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REGULAR ARTICLE

Thrombopoietin levels increased in patients with severe acute respiratory syndrome

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Abstract

Hematological changes in patients with Severe Acute Respiratory Syndrome (SARS) are common and frequently include thrombocytopenia. Using a ELISA method, we found an increase in thrombopoietin (TPO) levels in the plasma of convalesced SARS patients (290 ± 53 pg/ml) and active SARS patients (251 ± 23 pg/ml) comparing to that from normal control patients (228 ± 17 pg/ml). In addition, the plasma from active SARS patients had an inhibitory effect on CFU-MK formation, which could be neutralized by anti-TGF- β antibodies. In the experiment to determine whether SARS-CoV can directly infect hematopoietic stem cells and megakaryocytic cells, incubation of the cells with SARS-CoV did not show active infection. Our findings of increased TPO levels in the plasma of SARS patients provide a possible explanation for the genesis of thrombocytosis, which frequently develops from thrombocytopenia in SARS patients.

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Introduction

Severe Acute Respiratory Syndrome (SARS) is a recently emerged human disease caused by the infection of a novel coronavirus (SARS-CoV) [1]. The

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sequence of the complete genome of SARS-CoV has been determined [2,3], which is 29,727 nucleotides in length and has at least 11 open reading frames. The genome organization is similar to those of other coronaviruses [2]. The most common presenting symptoms in SARS patients are fever, chills, rigor, myalgia, cough, headache, and dizziness [4]. Less common symptoms include sputum production, sore throat, coryza, nausea and vomiting, and diarrhea [4].

Hematological changes in patients with SARS are common. For example, thrombocytopenia is found in 55% of SARS patients [4–7]. Previously, a platelet count of $<150 \times 10^9/l$ was documented in 44.8% of the SARS patients ($n=138$) in our hospital [4]. A prolonged activated partial-thromboplastin time greater than 38 s was noted in 42.8% of the SARS patients, whereas the prothrombin time remained normal in most cases. In 45.0% of the SARS patients, the D-dimer level was also elevated [4]. In one of our later reports, the common hematological changes found in SARS patients ($n=157$) included thrombocytopenia (55%), thrombocytosis (49%), and isolated prolonged activated partial-thromboplastin time >40 s (63%) [5]. The mechanism of how coronavirus causes thrombocytopenia and subsequent thrombocytosis have not been well understood, neither does the role TPO play in the process. In this study, TPO levels in plasmas from SARS patients, the effect of these plasmas on *in vitro* megakaryocytopoiesis, and whether SARS-CoV can directly infect hematopoietic stem cells and megakaryocytic cells are investigated.

Patients and methods

Patients

Plasma were collected from patients with SARS at the Prince of Wales Hospital, Hong Kong. These patients were divided into three groups: SARS patients in convalescence ($n=10$), active SARS patients ($n=7$), and normal control patients ($n=10$, laboratory staff and research students). The study was approved by the Committee on Clinical Research of the Institution.

ELISA

Plasma TPO levels were measured by an ELISA kit (R&D, Minneapolis, USA) following the manufacturer's instructions. Briefly, a monoclonal antibody specific for TPO was pre-coated onto a microplate. The standards and test samples were then pipetted into the wells and any TPO present was bound by the immobilized antibodies. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TPO was added to the wells. Following a wash that removed any unbound antibody–enzyme reagent, a substrate solution was added to the wells and color was developed, which is in proportion to the amount of TPO bound to the well. The optical density of each well was measured at the wave length of 450 nm by a microreader (Bio-tek, Instruments, Winooski, USA).

Human CFU-MK assay

Mononuclear cells from cord blood were separated by centrifugation using Ficoll–Hypaque (density 1.077) and added to a plasma clot culture system [8]. After incubation with plasma (5%) of SARS patients or normal control patients for 12 days, the megakaryocyte colonies were stained with an immunocytochemical method using biotin/alkaline phosphatase and detected with naphthol AS-MX phosphate and fast red. The cells were counterstained with haematoxylin and examined under an inverted-microscopy. The anti-TGF- β antibodies were obtained from R&D Systems (Minneapolis, MN) [9].

Infection of megakaryocytic cells and CD34+ cells by SARS-CoV

Human megakaryoblastic cell line Meg-01 cells were obtained from ATCC (Rockville, MD) [8]. Megakaryocytic progenitor cells were collected from human CFU-MK colonies. Haematopoietic CD34+ cells were purified from human cord blood [8]. These megakaryocytic cells and CD34+ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Calf Serum (FCS) (CSL, Australia), Penicillin (100 U/ml) and streptomycin (50 $\mu\text{g/ml}$) with or without SARS-CoV for 7 days at 37 °C in a humidified incubator with 5% CO₂ [8].

Immunofluorescence methods

The expression of SARS-CoV antigens on cultured human megakaryocytic cells and CD34+ cells were investigated by an immunofluorescence method using serums of SARS-CoV patients [10].

Statistical analysis

The statistical significances of the TPO levels among different groups were evaluated by Tukey–Kramer HSD (honestly significant difference) test, an ANOVA multiple comparison test, using software package JMP (SAS Institute, Cary, North Carolina, USA). The data are presented as the mean \pm SD. A p value less than 0.05 was considered statistically significant. The correlation of platelet counts and TPO levels in the three groups were analyzed by linear regression using JMP.

Results

Plasma TPO levels in SARS patients

The seven patients with active SARS were symptomatic patients admitted to the Prince of Wales Hospital in Hong Kong for acute management during the SARS outbreak from March to May in 2003. The diagnosis was serologically confirmed and fulfilled the WHO case definition of SARS. Their plasma were collected for laboratory investigation and stored in -80 °C for research use [5,11]. Convalescent plasma was obtained from 10 patients in the same hospital who had recovered from the SARS virus infection. Specifically, "recovery" was defined as having an afebrile status for at least seven days, showing radiographic improvement of 25%, having no further need of an oxygen supplement and being at least fourteen days following the onset of the symptoms [12]. In these groups of patients, the changes of platelet count have been observed in 55% of the active SARS patients with thrombocytopenia (platelet count of $<140\,000/\text{mm}^3$), and in 49% of the recovered SARS patients with thrombocytosis (platelet count of $\geq 400\,000/\text{mm}^3$) have been documented [5].

Using a ELISA method, the levels of TPO in three groups of plasma were measured. There was an increase of TPO level in the plasma of convalesced SARS patients (290 ± 53 pg/ml, $n=10$, $p=0.01$) and active SARS patients (251 ± 23 pg/ml, $n=7$, $p=0.3$) comparing to that of normal control patients (228 ± 17 pg/ml, $n=10$) (Fig. 1). To study the correlation of platelet counts and the plasma TPO levels, linear regression analyses were carried out for the normal, convalesced SARS and active SARS patient groups respectively. It is found that the correlation coefficients are $R=-0.79$ for normal group, $R=0.89$ for the active SARS group and $R=0.62$ for the convalesced SARS group. While TPO levels in normal group are inversely correlated to the platelet number ($R=-0.79$), the TPO levels in the active and convalesced SARS patients showed a positive correlation with their platelet counts.

Effect of plasma from SARS patients on CFU-MK formation

We then studied the effects of plasma collected from SARS patients on *in vitro* megakaryocytopoiesis. It is found that plasma from active SARS patients had an inhibitory effect on CFU-MK formation (42 ± 8 colonies/ 2×10^5 cells, $n=7$), comparing to that from convalesced SARS patients (66 ± 10 colonies/ 2×10^5 cells, $n=10$, $p=0.01$) and normal control (58 ± 6 colonies/ 2×10^5 cells, $n=10$, $p=0.04$). Previous studies showed an increased TGF- β level in active SARS patients [13,14], suggesting that TGF- β related cytokine storm triggered by viral infection might be involved in the inhibition of megakaryocytopoiesis, which lead to thrombocytopenia. To test this hypothesis, anti-TGF- β antibodies were added to the plasma clot culture system. We observed that the inhibitory effect of the plasma from active SARS plasma on CFU-MK formation could be neutralized (55 ± 8 colonies/ 2×10^5 cells, $n=7$, $p=0.05$) by the antibodies, suggesting that TGF- β may be involved in this inhibitory process.

Infection of megakaryocytes and CD34+ cells by SARS-CoV

Molecular sequence of SARS-CoV was detected in plasma of SARS patients before [10,15,16]. However, it remains unknown

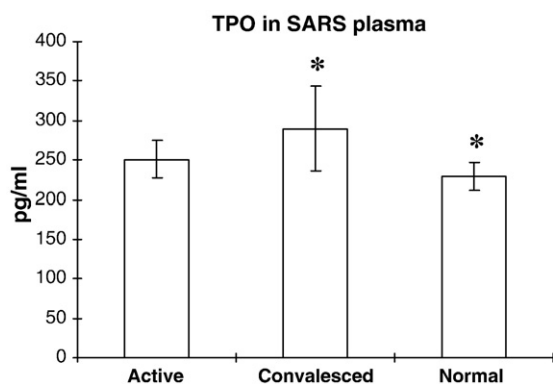


Figure 1 Using a ELISA method, the levels of TPO in the plasma from three groups of patients were detected and the data showed that there was a significant increase of TPO levels in the plasma of convalesced SARS patients ($n=10$, $p=0.01$) when compared with that from normal control ($n=10$), shown with “*”. The TPO levels between the plasma from active SARS patients ($n=7$) and that from normal control did not show significant difference ($p=0.3$).

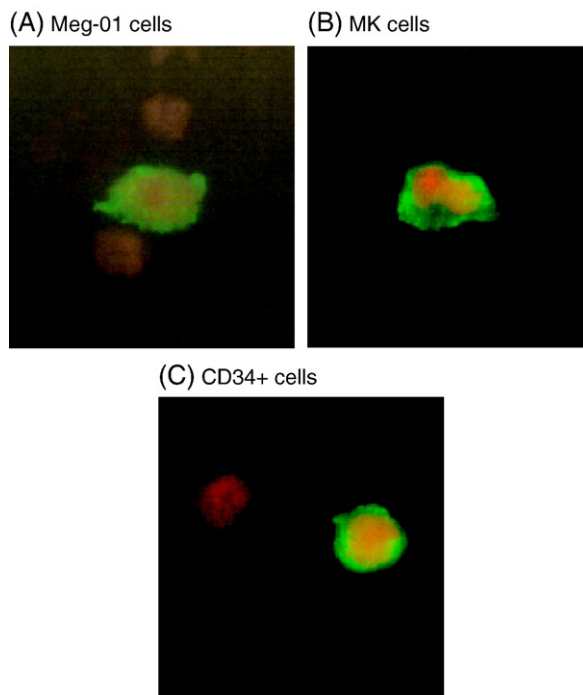


Figure 2 Antigen expression of SARS-CoV on cultured human megakaryocytic cells and CD34+ cells were investigated by an immunofluorescence method using serums of SARS-CoV patients. After 7 days of incubation with this SARS-CoV, infected cells were rarely detectable: (A) human megakaryocytic cell line Meg-01; (B) human megakaryocytic progenitor cells; and (C) human CD34+ cells.

whether SARS-CoV can establish infection in blood cells. After seven days of incubation with the SARS-CoV virus, only very weak infection on human megakaryocytic cell line Meg-01 cells (2–5%), human megakaryocytic progenitor cells (1–3%) and human CD34+ cells (1–2%) ($n=3$) could be found (Fig. 2). In summary, our data did not support an extensive infection of these blood cells by SARS-CoV.

Discussion

The present study showed that there was an increase of TPO levels in the plasma of convalescence SARS patients comparing to those from normal control and active SARS patients. The plasma from active patients of SARS also had an inhibitory effect on *in vitro* megakaryocytopoiesis and this inhibitory effect could be neutralized by anti-TGF- β antibodies. Thus, the involvement of TGF- β may provide a possible patho-physiological explanation for thrombocytopenia that usually is followed by thrombocytosis in SARS patients.

A number of mechanisms that control TPO production have been proposed [17]. Under normal conditions, TPO levels in blood are inversely correlated to the number of platelets. This is likely due to the fact that platelets express TPO receptor *c-mpl*,

through which TPO can be removed from the circulation [17]. Under disease conditions, the production of TPO can be enhanced by inflammatory mediators, which increase TPO transcription and production from the liver and bone marrow stromal cells directly [17], suggesting that the TPO level is more under the influence of productional control than the platelet numbers. Consistent with these observations, we also found an increase of TPO levels in SARS patients with an increase of platelet counts. The plasma with increased TPO promoted *in vitro* megakaryocytopoiesis, explaining the frequent occurrence of thrombocytopenia and subsequent thrombocytosis in SARS patients, possibly after the release from TGF- β related inhibition during the recovery phase.

There was no active infection on human megakaryocytic cells and hematopoietic stem cells after incubation with SARS-CoV in this study. ACE2 is a functional receptor for the SARS coronavirus [18]. However, we could not detect the expression of ACE2 on human bone marrow CD34+ cells, platelets and megakaryocytic cells (data not shown), which might explain why these cells are not infected by the SARS virus. Our results support the hypothesis that SARS-CoV may not invoke an active infection in blood cells. In stead, we demonstrated that there was a plasma megakaryocyte inhibitory factor (possibly TGF- β) that suppresses megakaryocyte development resulting in thrombocytopenia [19].

SARS is an emerged infection disease and its hematological changes are common. Elucidation of the mechanisms of how coronavirus infection causes cytopenias is important for better management of this disease. In general, virus may directly infect hematopoietic stem/progenitor cells, inducing their growth inhibition and apoptosis, although we were unable to observe an active infection of blood cells by the virus in our study. Furthermore, a general virus infection may cause immune damages to the blood cells by inducing auto-antibodies and immune complexes [6]. In the case of thrombocytopenia associated with SARS-CoV infection, another potential mechanism may also be involved. Specifically, the lungs of SARS patients showed diffused alveolar damage [4]. The damaged lung tissue and pulmonary endothelial cells may cause the activation and entrapment of platelets in the lungs, along with the thrombi formation at the sites of the injury, may lead to the consumption of platelets subsequently [20,21]. In addition, the lungs may also be the sites of platelet release from mature megakaryocytes. In this case, the damaged lungs may result in pulmonary fibrosis and pathological changes, affecting the lung megakaryocyte fragmentation and platelet pro-

duction [20,21]. Taken together, the increased consumption of platelet or/and the decreased production of platelet may lead to thrombocytopenia.

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