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Role of vascular cell adhesion molecules and leukocyte apoptosis in the lymphopenia and thrombocytopenia of patients with severe acute respiratory syndrome (SARS)

Rong-Fu Chen a, Jen-Chieh Chang a, Wen-Tien Yeh a, Chen-Hsiang Lee b, Jien-Wei Liu b, Hock-Liew Eng c, Kuender D. Yang a,∗

a Department of Medical Research (12F12L), Chang Gung Memorial Hospital at Kaohsiung, 123 Ta-Pei Road, Niau-Sung, Kaohsiung 833, Taiwan, ROC
b Division of Infectious Diseases, Chang Gung Memorial Hospital at Kaohsiung, Kaohsiung 833, Taiwan, ROC
c Division of Clinical Pathology, Chang Gung Memorial Hospital at Kaohsiung, Kaohsiung 833, Taiwan, ROC

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Abstract

The immunopathogenesis of leukopenia and thrombocytopenia in patients with severe acute respiratory syndrome (SARS) is unclear. In order to explore the leukopenia mechanism, we studied 15 SARS patients who were previously healthy, and 15 age-matched normal controls in a paired design. Soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble Fas ligand (sFasL) in plasma were measured by ELISA, and intracellular activated caspase-3 fragment in different leukocytes was determined by flow cytometry. Patients with SARS had significantly lower lymphocyte and platelet counts and significantly higher sVCAM-1 and sFasL levels compared to healthy controls. sVCAM-1 levels correlated negatively with total leukocytes and platelet counts, but positively with plasma sFasL levels. Intracellular cleaved caspase-3 expression was also significantly higher in lymphocytes from SARS patients in acute phase than in convalescent stage. Lymphopenia and thrombocytopenia in SARS patients may be caused, in part, by enhanced vascular sequestration associated with increased sVCAM-1 levels. However, lymphopenia may be due to enhanced cell death. Inhibition of cell adhesion and caspase-3 activation could, therefore, have prevented SARS patients from developing thrombocytopenia and lymphopenia.

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1. Introduction

The severe acute respiratory syndrome (SARS) caused by a human coronavirus originating in Guangdong Province of China spread to more than 30 countries in 2003 [1]. The immunopathogenesis of SARS is not clear, while the transmission route of SARS has been clarified. The treatment of SARS patients with anti-virus therapy or with anti-inflammatory corticosteroids remains highly controversial [2–6]. Whether there is a direct viral pneumonitis (virus cytopathic tissue damage) or indirect immune-mediated tissue damage in SARS infections remains obscure. Patients with SARS tended to have prolonged fever, rapid course of pulmonary infiltration, leukopenia, thrombocytopenia and lymphopenia, frequently complicated with co-infections [3,4]. A histology of lung necropsy showed abundant foamy macrophages and multinucleated syncytial cells [4,7]. This suggests that leukocyte sequestration in interstitial tissue or apoptosis of circulating leukocytes is implicated in the pathogenesis.

Earlier studies have shown that SARS patients with elevated polymorphonuclear leukocytes (PMNs), but not lower lymphocytes, had worse outcome [4]. It is uncertain how the SARS patients had lower lymphocytes and platelets but normal or higher PMNs. We have recently shown that SARS patients appear to have altered mitogen-activated protein kinase activation patterns in different leukocytes, associated with increment of immunosuppressive mediators [8]. This, however, does not answer why lymphopenia occurs in SARS patients. O’Donnell et al. [9] have proposed that apo-
ptosis may explain lymphopenia of SARS. Leukocyte apoptosis plays a critical role in various immune reactions and infectious diseases [10,11]. One of the major apoptotic pathways is the Fas ligand (FasL)/Fas pathway [12,13], in which caspase-3 is a key molecule in executing Fas-mediated apoptosis [14–16]. Soluble vascular cell adhesion molecule-1 (sVCAM-1) has been implicated in many vasculitis disorders [17,18] and leukocyte adhesion and migration [19]. Patients with SARS had lymphopenia [9] and extensive lung tissue damage [20]. Thus, we proposed that plasma sVCAM-1 levels might be correlated to vascular sequestration, resulting in lymphopenia or tissue damage in SARS infections. An alternative hypothesis is that lymphopenia could be related to an increase in plasma FasL and intracellular caspase-3 cascade. In an age-matched case–control design, we measured plasma sVCAM-1 and sFasL, and intracellular cleaved caspase-3 expression of different leukocytes in 15 SARS patients. We sought to explore the relationship of lymphopenia, thrombocytopenia and clinical manifestations to plasma sFasL and sVCAM-1 levels, as well as intracellular cleaved caspase-3 levels in SARS patients.

2. Materials and methods

2.1. Hospital outbreak of SARS and the design of this study

On April 26, 2003, an index case of SARS in a patient from Taipei who initially presented with flank pain and fever was admitted to our hospital at Kaohsiung, southern Taiwan. This patient was not suspected of SARS until April 30. From May 2 to 17, 2003, a cluster of SARS patients including in patients, family caregivers, and health care workers developed probable SARS. We recruited those who were previously healthy for this study in a case–control design. Once we had recruited one to three SARS patients for immune studies, we simultaneously included one to three age-matched healthy adults that had not been exposed to SARS patients as controls. Confirmation of SARS infections was determined by a definite exposure history and positive RT-PCR detection of coronavirus in acute stage or detectable coronavirus specific antibody in convalescent stage [8].

2.2. Preparation of plasma and leukocytes under safety procedures

Studies proceeded with a safety protocol. The blood collection and safety procedure for this study were approved by the Institute Review Board of this hospital. The progression of SARS can be summarized in three stages: (i) incubation period, (ii) febrile pneumonitis stage, and (iii) recovered stage or fulminant hypoxemia associated with adult respiratory distress syndrome (ARDS). We therefore collected whole blood (4 ml) into an ethylenedinitrilo-tetraacetic acid (EDTA)-containing blood collection tube in the first week of admission (acute phase) as well as in the third week of admission (convalescent phase). The blood samples were separated into plasma and blood cells by centrifugation at 1500 \( \times g \) for 15 min. The upper plasma layer was harvested for cytokine determination after heat inactivation (56 °C, 30 min) of potential viruses, and the bottom blood cells were separated into red blood cells and white blood cells (WBCs) by dextran sedimentation [21]. The WBCs were then drawn into a 2% formaldehyde-containing Eppendorf tube at 1/1 (v/v) for inactivation of any possibly viable organisms and fixation of leukocytes. During the whole procedure, we did not open the original blood collection tube, operated the procedure in a P2 laboratory, and had the blood collection tubes autoclaved before undertaking standard bio-hazard waste management.

2.3. Measurement of plasma sVCAM-1 and soluble FasL

The levels of sVCAM-1 were measured by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN). Soluble FasL levels were also measured by ELISA kit (Bender Medsystems, Vienna, Austria) in plasma. Plasma aliquots at 0.1 ml were used for each individual ELISA assay as previously described [22].

2.4. Flow cytometric analysis of intracellular cleaved caspase-3 in different leukocytes

Flow cytometric detection of intracellular cleaved caspase-3 levels was performed as previously described [23]. For experiments, 1% formaldehyde-fixed leukocytes were subjected to cell permeabilization by methanol at 1/4 (v/v) for 30 min after washing twice in PBS. This method has been shown to be more rapid and sensitive than other classical methods in human T cells [24]. The formaldehyde-fixed leukocytes were suspended to 1 \( \times 10^6 \) cells per ml for studies. The permeabilized leukocytes in 0.1 ml aliquots were subjected to dual staining of cell surface molecules by PE-conjugated mouse anti-human CD4, CD8 or CD14 antibodies (BD Pharmingen, Inc., Franklin Lakes, CA); and staining of intracellular signal molecules by rabbit anti-human cleaved caspase-3 antibodies (Cell Signaling Technology, Inc., Beverly, MA) for 30 min. This was followed by recognition with FITC-conjugated goat anti-rabbit IgG antibodies (Serotec, Ltd., Oxford, UK) for another 30-min staining. After washing twice with PBS, the reactions were suspended in 0.3 ml PBS for flow cytometric analysis of different cleaved caspase-3 in different leukocyte populations.

2.5. Statistical analysis

Data related to quantity of sVCAM-1 and sFasL are presented with mean ± standard error, and \( P \) values are analyzed by Student’s \( t \)-test. Data regarding the geometric mean intensities and percentage of positive staining cells of intracellular cleaved caspase-3 levels between the patient and control groups were analyzed using the Mann–Whitney \( U \)-test. Spear-
man rho correlation was used to test the correlation of lymphopenia or thrombocytopenia with sVCAM-1, sFasL and intracellular cleaved caspase-3 levels.

3. Results

3.1. Clinical features of the subjects studied

Fifteen patients aged 23–45, and 15 normal controls aged 29–41 were studied. The 15 SARS patients, who were previously healthy, all had fever (> 38 °C) and different extent of pneumonia on chest radiography. As shown in Table 1, they initially presented profound lymphopenia (range, 0.3–1.3; mean 0.6 × 10^9 per l, P < 0.001) and thrombopenia (range, 96–228; mean 145.5 × 10^9 per l, P < 0.001). The total WBCs (range, 3.1–14.8; mean 4.4 × 10^9 per l) and neutrophil count (range, 2.3–14.1; mean 4.5 × 10^9 per l) were within normal limits. Most of the patients (55.6%, P < 0.001) had prolonged activated partial thromboplastin time (APTT; defined by the clotting time longer than 130% of normal control); but normal PT (prothrombin time), suggesting vascular insult but not hepatic insufficiency. Normal or elevated lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) were also noted. These patients were treated under a protocol with initial institution of ribavirin 400 mg/m^2 per day in the first 7 days after a loading dose of 2 g. These patients did not receive steroid (methylprednisolone, 1 mg/kg per day with and without pulse therapy 500 mg every 12 h for 2 days) until progression to pneumonitis or ARDS that usually occurred between 7 and 21 days of the disease.

3.2. Elevated levels of sVCAM-1 in the plasma of patients with SARS

It was found that plasma sVCAM-1 levels were significantly higher in SARS patients than in normal controls in the first week of illness (1240.04 ± 160.67 vs. 547.54 ± 52.90 ng/ml; P = 0.003, Fig. 1A). The elevated sVCAM-1 levels returned to normal in the third week (Fig. 1A). Higher sVCAM-1 levels were significantly correlated to lower total leukocytes and platelets (P = 0.047 and 0.031, respectively; Fig. 1B, C), but not lymphocytes (P = 0.417).

3.3. Elevated sFasL levels in the plasma of patients with SARS

To investigate the role of apoptosis in the lymphopenia of patients with SARS, we measured the plasma sFasL in patients with SARS and those in normal controls. Plasma sFasL levels were significantly higher in SARS patients than normal controls in early stage (0.24 ± 0.04 vs. 0.11 ± 0.02 ng/ml; P = 0.039, Fig. 2A). The plasma sFasL levels were negatively correlated to patients’ lymphocytes tested by Spearman’s rho correlation (P = 0.047). (C). Plasma sVCAM-1 levels were negatively correlated to patients’ platelet counts (P = 0.031).

3.4. Altered cleaved caspase-3 activation in different leukocytes

Caspases are known to play a major role in the execution of apoptosis in mammalian cells [25]. Using formaldehyde-
fixed peripheral blood leukocytes, we measured the intracellular cleaved caspase-3 expression in total leukocytes from patients with SARS and age-matched normal controls. As shown in a representative experiment (Fig. 3A), it was found that SARS patients had an increase in leukocytes with positive intracellular cleaved caspase-3 staining. In a summary calculated from the results of 15-paired experiments, leukocytes with positive intracellular cleaved caspase-3 staining were significantly higher in SARS patients than in controls (Fig. 3B). In order to identify which subtypes of leukocytes had higher cleaved caspase-3 expression in SARS patients, we used a dual staining of anti-cleaved caspase-3 antibody plus anti-CD14, anti-CD4, or anti-CD8 antibody for this purpose. It was found that SARS patients tended to have higher cleaved caspase-3 expression in CD4 and CD8 cells than healthy controls (Fig. 4), although it did not reach a significant difference. The cleaved caspase-3 expression in CD4 and CD8, but not CD14, cells from SARS patients was significantly higher in acute phase than in convalescent phase (Fig. 4).

4. Discussion

Patients with SARS usually have a rapid progression of pneumonitis, even develop ARDS and pulmonary fibrosis in the second and third weeks of disease [3–5,26]. Histological examinations of lung necropsy from SARS patients have demonstrated infiltration of inflammatory cells associated with foamy macrophages, multinuclear syncytial cells and occa-

Fig. 2. Plasma sFasL levels in patients with SARS and age-matched controls (ctrl). (A). Plasma sFasL levels were significantly higher in SARS patients than in age-matched controls in the early stage (mean ± S.E.: 0.24 ± 0.04 vs. 0.11 ± 0.02 ng/ml; P = 0.039). The sFasL levels were significantly elevated in the late stage (second and third weeks) compared with the early stage (mean ± S.E.: 0.46 ± 0.09 ng/ml, P = 0.015). (B). Plasma sFasL levels in patients with SARS were correlated to sVCAM-1 levels (P = 0.025).

Fig. 3. Flow cytometric analysis of cleaved caspase-3 expression in peripheral blood leukocytes from SARS patients and controls (ctrl). (A). Expression of intracellular cleaved caspase-3 in total leukocytes from SARS patients was higher than controls in a representative case–control experiment. (B). In a summary calculated from the results of 15-paired experiments, leukocytes with positive intracellular cleaved caspase-3 staining were significantly higher in the SARS patients than in normal controls (% of positive cells: 66.65 ± 4.19 vs. 27.78 ± 7.54; P = 0.046).

Fig. 4. Flow cytometric dual staining of cleaved caspase-3 expression in CD14, CD4, and CD8 cells from SARS patients and controls (ctrl). (A). In a summary calculated from the results from 15-paired experiments, CD14 cells with positive intracellular cleaved caspase-3 staining were not significantly different between SARS patients and controls, or between early (acute) and late (convalescent) phases. (B). CD4 cells with positive intracellular cleaved caspase-3 staining were higher in early phase of SARS than those in late phase (P = 0.034). (C). CD8 cells with positive intracellular cleaved caspase-3 staining were also marginally higher in the early phase of SARS than those in the late phase (P = 0.050). Results were analyzed by Mann–Whitney U-test.
sional hemophagocytic phagocytes [4,7]. This has raised the possibility of immunopathological damage of lung tissues due to exaggerated host response but not uncontrolled virus replication in the lung [3]. As previously reported, apoptosis of lung epithelial cells by Fas ligation may be involved in lung injury and fibrosis [27,28]. We did find the plasma sFasL levels were significantly higher in SARS patients. Whether the higher plasma sFasL levels were related to apoptosis of circulating leukocytes or lung tissue damage remains to be determined. The fact that sFasL levels were correlated to sVCAM-1 levels but not total leukocytes or platelet counts suggest the elevated sFasL in plasma may be not related to lymphopenia but tissue damage.

Soluble VCAM-1 is generated at sites of vascular insult or inflammation [29,30]. Elevation of sVCAM-1 levels may originate in shedding from vascular or tissue inflammation. sVCAM-1 perhaps acts as a competitive ligand to inhibit leukocyte adhesion to inflamed vascular endothelium. Kitani et al. [31] have shown that sVCAM-1 inhibits T cell proliferation possibly because of the suppression of IL-2 production. Rose et al. [19] showed that sVCAM-1 could actively bind T cells but the binding is disrupted in apoptosis. This suggests that sVCAM-1 may be involved in adhesion and chemotaxis toward endothelium of circulating lymphocytes. Patients with SARS had significantly higher sVCAM-1 levels in the first week of illness but returned to normal levels in the third week. The elevated sVCAM-1 might contribute to early vascular insult and suppression of T cell function. Assessment of vascular adhesion molecules to predict sepsis or systemic infections has been recently extensively reviewed [32]. Results showed that sVCAM-1 levels correlated well with the severity of inflammation and diseases, but lacked specificity of certain infection induced endothelial damage. It remains unclear whether vascular insult or immunosuppression associated with overwhelming lung infection or co-infection is the cause of morbidity or mortality.

Lymphopenia has been found in many infections including SARS [9,33–35]. Patients with measles tend to have lymphopenia due to inhibition of lymphocyte proliferation [34], and herpes and vaccinia infections tend to inhibit maturation of CD8 lymphocytes [33]. Extensive leukocyte apoptosis has been implicated in Ebola virus infections [36]. It has been proposed that lymphopenia in SARS patients is related to cell apoptosis [9]. We did find that SARS patients appeared to have higher plasma FasL levels, associated with higher intracellular cleaved caspase-3 positive CD4 and CD8 lymphocytes in the acute phase of SARS. Although the FasL levels were not significantly correlated to lymphocyte counts in blood, this may be because T cell apoptosis usually requires a two-step mechanism [35]. Inhibition of cell apoptosis has recently been shown to improve survival of septic animals [37]. Taken together, this suggests that inhibition of lymphocyte apoptosis could prevent lymphopenia in SARS. Whether correction of lymphopenia would improve the outcome of SARS may never be known.

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