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ORIGINAL ARTICLE

The role of CD14 gene promoter polymorphism in tuberculosis susceptibility

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Background: CD14 is expressed principally by cells of monocyte/macrophage lineage and plays a pivotal role in the innate immunity to intracellular infections. Recent research findings have revealed an association between the CD14 gene promoter polymorphism and several major infectious diseases.
Objective: The aim of the present study was to investigate the association between the CD14-159C/T polymorphism and tuberculosis in a Turkish population.
Methods: For this purpose, 88 consecutive patients with tuberculosis (63 pulmonary, 25 extrapulmonary) and 116 control subjects were enrolled into a prospective study. We determined CD14-159 genotypes by polymerase chain reaction - restriction fragment length polymorphism analysis and also measured serum concentrations of soluble CD14 (sCD14) by using a quantitative sandwich enzyme immunoassay technique.
Results: There was no significant difference in terms of genotype distribution between patients with tuberculosis (CC 18.2%, CT 48.9%, TT 33.0%) and controls (CC 12.9%, CT 50.9%, TT 36.2%) or between patients with pulmonary and extrapulmonary tuberculosis. Serum levels of sCD14 were significantly increased in patients with active tuberculosis compared to those with inactive tuberculosis and healthy controls ($p < 0.001$). However, levels of sCD14 were not associated with any genotypes of CD14-159.
Conclusion: The genotyping findings of the present study do not support a role for the CD14-159C/T polymorphism in the development of tuberculosis, at least in the geographical

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region of central Anatolia. Significantly elevated serum sCD14 levels in patients with active disease reflect the importance of the mononuclear phagocytic system activation in tuberculosis.

Introduction

Activation of macrophages represents one of the initial events in the innate immunity to intracellular infections. CD14 is expressed principally by cells of monocyte/macrophage lineage and plays a pivotal role in the innate recognition of bacterial cell wall components. The binding of a microbial component to CD14, as an accessory receptor for toll-like receptor (TLR), results in cellular activation and initiates a variety of effector functions including cytokine secretion and proliferation.[1–3] CD14 exists in both membrane-bound and soluble forms. Soluble CD14 (sCD14) is present in the circulation and other body fluids, and its levels in plasma increase during inflammation and infection.[4,5]

Exposure to *Mycobacterium tuberculosis* (*M tuberculosis*) can result in numerous different clinical outcomes. Only about 25% of those exposed to *M tuberculosis* become infected. While most infections result in a latent infection, a minority of these cases eventually progresses to active disease. Active tuberculosis may manifest a broad spectrum of clinical presentations varying from lung involvement to dissemination of the disease and multiorgan involvement.[6–9] Host genetic factors play a major role in determining differential susceptibility to major infectious diseases.[9,10] Genetic polymorphisms in the immune response to *M tuberculosis* may explain interindividual differences in both susceptibility to disease and the course of infection.

Innate immune receptors and recognition of bacterial products play a central role in the immune response to infectious organisms. Polymorphisms involving these innate immune receptors and related molecules, have been described to be associated with susceptibility to infectious diseases.[3,11–14] A possible relationship between CD14-159C/T polymorphism and several infections such as brucellosis, sepsis, chronic *Chlamydia pneumoniae* infection, and bacterial diarrhea, has been implicated previously.[11,12,15,16] The impact of the CD14-159C/T polymorphism on the susceptibility to tuberculosis has also been examined in several studies, providing controversial results among the different populations.[3,17,18] The aim of the present study was to analyze the association between the CD14-159C/T polymorphism and tuberculosis in a Turkish population.

Methods

Subjects

This prospective study included 88 unrelated tuberculosis patients from central Anatolia who presented to the Infectious Diseases and Clinical Microbiology Department of Kırıkkale University Faculty of Medicine, or were admitted to the Ataturk Chest Disease and Surgery Center, Department of Respiratory Medicine, between September 2008 and March 2010. It was approved by the ethical committees of these two hospitals, and written informed consent was obtained from the study participants.

The inclusion criteria were as follows: (1) having a diagnosis of tuberculosis; (2) age $\geq$ 16 years; and (3) consented to be included into the study. The healthy group consisted of subjects with no known diseases. The exclusion criteria for both patient and healthy groups were: (1) having any infectious diseases other than tuberculosis in the last 6 weeks; (2) having significant chronic systemic diseases which could lead to immunosuppression; (3) being pregnant; and (4) being HIV positive (testing positive for HIV).

Pulmonary tuberculosis (n = 63) group

There were 63 patients in this group. The diagnosis of pulmonary tuberculosis was based on the presence of acid-fast bacilli by sputum smear or *M tuberculosis* by culture of sputum and bronchoalveolar lavage fluid. In patients with negative smears and cultures, the diagnosis of tuberculosis was based on symptoms, chest radiographic infiltrates in the upper lobes, and clinical and radiographic responses to antituberculosis drugs. The patients were divided radiologically into three categories according to the severity of the pulmonary lesions, as minimal, moderate-advanced, and far-advanced.[19]

Extrapulmonary tuberculosis (n = 25) group

This study group included 25 cases of extrapulmonary tuberculosis. Nine patients had pleural involvement; six of them suffered from concomitant pulmonary tuberculosis, whereas the other three patients had isolated pleural involvement (tuberculous pleuritis). Four patients had cervical and/or submandibular lymphadenitis, and two of them were reported as recurrent lymphadenitis. Four patients were diagnosed as urogenital tuberculosis (two had renal involvement, two had epididymitis). Six patients had tuberculous meningitis, with cranial nerve involvement (2), osteoarticular involvement (2), mediastinitis (1), and peritonitis (1). One patient was diagnosed with lupus vulgaris involving the left upper eyelid, and the last case had disseminated tuberculosis with splenic and scrotal abscesses. The majority of extrapulmonary tuberculosis cases (15) were diagnosed with histopathological examination of the pleura, lymph node, peritoneum, and bone biopsy samples; culture was positive in eight cases with extrapulmonary tuberculosis [tuberculous pleuritis (3), lymphadenitis (3), peritonitis (1), and scrotal abscesses

(1)]. The diagnosis of extrapulmonary tuberculosis in the five patients with negative culture and histopathological examination was based on the presence of compatible clinical findings in conjunction with radiological and laboratory findings or response to antituberculosis drugs.

The patient group included both new and previously diagnosed cases of tuberculosis. Fifty-four patients had newly diagnosed active tuberculosis and were assigned as the active tuberculosis group. Thirty-four patients were under antituberculous treatment. After completion of the treatment, they were included into the study and assigned as the inactive tuberculosis group. One hundred and sixteen healthy individuals (mean age $= 32.96 \pm 10.48$ years, M/F $= 59/57$) were assigned as the control group. Healthy subjects were selected from the adult population who had no underlying comorbidity and no diagnosis of tuberculosis. All subjects were tested for tuberculin skin test (TST) with purified protein derivative (PPD). Subjects with a recent history of tuberculosis contact and a positive PPD were screened by chest-x ray and sputum culture to exclude active disease.

Genotyping of CD14-159 polymorphism

Isolation of the genomic DNA from the peripheral blood leukocytes was carried out by using phenol-chloroform extraction and the ethanol precipitation method. Polymerase chain reaction (PCR) was carried out in a 25 μL volume containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Promega, Madison, WI, USA), and 5 pmol of each primer. The sequence of primers was as follows: Forward 5'-TAGATTCTCTGGGATATAAGG-3' and Reverse 5'-CTGACAGTTTATGTAATCCTG-3'. The DNA was denatured at 95°C for 5 minutes, and cycling was set at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds for 35 cycles, followed by a final extension at 72°C for 5 minutes. Aliquots of PCR products were digested with restriction endonuclease, Eco47I (Ava II) (Fermentas Life Sciences, Vilnius, Lithuania) at 37°C. Digestion products were electrophoresed on 3% agarose gels and visualized with ethidium-bromide staining and ultraviolet illumination. The sizes of the generated digestion products were 357 bp for CC allele, 357 bp-217 bp-140 bp for heterozygotes and 217 bp-140 bp for TT allele.

Serum sCD14 measurement

Serum sCD14 levels were determined for 82 tuberculosis patients and 99 normal controls using Quantikine kits (Quantikine R&D Systems, Inc., Minneapolis, MN, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. All procedures were applied according to manufacturer's instruction.

Erythrocyte sedimentation rate (ESR) was measured by Westergren's method, and C-reactive protein (CRP) was tested by immunoturbidimetric assay.

Statistical analysis

Statistical analysis was performed with the SPSS-16.0 statistical package. Hardy–Weinberg equilibrium was tested with the Chi-square statistic. Comparisons of sCD14 levels between groups were made using univariate analysis of variance (ANOVA) or analysis of covariance (ANCOVA), using age and gender as covariates. Categorical variables were compared by the Chi-square test. Spearman correlation coefficient was performed to establish a correlation between sCD14, CRP and ESR values. A p value < 0.05 was considered significant.

Results

This study included 88 consecutive patients with tuberculosis (63 pulmonary, 25 extrapulmonary). The mean age of the patients was 49.93 ± 16.15 years (range $= 16$ to 78 years), and 65 (73.9%) were males. Genotyping was done in all study populations and healthy controls. The distribution of each of the genetic variants met the conditions of the Hardy-Weinberg equilibrium.

There was no significant difference in the genotype distributions between patients with tuberculosis (CC 18.2%, CT 48.9%, TT 33.0%) and controls (CC 12.9%, CT 50.9%, TT 36.2%), or between patients with pulmonary and extrapulmonary tuberculosis (Figs. 1 and 2). There was also no association between allelic frequencies. About half of the individuals in both groups were CT heterozygous.

The severity of the pulmonary lesions was graded in 73 patients, including patients with extrapulmonary tuberculosis with lung involvement. Far-advanced, moderate-advanced and minimal involvement was detected in 31, 26 and 16 patients, respectively. The genotype frequencies observed in these three groups were similar. The genotype frequencies observed in patients with far-advanced (CC 25.8%, CT 41.9%, TT 32.3%), moderate-advanced (CC 11.5%, CT 57.7%, TT 30.8%), and minimal involvement (CC 18.8%, CT 43.8%, TT 37.5%) were similar. No association was found between CD14-159C/T polymorphism and the severity of pulmonary involvement.

Serum sCD14 levels were determined for 80 tuberculosis patients (49 active, 31 inactive) and 99 healthy controls. Serum levels of sCD14 were significantly increased in patients with active tuberculosis compared to those with inactive tuberculosis and healthy controls ($p < 0.001$) (Fig. 3).

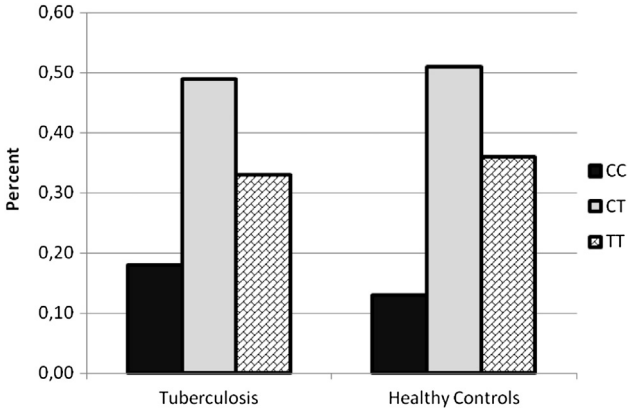

Figure 1. Percentages of the genotypes (CC, TT, CT) in the control and tuberculosis groups.

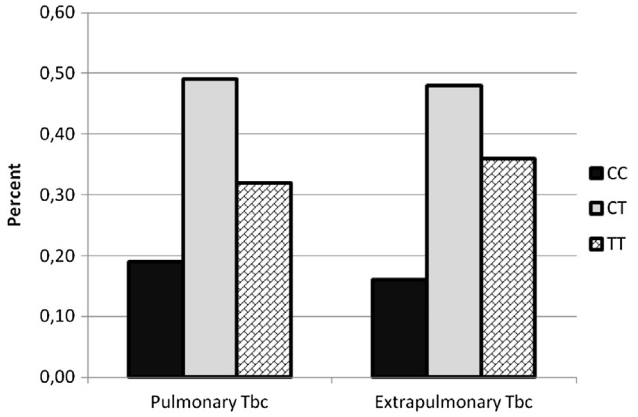

Figure 2. Percentages of the genotypes (CC, TT, CT) in the tuberculosis and extrapulmonary tuberculosis groups.

A possible association between CD14 gene polymorphisms with the levels of serum sCD14 in the tuberculosis patients and controls was also tested. However, levels of sCD14 were not associated with any genotypes of CD14-159, (CD14-159 TT $= 8.27 \pm 4.25$ ug/mL, CD14-159 CT $= 8.21 \pm 4.13$ ug/mL, CD14-159 CC $= 9.13 \pm 5.37$ ug/mL).

ESR and CRP, markers of acute phase response, were measured in patients with tuberculosis. Because sCD14 is also regarded as an acute phase protein, a possible correlation between the levels of serum sCD14, ESR and CRP were investigated. The median levels of CRP and ESR were 2.65 (5.86) versus 0.56 (1.37) mg/dL and 61.50 (53.75) versus 14.00 (28.00) mm/hour in patients with active and inactive tuberculosis, respectively. ESR and CRP values were elevated in patients with active tuberculosis as compared with those of inactive cases ($p < 0.001$). There was a significant correlation between ESR and CRP values in patient group ($r = 63$, $p < 0.001$). However, ESR or CRP values did not correlate with sCD14 levels.

Discussion

There are a few studies investigating the role of CD14-159C/T polymorphism in tuberculosis and they revealed conflicting results.[3,17,18] A Mexican study evaluating CD14-159C/T and TLR4 Asp299Gly polymorphisms in tuberculosis claimed that the CD14-159 TT genotype was a risk factor for development of pulmonary tuberculosis.[17] Similarly, a study conducted in a Korean population reported a significant association between this polymorphism and tuberculosis.[18] However, the study by Pacheco et al failed to detect a significant association between CD14-159C/T polymorphism and susceptibility to tuberculosis and its clinical forms in a Colombian population.[3] In this present study, we investigated the association between CD14-159C/T polymorphism and tuberculosis in a Turkish population. There was no significant difference between the genotype distribution of patients with tuberculosis and controls. The percentage of TT homozygotes was similar in both groups. We are unable to find an association between the CD14 promoter polymorphism neither with the development of tuberculosis nor with the severity of pulmonary involvement. Many factors could contribute to these conflicting results reported in these studies. Our failure to demonstrate an association between the CD14 promoter polymorphism and tuberculosis may have been due to the relatively small size of this study. Tuberculosis is a complex disease and susceptibility is likely influenced by several genetic and environmental factors.[8,20] The racial and environmental differences among the populations might account for these conflicting results.

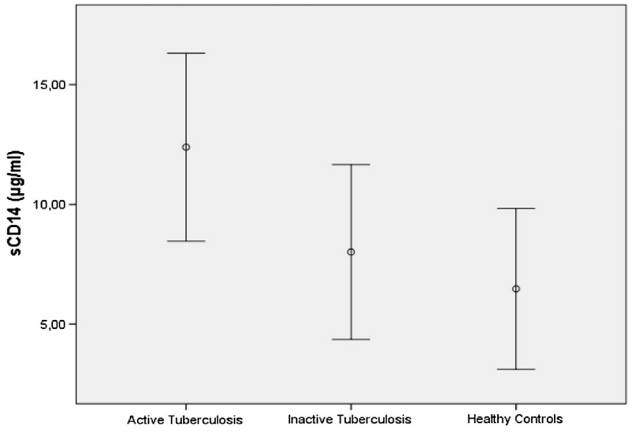

Figure 3. Serum levels of sCD14 in active tuberculosis, inactive tuberculosis and healthy controls. (The points represent mean values and the vertical lines represents plus/minus one standard deviation).

CD14 plays an important role in the induction of inflammatory responses evoked by a variety of microorganisms.[1,10] Several studies have been conducted to show the impact of the CD14 C-159T single nucleotide polymorphism on the risk of infectious diseases. A multiple-center study by Gibot et al provided suggestive evidence of an association between the CD14 gene polymorphism and sepsis.[11] They found that the prevalence of the TT genotype was significantly higher in patients with sepsis than in controls and that the mortality rate in patients with the TT genotype was significantly increased. Sutherland et al suggested that a single nucleotide polymorphism (SNP) within the *CD14* gene was associated with an increased prevalence of gram-negative bacterial infections in critically ill adults.[21] However, Agnese et al and Jessen et al found no association between CD14 SNP and gram-negative infection.[22,23] Recently, a SNP within the *CD14* gene was demonstrated to be associated with the risk of developing bacterial diarrhea.[16] A possible relationship between the CD14 C-159T polymorphism and several intracellular infections such as brucellosis and chronic *C pneumoniae* infection, has also been implicated in different populations.[12,15]

Tuberculosis is a broad-spectrum disease which may involve both pulmonary and extrapulmonary locations.[6] A failure of some of the immune response genes involved in controlling the dissemination of *M tuberculosis* from the lung to other tissues may be responsible for the extrapulmonary involvement. Our study included 25 cases of non-HIV tuberculosis with different extrapulmonary involvements, including pleural, lymph nodal, urogenital, osteoarticular, and meningeal tuberculosis. There was no significant difference in terms of CD14 genotype distribution between patients with pulmonary and extrapulmonary tuberculosis and controls. The CD14 gene promoter polymorphism was also investigated in different clinical forms

of tuberculosis by Pacheco et al[3] and they found no association between the CD14 polymorphism and clinical forms of extrapulmonary tuberculosis. The findings of these two studies did not reveal any relationship between the CD14 gene promoter polymorphism and non-HIV extrapulmonary tuberculosis. It is plausible that the CD14 gene promoter polymorphism genotype does not influence the development of extrapulmonary tuberculosis. Innate immune mechanisms are undoubtedly essential in the initial phase of the infection.[24] There may be some defects in acquired immune mechanisms and their molecules during the development of extrapulmonary involvement.

The soluble form of CD14 is found in normal human serum and is increased in several clinical conditions characterized by local or systemic activation of monocytes/macrophages.[23–30] Although the function of sCD14 in human disease is not yet clarified, a potential pathogenic role has been proposed for several infectious diseases. A vast majority of evidence suggests that CD14 signaling is a protective host response to intracellular bacterial pathogens such as *M tuberculosis*, and increased sCD14 levels were documented in the sera and bronchoalveolar lavage fluid of patients with active tuberculosis.[25,29,30] Consistent with previous reports, serum levels of sCD14 were found to be significantly increased in active tuberculosis patients compared with those with inactive tuberculosis and healthy controls. These findings may implicate that CD14 signaling is an important component of the initial anti-mycobacterial host response. We also investigated the association between the CD14 polymorphism and sCD14 levels. However, we were unable to find any correlation between the CD14 polymorphism and the level of sCD14 in tuberculosis.

CD14 is a molecule with a wide range of functions. CD14-induced activation of macrophages results in the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1 and IL-6.[1,4] These inflammatory cytokines lead to the production of acute phase reactants. IL-6 induces sCD14 expression in liver cells and is regarded as an acute phase protein.[1] An increased sCD14 level is not specific to infectious diseases; its concentration also increases during several noninfectious inflammatory conditions.[26–28]Although sCD14 levels were significantly elevated in our patients with active tuberculosis, the levels did not correlate with CRP and ESR values. The lack of a positive correlation is not surprising, given the fact that several other accessory molecules are involved in CD14 signaling and in the subsequent release of inflammatory cytokines.[1,4,31,32]

There are conflicting results regarding the association between the CD14-159C/T polymorphism and tuberculosis among different populations. The genotyping findings of the present study do not support a role for the CD14-159C/T polymorphism in the development of tuberculosis, at least in the geographical region of central Anatolia. However, significantly elevated serum sCD14 levels in patients with active disease reflect the importance of the mononuclear phagocytic system activation in tuberculosis and may implicate that CD14 signaling is an important component of the initial anti-mycobacterial host response. Since our results are based on a relatively low number of subjects, further large-scale studies are needed to demonstrate the role of the CD14-159 gene polymorphism in susceptibility to tuberculosis disease.

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