INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects several organs, and it is also associated with the destruction of joint connective tissues and bones. The joints become swollen, painful, and stiff on aggravation, which ultimately is liable to cause immobility and physical deformity. The most common chronic arthritis in rheumatology is RA. The disease is characterized by chronic inflammation starting in joint synovial membranes and spreading to cartilage and bone gradually leading to the destruction of these joints. RA is found 3-4 times more frequently in women than men [1]. The prevalence of RA is 1% in the world population. The majority of the prevalence studies carried out in Northern European, and North American countries estimate a prevalence of 0.5-1.1%, whereas the annual incidence varies between 20 and 50 cases per 100,000 inhabitants [2]. Anticyclic citrullinated peptide/protein antibodies (ACPAs) appear early in the rheumatic disease process and remain throughout the course of RA [3]. Cyclic citrullinated peptide (CCP) is the most widely used antigen to detect ACPA [4]. The ACPAs bind to the citrulline epitope of the CCPs. Citrullination is a process causing modifications of positively charged arginine to neutrally charged citrulline at certain positions of autologous proteins/peptides. The change is catalyzed by the enzyme peptidylarginine deiminases (PADI), which requires Ca\(^{2+}\) ions for its activation. During inflammation and apoptosis cell death, cell membranes become porous and penetrable to a number of small molecules. Ca\(^{2+}\) ions move inside the cell increasing the intracellular concentration of Ca\(^{2+}\) ions a 100-1000 fold. This activates PADI resulting in citrullination of different proteins in the inflamed joints.

TUMOR NECROSIS FACTOR-ALPHA LEVEL IN SERA OF SOUTH INDIAN PATIENTS WITH RHEUMATOID ARTHRITIS: CORRELATION WITH ANTICYCCL CTRULLINATED PEPTIDE ANTIBODY LEVEL

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Received: 23 July 2016, Revised and Accepted: 04 October 2016

ABSTRACT

Objective: The present study was aimed to find out the anticyclic citrullinated peptide (CCP) antibody level and expression level Th2 cytokine-like tumor necrosis factor-alpha (TNF-α) in patients with rheumatoid arthritis (RA) from South India.

Methods: The patients attending the Arthritis and Rheumatism Care Centre, Vadapalani, Chennai and healthy individuals from the Presidency College, Chennai, were enrolled for this study. The study group included 74 patients with RA and 50 healthy individuals without history of RA. 3-5 ml of blood samples were aseptically collected using Vacutainer, and the separated serum samples were transported to the Department of Microbiology, Presidency College, Chennai, Tamil Nadu, in cold chain. Anti-CCP antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Serum concentrations of TNF-α were studied in patients with RA and in healthy controls, using an ELISA method.

Results: The results of anti-CCP enzyme immunoassay revealed that out of 74 patients, all were anti-CCP positive, which included 65 females and 9 males. Higher levels of anti-CCP (456 IU/ml) were present in the age group between 41 and 50 followed by 21-30 years age group which shows 335.28 IU/ml of anti-CCP antibody level. The level of serum TNF-α was measured in the range of 4.6-1082.84 pg/ml for RA patients and 6.630-459.74 pg/ml for the healthy control group.

Conclusion: TNF-α levels were significantly increased in RA patients compared to healthy individuals. A negative correlation was found between anti-CCP antibody and TNF-α level in RA patients.

Keywords: Rheumatoid arthritis, Tumor necrosis factor-alpha, Enzyme-linked immunosorbent assay, Anticyclic citrullinated peptide antibodies.

INTRODUCTION

Cytokines play a critical role in the pathogenesis of RA [5]. Development of sensitive immunoassays for cytokines has made it possible to show increased levels of some cytokines in the blood of patients with RA [6]. Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine involved in the pathogenesis of RA, and antagonism of TNF may reduce the activity of the disease [7]. Treatment with a chimeric mAb to TNF-α has been shown to suppress inflammation [9] and improve patient well-being in RA, but the mechanisms of action of such treatment have not been fully explored [9,10]. The present study aims to find out the anti-CCP antibody level and expression level of Th2 cytokine-like TNF-α in patients with RA from South India.

METHODS

Study population

Patients details such as age, sex, history of RA, and autoimmune markers were recorded in the prescribed pro forma formatted for this study. The patients attending the Arthritis and Rheumatism Care Centre, Vadapalani, Chennai and healthy individuals from the Presidency College, Chennai were enrolled for this study. The study group included 74 patients with RA and 50 healthy individuals without history of RA. This study was approved by the Ethical Committee (No: 15102013) of Madras Medical College and Hospital, Chennai, Tamil Nadu, and an informed written consent was obtained from each participant.
Collection, transport, and storage of clinical specimens
For laboratory diagnosis of RA, 3-5 ml of blood samples was aseptically collected using Vacutainer (BD Bioscience), and the separated serum samples were transported to the Department of Microbiology, Presidency College, Chennai, Tamil Nadu, in cold chain. The serum samples were labeled and stored in standard screw capped leak proof vials and frozen at −20°C until further processed.

Anti-CCP antibody
Anti-CCP antibodies were detected by enzyme-linked immunosorbent assay (ELISA) kit obtained from Orgentec, Germany. This assay was performed with the patient’s sera according to the manufacturer’s instructions. CCP is bound to microwells. Antibodies against this antigen, if present in diluted serum, bind to the respective antigen. Washing of the microwells removes unspecific serum components. Horseradish peroxidase conjugated with antibody against anti-CCP antibody immunologically detects the bound patient ACPA forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end product. The intensity of this yellow was measured photometrically at 450 nm. This intensity of color was directly proportional to the concentration of anti-CCP antibodies in the original sample.

TNF-α ELISA
Serum concentrations of TNF-α were studied in patients with RA at various degrees of disease activity and in healthy controls, using an ELISA kit obtained from eBioscience, USA. The human TNF-α kit is a solid phase sandwich ELISA. A polyclonal antibody specific for human TNF-α has been coated onto the wells of the microtiter plate. Samples, including standards of known human recombinant TNF-α concentrations, and unknown were pipetted into these wells. During the first incubation, the human TNF-α antigen and a biotinylated polyclonal antibody specific for human TNF-α were simultaneously incubated. After washing, the enzyme (streptavidin-peroxidase) was added. After incubation and washing to remove the entire unbound enzyme, a substrate solution which is acting on the bound enzyme was added to induce a colored reaction product. The intensity of this colored product was directly proportional to the concentration of human TNF-α present in the samples.

Statistical analysis
Data are presented as mean±standard deviation. Statistical Package for the Social Sciences package 20 for Windows was used. Differences in mean levels between the groups were determined by Chi-square test. A p<0.05 was considered statistically significant. Furthermore, correlation study was performed between the TNF-α and anti-CCP antibody level in RA patients.

RESULTS
Age-wise distribution of RA patients
Table 1 shows the demographic characteristics of RA patients. Age group analysis of RA patients revealed the maximum number of positive cases among the age group 41-50 (31%), followed by 31-40 (30%) and 21-30 (19%) years age groups. The number of RA cases has been drastically declined after 51-60 years (14%), and the minimum positivity was present in 71-80 years (1.3%) (Table 2).

Gender-wise distribution of RA patients
In the gender-wise distribution of RA, there was a preponderance of females 65 (87.83%) when compared with males 9 (12.16%).

Anti-CCP antibody levels
Samples from 74 patients with symptoms more likely for RA were collected. Out of the 74 samples, 65 (89.9%) were of females and 9 (12.1%) of males. The results of anti-CCP enzyme immunoassay revealed that out of 74 patients, all were anti-CCP positive, which included 65 females and 9 males. Higher levels of anti-CCP (456 IU/ml) were present in the age group between 41 and 50 followed by 21-30 years age group which shows 335.28 IU/ml of anti-CCP antibody level (Table 3).

TNF-α levels
The levels of TNF-α in serum were estimated in 74 RA patients and 50 healthy volunteers. The level of serum TNF-α was measured in the range of 4.6-1082.84 pg/ml for RA patients and 6.630-459.74 pg/ml for healthy control group. TNF-α level was highest in patients with RA when compared to normal individuals. The mean level of TNF-α in RA patients and healthy individuals were 358±156 and 85±95 pg/ml, respectively. A negative correlation was noted between anti-CCP antibody and TNF-α levels in RA patients (r=−0.008) (p=0.947) (Table 4). TNF-α levels were significantly increased in SLE patients compared with healthy individuals (p=0.0001) (Table 5). A significant inverse correlation was obtained between erythrocyte sedimentation rate and TNF-α levels (r=−0.2; p=0.03) (Table 6 and Fig. 1).

DISCUSSION
In the present study, 74 patients were positive for anti-CCP antibodies ranged from 21.1 to 804.7 IU/ml. Higher mean values of anti-CCP antibody level shows 335.28 IU/ml of anti-CCP antibody level (Table 3).

Table 1: Demographic characteristics of RA patients
| Findings                                      | Mean±SD values |
|----------------------------------------------|----------------|
| Age                                          | 40±9 years     |
| Gender (female/male)                         | 65/9           |
| ESR level                                    | 60±20 mm/h     |
| CRP level                                    | 15±8 mg/dl     |
| Anti-CCP antibody level                      | 245±205 IU/ml  |
| TNF-α level                                  | 358±156 pg/ml  |

Table 2: Age-wise distribution of RA cases
| S.No. | Age group in years | RA cases | Percentage |
|-------|--------------------|----------|------------|
| 1     | 1-10               | -        | -          |
| 2     | 11-20              | 3        | 4.05       |
| 3     | 21-30              | 14       | 18.9       |
| 4     | 31-40              | 22       | 29.7       |
| 5     | 41-50              | 23       | 31.0       |
| 6     | 51-60              | 11       | 14.8       |
| 7     | 61-70              | -        | -          |
| 8     | 71-80              | 74       | 100        |

RA: Rheumatoid arthritis

Fig. 1: Correlation between erythrocyte sedimentation rate and tumor necrosis factor-alpha levels in rheumatoid arthritis patients
Inui N, Enomoto N, Suda T, Kageyama Y, Watanabe H, Chida K. Anti-CCP antibodies in RA patients. J Rheumatol 2003;30:731-4.

RA: Rheumatoid arthritis, CCP: Cyclic citrullinated peptide, TNF-α: Tumor necrosis factor-alpha, SD: Standard deviation

Table 3: Mean values of anti-CCP antibodies in RA patients

| Age group in years | Total number of subjects | Mean±SD of anti-CCP | Mean±SD of TNF-α |
|--------------------|--------------------------|---------------------|------------------|
|                    | Female                   | Male                | Female           | Male            |
| 11-20              | 3                        | 95.3±9.35           | 0                | 81±96           |
| 21-30              | 14                       | 292.78±25.688       | 42.5±0           | 66±67           |
| 31-40              | 22                       | 291.68±21.538       | 30.5±0           | 44±26           |
| 41-50              | 23                       | 231.22±24.78        | 22.5±0.5         | 30±23           |
| >50                | 12                       | 198.12±25.17       | 274.9±29.32      | 44±43           |

RA: Rheumatoid arthritis, CCP: Cyclic citrullinated peptide, TNF-α: Tumor necrosis factor-alpha, SD: Standard deviation

Table 4: Correlation of anti-CCP antibodies and TNF-α in RA patients

| Age group in years | Total number of patients | Mean±SD of anti-CCP | Mean±SD of TNF-α | p values |
|--------------------|--------------------------|---------------------|------------------|---------|
|                    | Female                   | Male                | Female           | Male            |
| 11-20              | 3                        | 95.3±9.35           | 0                | 81±96           |
| 21-30              | 14                       | 292.78±25.688       | 42.5±0           | 66±67           |
| 31-40              | 22                       | 291.68±21.538       | 30.5±0           | 44±26           |
| 41-50              | 23                       | 231.22±24.78        | 22.5±0.5         | 30±23           |
| >50                | 12                       | 198.12±25.17       | 274.9±29.32      | 44±43           |

S: Significant, NS: Nonsignificant, RA: Rheumatoid arthritis

Table 5: TNF-α levels in RA patients and healthy individuals

| Variable      | RA patients | Healthy individuals | t value | p value |
|---------------|-------------|---------------------|---------|---------|
| TNF-α levels  | 358±156 pg/ml | 85±95 pg/ml | 0.0001 (S) |         |

RA: Rheumatoid arthritis, CCP: Cyclic citrullinated peptide, TNF-α: Tumor necrosis factor-alpha, SD: Standard deviation

Table 6: Correlation between anti-CCP antibody and TNF-α levels in RA patients

| Number of RA patients | Mean±SD of anti-CCP antibody | Mean±SD of TNF-α levels | r value | p value |
|-----------------------|------------------------------|-------------------------|---------|---------|
| 74                    | 245±205 IU/ml                | 358±156 pg/ml           | -0.008  | 0.947 (NS) |

NS: Nonsignificant, RA: Rheumatoid arthritis, CCP: Cyclic citrullinated peptide, TNF-α: Tumor necrosis factor-alpha, SD: Standard deviation

In the present study, the level of serum TNF-α was measured in the range of 8.6-1082.84 pg/ml for RA patients and 6.630-459.74 pg/ml for the healthy control group. TNF-α level was highest in patients with RA when compared to normal individuals. Straub et al. from Germany reported increased serum levels of TNF-α level (450 pg/ml) in 22 patients with RA, whereas low level in healthy controls (200 pg/ml) [13]. In another study, Danis et al. reported TNF-α level in 27 patients with RA. Their results showed that the median level of TNF-α was 1.2 ng/ml. Thus, the present study differs from earlier findings in the TNF-α level [6].

CONCLUSION
The result of our study suggests that TNF-α levels were significantly increased in RA patients compared to healthy individuals. Negative correlation was noted between anti-CCP antibody and TNF-α levels in RA patients.

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