

TITLE:

Acquired but reversible loss of erythrocyte complement receptor 1 (CR1, CD35) and its longitudinal alteration in patients with severe acute respiratory syndrome

ABSTRACT:

This longitudinal study investigates the change of erythrocyte complement receptor (E-CR1) expression in patients with severe acute respiratory syndrome (SARS). Circulating E-CR1 expression was semiquantified by flow cytometric analyses in 54 SARS patients and in 212 healthy individuals as a control. Since E-CR1 expression is influenced by the genetic polymorphisms in the CR1 gene, a major genetic polymorphism located within intron 27 of the CR1 gene was simultaneously analysed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The results showed that the expression level of E-CR1 (referred to as net fluorescence intensity values, NFI) was statistically correlated with the relevant genetic genotypes among the Chinese population including the healthy individuals (NFI: 5.14 ± 0.82 , 3.57 ± 0.66 and 2.67 ± 0.32 for HH, HL and LL genotypes, respectively) and SARS patients (NFI: 3.52 ± 0.91 and 2.63 ± 0.70 for HH and HL genotypes, respectively). Interestingly, the expression density of E-CR1 was found to fall significantly during the initiation and progressive phases (weeks 1 and 2 after the disease onset) and gradually returned close to normal through their whole convalescent phase (beginning from weeks 2 or 3 to weeks 7 or 8) in SARS patients irrespective CR1 genotype. In conclusion, our findings, at least, suggest that E-CR1 is likely involved in immune pathogenesis of SARS disease.

Introduction:

Severe acute respiratory syndrome (SARS) is a new emerging disease caused by a novel coronavirus (SARS-CoV) belonging to family Coronaviridae [1,2]. During the worldwide outbreaks of catastrophic SARS epidemic in 2003, SARS disease affected a cumulative number of 8098 subjects and claimed 774 deaths as recorded by the World Health Organization [3,4]. Clinically, SARS is characterized by persistent high fever (temperature $>38^{\circ}\text{C}$), nonproductive cough or dyspnoea, rapidly progressing abnormal results on radiography and laboratory evidence of SARS-CoV RNA and existence of specific antibodies. Most of the SARS subjects exhibited a typical course of acute self-limited illness and around 10% died from SARS disease or associated complications [5]. To date, the considerable findings in early clinical manifestation, haematological, radiological, and microbiological aspects have showed that SARS-CoV infection resulted in severe cytopathic changes [6–9]. The histological pathology in the lung of patients at autopsy, clinical manifestation and the abnormality showed by chest radiograph suggest that these changes are likely to be induced by the acute overwhelming immune response in vivo, but the underlying mechanism in the process of SARS-CoV infection is poorly understood yet [1, 5, 6]. A recent study indicated that complement receptor one (CR1, C3b receptor or referred to CD35 molecule) expressed on erythrocytes (E), can vary as much as 10-fold among the healthy individuals [10]. The CR1 gene is highly polymorphic with some single nucleotide polymorphism (SNP) sites which may contribute to variation of E-CR1 expression. Interestingly, Wilson et al. identified the genomic Hind III restriction fragment length polymorphism (RFLP) in intron 27 of the CR1 gene that, probably acting as a principle genetic polymorphism, had been found to correlate with E-CR1 expression in Caucasians. This gives rise to three genotypes including homozygosity of the HH (high) allele, heterozygosity of HL allele and homozygosity of the LL (low) allele that genetically regulate the high, intermediate and low expression of CR1, respectively [11]. E can bind complement opsonized microbe pathogens or immune complex (IC) through surface CR1 molecules and deliver them to tissue macrophages fixed at the reticuloendothelial system where they are eliminated [12]. As a result, CR1 on E (E-CR1) functions as one of the multiple components in host innate immunity and plays a crucial role against invading pathogens [13]. E lose CR1 as they age in the circulation and, in particular, this process is markedly accelerated in patients with a variety of inflammatory diseases characterized by chronic viral infections (HIV or HCV), lepromatous leprosy and specific autoimmune diseases like systemic lupus erythematosus (SLE) [11,14–17]. This suggests that E-CR1 molecules are likely to participate in the pathogenesis of immune-complex mediated diseases [18–21]. Beijing, as the most affected area by the SARS epidemic in early 2003, claimed 2521 cases and 191 deaths [22]. The viral infection may produce various haematological changes [6]. Our previous work and other findings motivated us to seek how E-CR1 molecules in peripheral blood change in

patients with SARS-Cov infection. To address the question, we longitudinally recorded the alteration of circulating E-CR1 expression throughout the course of SARS illness in 54 SARS patients. In addition, we simultaneously analysed the distribution of the Hind III expression polymorphism in the CR1 gene among the SARS patients and healthy individuals. Our findings suggest E-CR1 molecules, as one of the important components in innate immunity, are involved in the process of the deadly SARS disease.

Study patients :: Materials and methods:

We employed 54 confirmed SARS patients (34 males, 20 females; age range 13–74 years; 79.6% (43/54) in age range 20–50 years) that were admitted to the Beijing 302 hospital during the period of March 5th and June 20th, 2003. All the patients fulfilled the accepted definition of SARS [2]. Briefly, the incubation period for all the patients was within the range of 2–14 days. In early prodromal period, all patients had fever $\geq 38^{\circ}\text{C}$ over 48 h, a nonproductive cough or shortness of breath, new pulmonary infiltrates on chest radiography or high-resolution CT scans in the absence of an alternative diagnosis to explain the clinical presentation. Each patient was nursed in the isolation ward with other SARS patients.

The study protocol was approved by the Ethics Committee of this institution, and written informed consent was obtained from each participant. Fresh EDTA-anticoagulated peripheral blood (5.0 ml) was obtained from each of the SARS-diagnosed patients at enrolment and at weekly intervals. The genomic DNA samples were extracted from 0.2 ml of whole peripheral blood by using the QIAgen DNA purification kit (Qiagen, CA, USA) as the previous report [21]. The remaining blood was used for the analyses of E-CR1 by flow cytometry. We simultaneously recruited 212 healthy subjects who received a health examination in our hospital during the same period. Both the enrolled SARS patients and healthy individuals were excluded from HIV, HBV, HCV infections as well as free from the diseases such as influenza and pneumonia different from SARS. We randomly selected 39 sex- and aged-matched healthy individuals as a control for establishment of the reference values (NFI) of E-CR1. Both SARS patients and healthy subjects are from the Chinese Han ethnic origin.

Genotyping of CR1 alleles by PCR-RFLP :: Materials and methods:

All the genomic DNA samples were used as the PCR templates in our study. We synthesized the PCR primers as previously reported by Cornillet et al. [14]. Primer 1: 5'-CCTTCAATGGAATGGTGCAT-3' (4415nt – 4435nt); Primer 2: CCCTTGTAAGGCAAGTCTGG location 25nt – 55nt upstream 3' end of CR1 intron containing the Hind-3' (cleavage site III), the full length of amplified product is a 1.8 kb cDNA fragment in size. CR1 genotypes were determined on the basis of fragments sizes of Hind III digested PCR products. Homozygous HH individuals exhibit only one fragment of 1.8 kb, heterozygotes (HL) show three fragments of 1.8 kb, 1.3 kb and 0.5 kb, respectively, and the mutant homozygous LL has two bands of 1.3 kb and 0.5 kb in sizes as shown in Fig. 1. The three different genotypes represent the high, intermediate and low expression levels of E-CR1, respectively.

Analysis of E-CR1 expression using flow cytometric assay :: Materials and methods:

According to the previous reports [18,23], E-CR1 expression was semiquantitatively determined by using a flow cytometric analysis for all the SARS patients and healthy individuals, weekly if possible. Briefly, EDTA-anticoagulated venous blood was suspended in phosphate-buffered saline (PBS) and then layered onto a Ficoll-Hypaque gradient solution. After being centrifuged at $1000\times g$ for 10 min, erythrocytes were gently collected in the bottom layer and washed three times with saline. 106 cells were suspended in 50 μl of PBS plus 1% bovine serum albumin (BSA) and incubated for 1 h at 4°C with 10 μl of fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antihuman CR1 antibody (E11 Clone, mouse IgG1) from BD-Pharmingen (Becton-Dickson, Mountain View, CA) [12,17]. As a control, 10 μl of mouse purified isotypic antibody conjugated with FITC or PBS were substituted for anti-CR1 antibody. After the samples had been washed, they were fixed with PBS containing BSA and 1% formaldehyde. Finally, the samples were subjected to immunofluorescent analysis in duplicate in FACScalibur (Becton-Dickson, Mountain View, CA, USA) within 12 h after fixation. The mean fluorescence intensity (MFI) of stained erythrocytes (MFIs) and that of controls (MFIC) were measured using the Cellquest software program (Becton Dickinson). The degree of E-CR1 expression was estimated as the ratio of MFIs to MFIC (MFIs/MFIC) and was expressed as the net fluorescence intensity (NFI) according to the previous reports [15,23]. To confirm the reproducibility of E-CR1 measurements, the

fluorescence intensity of FITC-conjugated Calibrite Beads was measured before FACS analysis every time on a separate day, the coefficient of variance of MFI of Calibrite Beads was within 3%.

SARS laboratory testing for analyses of **plasma SARS-CoV RNA** and specific antibodies :::

Materials and methods:

The method was the same as described in our previous reports [22,24]. In brief, RNA was directly extracted from plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The viral RNA was amplified through nested reverse-transcription polymerase chain reaction (RT-PCR). The products were analysed by electrophoresis in 2.5% agarose gel containing 0.5 µg/ml of ethidium bromide. The viral sequence was confirmed by DNA sequence analysis. At the same time, plasma anti-**SARS-CoV** IgG antibodies was detected according to the instruction of commercial available enzyme-linked immunosorbent assay (ELISA) kit (Huada-Jibiai Biotech, Beijing). Indirect assay was employed by the kit using culture-obtained **SARS-CoV** antigen for the capture of antibodies. The OD value ≥ 0.105 was judged as positive. The routine analysis for urine and faeces samples, and complete differential blood count included total leucocytes, granulocytes, lymphocytes, monocytes, erythrocytes, haemoglobin, and platelets for **SARS patients** was simultaneously recorded, respectively.

Chest radiograph examination ::: Materials and methods:

In order to identify any abnormal changes occurring in the lungs, all patients underwent examination by chest radiography once daily for patients at the progressive disease stages or critical **SARS status**, and every 2–4 days for patients with stable or general disease status. According to a recent report [25], we retrospectively calculated the mean chest radiograph score (referred as MCR score representing the severity of pneumonia in **SARS patients**) on the basis of average percentage of area manifesting the ground glass opacification, consolidation or nodular shadow, in each lung with a maximal score of 10 (equivalent to 100% area involved).

Treatment protocols ::: Materials and methods:

All patients received ribavirin and steroid combination therapy. Ribavirin, a broad-spectrum antiviral agent, was empirically used to treat **SARS**, although its efficacy is not sure because the emergent outbreak of **SARS** did not permit physician to conduct any randomized controlled trials on the treatment of **SARS**. Also on the basis of early anecdotal experience and its radiologic resemblance to acute respiratory distress syndrome and other immunologically mediated lung diseases, different corticosteroid regimens were also employed for the treatment of **SARS**. The timing to commence corticosteroid and ribavirin therapy varied for each individual patient. The different steroid regimens were employed in clinic for **SARS patients**, the therapeutic dose of methylprednisolone includes ≤ 40 mg/day, 80–160 mg/day or ≥ 240 mg/day (intravenously once a day) based on the clinical manifestation, radiograph deterioration, PO₂ decrease, high fever and ventilation of patients.

Statistical analysis ::: Materials and methods:

The distribution difference of CR1 allelic genotypes among the **healthy subjects** and the **SARS patients** was analysed using Pearson's χ^2 test. The statistical difference of **CR1 densities on E** between the two groups was compared by using the SAS6.21 software program (t-test). The value of $P < 0.05$ represents a statistical difference.

Distribution of CR1 genotyping in **SARS patients** and **healthy subjects** ::: Results:

The distribution of the CR1 Hind III polymorphism was analysed using PCR-RFLP for **54 SARS patients** and **212 healthy subjects** (Table 1). The percentages of HH, HL and LL genotypes were 66.67%, 31.48% and 1.85% for SARS patients, and 66.92%, 27.83% and 4.25% for healthy subjects, respectively. The H and L allele frequencies in both groups are consistent with the Hardy–Weinberg equilibrium. Statistical analysis showed that there was **no significant difference between the healthy controls and SARS patients ($P > 0.05$)**. Therefore, these findings suggest that CR1 molecules are unlikely to have a direct influence on host genetic susceptibility of SARS-CoV infection entering target cells and viral replication.

Comparison of **E-CR1 expression levels** among the **healthy subjects** and **SARS patients** :::

Results:

On admission into our unit, we observed that all the patients had a high fever ($\geq 38^\circ\text{C}$) and mild or severe progressive consolidation change on serial chest radiographs in one or both sides of

lungs. Using flow cytometric analysis, we semiquantitatively measured the E-CR1 expression levels in SARS patients, for which the NFI value of fluorescent density was used to indirectly indicate relative E-CR1 expression levels [15,23]. Figure 2 shows the representative differences of fluorescent densities of E-CR1 in a representative healthy individual and a SARS patient both of whom are with the HH genotype. In the samples from the SARS individual, MFIs and MFIc were 11.5 and 2.9, respectively. Thus NFI value of fluorescent density for the SARS patient was determined to be 3.96 (11.5/2.9). In the same way, NFI value for the healthy individual was determined to be 6.36 (14/2.2). Our results were summarized in Tables 2 and 3, which show that the measurements using flow cytometric analysis are stable and reproducible. The data in Table 2 seems to support the following notion:

Even though the number of enrolled SARS patients in different groups as shown in Tables 2 and 3 is limited, our observations, at least in part, suggest that the magnitude of decrease in E-CR1 was consistent with the severity of SARS disease as related to lung damage with progressive changes as shown by chest radiograph examinations.

Among the 54 patients, 52 cases survived the disease through a hospitalization for 27–60 days (average hospitalization duration 40.5 ± 12.6 days). However, 2 of the 54 patients (males, and 73 years old, respectively) were critical and succumbed to the disease due to development of acute respiratory distress syndrome (ARDS) and aspergillosis as previously reported [26], resulting in a mortality rate of 3.7% (2/54). Even though the patients who died were identified as HH genotype, they had a dramatic decline level of E-CR1 expression compared to healthy subjects with the HH genotype (NFI: 2.44–2.52 versus 5.14 ± 0.82). In addition, the 2 patients also were older, more 50 years old, and probably had some severe underlying diseases like hypertension and diabetes [1,5] which may also contributed to the severity of SARS-Cov infection.

Longitudinal investigation of 5 representative individual SARS patients :: Results:

The quantitative analyses of E-CR1 were conducted weekly for each of the 54 patients during the entire period of their hospitalization from progressive to the convalescence stage. To obtain profiles of circulating E-CR1 molecules, clinical status (pneumonia severity), the viral RNA and specific antibodies, 5 representative individual SARS patients with HH, HL and LL genotypes were serially analysed in combination through the entire duration of the disease (Fig. 3). SARS-Cov RNA and specific antibodies were detected in the plasma in all the patients analysed (coexistence of the viral RNA and the antibodies was uniquely observed in patient 1, 13 and 17). The serum IgG became positive in 100% of the samples within 25 days following onset of the disease. We observed a decrease in the fluorescent densities of E-CR1 at the onset of clinical illness, which persisted from the 7th to the 15th day with variations occurring during the entire progressive stage accompanied with abnormal chest radiographs and the appearance of SARS-Cov in vivo. The lower E-CR1 levels slowly started increasing during the seventh or eighth week of the illness while the chest radiographs or high-resolution CT scans shown complete resolution in approximately three weeks after the onset of symptoms for patients in convalescence. The E-CR1 levels returned to normal for all of the patients within 9–14 weeks from the onset of illness.

Discussion:

The analysis of genetic mutations responsible for CR1 stability and expression level on E, its distribution, and dynamic alteration would provide a alternative approach to understanding the mechanism which regulates the severity of the inflammatory tissue damage and pathogenesis of SARS-Cov infection [14,17]. Our current observations confirmed a significant correlation between the Hind III RFLP and the relative expression of E-CR1 in Chinese Han ethnic population sample. This is consistent with an earlier report of 100 Southern Chinese and Taiwanese blood donors [27] and is also similar to results found in Caucasian populations. A recent study in Thailand also found a correlation between the Hind III RFLP and E-CR1 [28]. These findings further support the notion that variations of E-CR1 among healthy individuals are inherited and regulated by different HH, HL and LL genotypes [17,27]. We found that there was no significant difference in the frequency of HH, HL and LL genotypes between the SARS patients and healthy subjects in the Chinese Han population sample. Our limited data suggests that all the subjects have similar susceptibility to SARS viral infection. Unlike other complement regulatory proteins such as membrane control protein (MCP) and decay-accelerating factor (DAF), CR1 levels may have no direct influence on the ability of the virus to enter human target cells, and the viral replication process.

Different declining levels of E-CR1 have been found in patients not only with SLE, chronic HIV, and HCV infection, but also in patients with malaria, malignant tumour, and chronic diseases [15,

20, 21, 28, 29, 30, 31]. Interestingly, we also found a significant decrease of E-CR1 in SARS patients, which may suggest an acquired deficiency of host innate immunity [13]. SARS-CoV viral loads may be an important factor in the decrease of E-CR1 since the early rapid decline and sequential persistent lower levels of E-CR1 occurred during the periods when SARS-CoV is positively detected in patient's plasma, sputum, throat swab and/or stool samples. In this way, SARS viral infection may induce the reduction or damage of innate immunity.

As shown in Tables 2 and 3, the fluorescence intensity values of E-CR1 molecules varied over a broad range and some of them even partly overlapped between the individuals with HH, HL and LL alleles. One possibility is that the CR1 gene is highly polymorphic with a large number of single nucleotide polymorphism (SNP) sites [27], which may also influence the expression of E-CR1 independent of the H and L allele. The fluorescent density values of E-CR1 varies over a wide range for a definite genotype such as the HH, HL or LL alleles in various individuals, which may be influenced by the existing haplotypes as reported by Xiang et al. [17].

Finding of a dramatically decreased E-CR1 expression during the SARS disease progression in all the SARS patients compared with the healthy controls implies that the E-CR1 molecules are engaged in the pathogenesis of SARS. This is probably occurring during the process of forming antibody-SARS antigens and complement immune complexes at the same time when the host produces the specific antibodies to the SARS antigens. The evidence that coexistence of the specific antibodies and the direct viral RNA was found in some patients supports this hypothesis. Therefore, the aetiology of the E-CR1 reduction may be associated with the removal of the immune complex (IC) by tissue macrophages during which CR1 is proteolytically cleaved from the surface of E though its underlying mechanism is still unknown.

We would like to mention that our data should be carefully interpreted because of: (1) the limitation of a small sample size for some groups; (2) the factors including the case ageing and the steroid therapy may have also influenced the variation of E-CR1 expression in the SARS patients [25]. The different steroid regimens (doses ranging 80 mg –480 mg each day) were empirically administrated for the treatment of SARS based on pneumonia severity as determined by chest radiograph examinations. In addition, we were unable to design a randomized controlled investigation due to the rapid emergence of the SARS epidemic.

It is noteworthy that despite the clinical recovery and pulmonary damage resolution, the lower E-CR1 levels still were longitudinally sustained for a 7–8-week period and increased continuously and slowly to near normalization. Therefore, our findings suggest that the E-CR1 density undergoes an acute but reversal decrease, which correlates with the initiation, progression and convalescence stages of SARS disease [13, 15, 32, 33]. The density of the E-CR1 molecules in patients during the convalescence stage increased to almost as high a level as found in healthy subjects. This may be because the old erythrocytes with decreased density of CR1 molecules were gradually replaced by newly produced erythrocytes having normal density of E-CR1 molecules. This in turn increased the percentage of erythrocytes with normal density of E-CR1 molecules in the total peripheral population contributing to the slow rise of peripheral E-CR1 levels in SARS patients at convalescence stage. In fact, reticulocytes have shown to increase E-CR1 levels as compared to cells that have been in the circulatory system for a period of time (J.-M. Moulds, unpublished observation).

Collectively, our findings show that an acute but reversible acquired reduction of E-CR1 may represent a significant alteration in SARS disease, suggesting that E-CR1 molecules may be one of the important components in host innate immunity, which probably participates in the process of the deadly SARS disease. This knowledge continues to increase our understanding of the immune pathogenesis of SARS.