasculopathy, thrombopathy, shock, and disseminated intravascular coagulation.

A recent study has reported that dengue virus infection induced liver injury was a leading cause of acute hepatic failure in Thai children. Liver involvement is common in dengue virus infection with elevation of aminotransferases and with acute hepatitis, depending on the severity of liver damage. Accordingly, we revealed that the elevated levels of AST were higher than those of ALT in patients with dengue infection. This finding may be attributed to the release of AST during myocyte damage in dengue infection. Moreover, we showed that serum HA levels were significantly greater in dengue patients compared with those in healthy controls. We also observed serum HA levels in acute HAV-infected patients that were much lower than those found in dengue virus infected patients. This supports the hypothesis that dengue virus may infect and damage liver endothelial cells while HAV favorably induce hepatocyte damage by a direct cytopathic effect. Therefore, the increased serum HA levels in patients with dengue infection reflect impairment of HA degradation in liver sinusoidal endothelial cells.

The present study is the first to demonstrate that serum HA levels associate with disease severity in dengue infection. However, due to the small population of dengue patients in the current study, further studies with larger populations of dengue patients that include adults are necessary to verify that this finding is true for dengue patients. The mechanism that results in elevation of serum HA levels during dengue infection is still unclear, but it is possible that both virus-induced HA overproduction and/or reduced HA degradation may be responsible for the increase in its serum levels. An alternative explanation might be that elevated HA, and its associated water of hydration, is the body’s defense mechanism against acute stress injury. It may be the body’s own intravascular volume expander, to delay death from hypovolemic shock. The elevated circulating HA, while being a manifestation of viral infection may contribute to the body's defenses for maintaining intravascular volume. Our data suggest that the increment of HA production may be involved in the pathogenesis of DHF/DSS. Additional studies are needed to determine whether the increase in the production of HA, decrease in its clearance, or both is responsible for the elevated serum HA levels during dengue infection.

In conclusion, we have demonstrated in the present study that serum HA levels were elevated and associated with the severity of the disease in dengue patients. The serum levels of HA may reflect the degree of hepatic sinusoidal endothelial cell damage in patients with dengue infection. Further studies should address the relationships with HA levels among patients with DHF/DSS and dengue fever, effects of treatment, and morbidity outcomes.

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