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Original Contribution

Oxidative damage in dengue fever

Raymond C.S. Seet [a,b,*], Chung-Yung J. Lee [c], Erle C.H. Lim [a,b], Amy M.L. Quek [b], Leonard L.L. Yeo [b], Shan-Hong Huang [c], Barry Halliwell [c]

[a] Department of Medicine, Yong Loo Lin School of Medicine, National University Singapore 119074
[b] Department of Medicine, National University Hospital, Singapore 119074
[c] Department of Biochemistry, Yong Loo Lin School of Medicine, National University Singapore 119074

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ABSTRACT

Oxidative stress may be important in the pathogenesis of dengue infection. Using accurate markers of oxidative damage, we assessed the extent of oxidative damage in dengue patients. The levels of hydroxyeicosatetraenoic acid products (HETEs), F_2-isoprostanes (F_2-IsoPs), and cholesterol oxidation products (COPs) were measured in 28 adult dengue patients and 28 age-matched study controls during the febrile, defervescent, and convalescent stages of infection. We compared the absolute and the percentage change in these markers in relation to key clinical parameters and inflammatory markers. The levels of total HETEs and total HETEs/arachidonate, total F_2-IsoPs/arachidonate, and COPs/cholesterol were higher during the febrile compared to the convalescent level. Total HETEs correlated positively with admission systolic blood pressure ($r=0.52$, $p<0.05$), whereas an inverse relationship was found between 7β-hydroxycholesterol and systolic and diastolic blood pressure ($r=-0.61$ and -0.59, respectively, $p<0.01$). The urinary F_2-IsoP level was higher in urine during the febrile stage compared to the convalescent level. Despite lower total cholesterol levels during the febrile stage compared to convalescent levels, a higher percentage of cholesterol was found as COPs (7β-, 24-, and 27-hydroxycholesterol). The levels of platelet-activating factor–acetylhydrolase activity, vascular cellular adhesion molecule-1, tumor necrosis factor-α, and high-sensitivity C-reactive protein were higher during the febrile stage compared to their convalescent levels ($p<0.01$). Markers of oxidative damage are altered during the various stages of dengue infection.

Dengue, an arthropod-borne flavivirus, affects 2.5 million people in more than 100 countries worldwide [1–4]. After infection via the *Aedes aegypti* mosquito in humans, a high fever develops in the first 2–7 days (febrile stage), during which high viral replication takes place [1]. Thereafter, the fever ceases (defervescent stage) and within 2 days, the viral load falls to undetectable levels and clinical recovery to health begins (convalescent stage) [1]. Reports from cell culture studies [5,6] and observational studies in humans [7–11] suggest that oxidative stress may be important in the pathogenesis of dengue infection. Cell culture studies indicate that after inoculation of the dengue virus onto the cells, the extent of cytokine mRNA expression is modulated by *N*-acetyl-l-cysteine, nitro-l-arginine methyl ester, and pyrrolidine dithiocarbamate [5]. In vivo studies suggest that the release of reactive oxygen species during dengue infection is determined by the levels of tumor necrosis factor-α (TNF-α) and soluble cellular adhesion molecules (such as vascular cell adhesion molecule-1, or VCAM-1) [12,13] in the endothelium.

In humans, oxidative stress markers such as malondialdehyde, thiobarbituric acid-reactive substances, and lipid hydroperoxides have been used to suggest the involvement of oxidative stress in dengue infection [7,9]. Nevertheless, these markers can lack specificity and precision to detect oxidative stress in vivo [14,15]. In contrast, F_2-isoprostanes (F_2-IsoPs) are more reliable markers that reflect the extent of oxidative stress-induced lipid peroxidation [16,17]. F_2-IsoPs are products of the free radical-induced peroxidation of arachidonic acid and are present in an esterified form in phospholipids [17]. Their release in a free form is catalyzed by the activities of phospholipase A_2 (PLA_2) and platelet-activating factor–acetylhydrolase (PAF-AH) [18,19]. Arachidonic acid can also be oxidized both enzymatically and nonenzymatically to generate hydroxyeicosatetraenoic acid products (HETEs) [20–22]. Several isomers of HETEs have been identified (such as 5-, 8-, 9-, 11-, 12-, 15-, and 20-HETEs) and some are known to have vasoactive effects [20,21]. For example, 20-HETE is a potent vasoconstrictor in the cerebral circulation and 9-HETEs are elevated in the presence of coronary artery disease [20,21]. Cholesterol oxidation products (COPs) are a group of oxysterols produced from cholesterol oxidation through enzymatic cytochrome P450 (to give 24- and 27-hydroxycholesterol) and non-cytochrome P450 pathways (to give 7β-hydroxycholesterol) [23,24]. Studies that showed attenuation of 7β-hydroxycholesterol levels after long-term vitamin E supplementation [25] and higher plasma oxysterol levels in smokers [26] and after paraquat ingestion [27] suggest a potential role for COPs as markers of oxidative stress.

* Corresponding author. Fax: +65 67794112.
E-mail address: raymond_seet@nus.edu.sg (R.C.S. Seet).

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In this study, we investigated oxidative stress during dengue infection. We measured plasma HETE, F_2-IsoP, and COP levels in a cohort of adult dengue patients and aimed to determine the temporal change in these levels in relation to key clinical parameters of dengue infection.

Methods

Study population

Consecutive patients with acute dengue infection, and community-based age-matched controls, were recruited after patient consent and approval from the ethics committee of the National University Hospital, Singapore, were obtained. Clinical information (including age, gender, stage of illness, blood pressure) was obtained using standardized questionnaires [2,3] and samples were collected during the febrile (high fever), defervescent (fever cessation), and convalescent (recovery) stages of infection. Systolic and diastolic blood pressure was measured using an automated sphygmomanometer (Model SEM-1; Omron) and the average of two readings was recorded. Full blood count, renal and liver function tests, and additional tests such as lipid profile (total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein) and high-sensitivity C-reactive protein (hsCRP) were measured at the National Referral Laboratory (NRL), Singapore. Commercial assay kits were used to determine plasma PLA_2 and PAF-AH activities (Cayman Chemical, Ann Arbor, MI, USA) and VCAM-1 and TNF-α concentrations in serum (R&D Systems, UK).

Dengue severity was defined using the World Health Organization criteria [28]. Patients with acute dengue infection were identified by RT-PCR-based dengue gene identification or dengue-specific IgM capture ELISA and hemagglutination inhibition test [29]. Secondary dengue infection was considered in patients with dengue-specific IgM/IgG ratio < 1.8 or a greater than fourfold rise in hemagglutination inhibition antibody titer in paired acute and convalescent sera. Using a conservative standard deviation of 15, we calculated that 25 subjects in each group would allow us to detect a mean difference of 10% in oxidative stress markers during the febrile and convalescent stages of infection (90% power with a two-tailed 5% statistical significance).

Measurement of lipid oxidation products

Blood samples were collected into EDTA tubes from fasting patients. The blood was centrifuged and indomethacin and butylated hydroxytoluene were added into the plasma samples before they were stored at −80°C until analysis. Oxidized lipid products (which include F_2-IsoPs, HETEs, and COPs) were measured using methods described by Lee et al. [30]. Briefly, mixed heavy isotopes, 8-iso-$PGF_{2\alpha}$-d_4, $IPF_{2\alpha}$-VI-d_4, 5(*S*)-HETE-d_8, 12(*S*)-HETE-d_8, 15(*S*)-HETE-d_8, 20-HETE-d_6, and arachidonic acid-d_8 and 7β-OH cholesterol-d_7, 24-OH cholesterol-d_7, and 26(27)-OH cholesterol-d_5 were added into plasma for quantification of the oxidative stress markers. To measure the free and esterified forms of oxidized lipid products, 1 M potassium hydroxide prepared in methanol was added to plasma (1:1) and hydrolyzed at 37°C for 30 min. Thereafter, methanol, 5 M HCl, and 40 mM formic acid (pH 4.6) were sequentially added and mixed. The plasma mix was then purified using anionic exchange solid-phase extraction (SPE) [30]. To measure the free forms in plasma and urine, 40 mM formic acid (pH 4.5) was added to the plasma or urine sample, mixed, and then purified by SPE [30]. Urinary creatinine levels were measured to standardize urinary F_2-IsoP levels (Sigma Diagnostic Kit, St. Louis, MO, USA) and total cholesterol (NRL) to standardize COP levels.

The purified samples were derivatized [30]. Analyses of the derivatized samples were carried out using gas chromatography (Hewlett–Packard 6890; Agilent Technologies, Santa Clara, CA, USA) coupled to a mass selective detector (Hewlett–Packard 5973N; Agilent Technologies). For F_2-IsoP, HETE, and total arachidonate measurements, the negative chemical ionization mode was applied in selective ion monitoring and chromatographic separations were carried out on a fused silica capillary column coated with cross-linked 5% phenylmethylsiloxane (HP-5; Agilent Technologies). To determine COPs, the electron ionization mode was applied in selective ion monitoring and chromatographic separations were carried out on a fused silica capillary column coated with cross-linked 5% phenylmethylsiloxane (Ultra 2; Agilent Technologies, J&W, USA). Concentrations of the oxidized lipid products were calculated by comparing the peak area of each compound with deuterated internal heavy standard.

Statistical analysis

All statistical analyses were performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA) and statistical significance was set at $p<0.05$. Data are presented as means ± standard deviation for continuous measures and counts and percentages for categorical variables. Differences in data were compared using analysis of variance and Wilcoxon rank-sum tests for continuous measures and χ^2 and Fisher's exact tests for categorical variables. Repeated-measures analysis was used to assess the trend of each variable and logistic regression methods were used to obtain odds ratios with 95% confidence intervals. The differences in lipid oxidized products were considered as percentage change from their convalescent levels and these were compared with the demographics information, admission parameters, and measured variables of the study participants.

Results

Clinical characteristics

Twenty-eight patients (mean age 43, range 23–68 years; 14 males and 14 females) with confirmed dengue infection and 28 age-matched controls were included in this study. Mean (standard deviation) systolic and diastolic blood pressure of patients on admission was 120 (16) and 76 (15) mm Hg, respectively. A majority of the laboratory parameters on admission were comparable to those in the controls, except for white cell and platelet counts, aspartate transaminases, lactate dehydrogenase, and activated thromboplastin time (Table 1). There were no differences in the male/female ratio or in the proportions of those with hypertension, diabetes mellitus, atrial fibrillation, hyperlipidemia, previous stroke, or current smoking between dengue patients and study controls ($p>0.50$). The mean duration of symptoms for patients during the febrile stage was 3 ± 1

Table 1
Laboratory blood parameters of patients on admission and in study controls

	Patients ($n=28$)	Controls ($n=28$)
Hematologic parameters		
Hemoglobin (g/dl)	15.1 (1.9)	14.5 (1.3)
Hematocrit (%)	43.9 (5.4)	42.3 (3.9)
White cell count ($\times 10^9$/L)*	3.7 (2.0)	5.6 (1.3)
Platelet count ($\times 10^9$/L)*	59 (39)	280 (33)
Biochemical parameters		
Urea (mmol/L)	4.1 (1.9)	4.2 (1.2)
Creatinine (μmol/L)	76 (24)	83 (20)
Conjugated bilirubin (μmol/L)	5.5 (7.8)	2.3 (0.5)
Unconjugated bilirubin (μmol/L)	6.6 (5.5)	10 (2.3)
Aspartate transaminases (U/L)*	106 (66)	30 (8)
Alkaline transaminases (U/L)	81 (61)	45 (12)
Alkaline phosphatase (U/L)	66 (34)	80 (12)
Lactate dehydrogenase (U/L)*	855 (249)	300 (95)
Albumin (g/dl)	36 (4)	44 (8)
Prothrombin time (s)	13.6 (0.9)	13.8 (0.5)
Activated thromboplastin time (s)*	40.5 (5.5)	31.2 (3.4)

Values are expressed as mean (SD) or median (IQR).
* Significant difference, $p<0.05$.