Serum carbonic anhydrase I and II autoantibodies in patients with chronic lymphocytic leukaemia

AHMET MENTESE1, NERGIZ ERKUT2, SELIM DEMIR3, SERAP OZER YAMAN4, AYSEGUL SUMER5, MEHMET ERDEM4, AHMET ALVER4, MEHMET GIRAY SÖNMEZ2

1Program of Medical Laboratory Techniques, Vocational School of Health Sciences, Karadeniz Technical University, Trabzon, Turkey
2Department of Haematology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey
3Department of Nutrition and Dietetics, Faculty of Health Sciences, Karadeniz Technical University, Trabzon, Turkey
4Department of Medical Biochemistry, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey
5Department of Nursing, School of Health Services, Recep Tayyip Erdoğan University, Rize, Turkey

Abstract

Cancer is the second most important cause of mortality, and millions of people either have or have had the disease. Leukaemia is one of the most common forms of cancer. Autoantibodies that have developed against the organism’s self-antigens are detected in the sera of subjects with cancer. In recent years carbonic anhydrase (CA) autoantibodies have been determined in some autoimmune diseases and carcinomas, but the mechanisms underlying this immune response have not yet been fully explained. The purpose of this study was to determine CA I and II autoantibodies in subjects with chronic lymphocytic leukaemia (CLL) and to provide a novel perspective regarding the autoimmune basis of the disease. Autoantibody levels were investigated using enzyme-linked immunosorbent assay (ELISA) in serum samples from 37 patients with CLL and 37 healthy peers. Anti-CA I titres in the CLL group were significantly higher compared with the control group (p = 0.0001). However, there was no significant difference between CLL and control groups in terms of anti-CA II titres (p = 0.278). The prevalences of CA I and II autoantibodies in patients with CLL in this study were 27% and 24.3%, respectively. Our results suggest that these autoantibodies may be involved in the pathogenesis of CLL. More extensive studies are now needed to reveal the entire mechanism.

Key words: autoantibody, cancer, carbonic anhydrase, chronic lymphocytic leukaemia.

Introduction

Chronic lymphocytic leukaemia (CLL) is characterised by the progressive accumulation of monoclonal lymphocytes with various phenotypes, such as CD5+, CD19+, CD20dim, CD23+, and SmIgdim in peripheral blood, bone marrow, and lymphoid tissues [1]. CLL is the most common form of leukaemia in adults, representing 25-30% of all leukaemias [2]. Approximately 18,960 new cases are diagnosed annually, and approximately 4660 deaths from CLL are predicted in adults in the USA in 2016. CLL is more common in men than in women, with a sex ratio of approximately 1 : 3 [3]. Compared with other chronic lymphoproliferative disorders, more severe immune disorders are seen in patients with CLL [2]. Autoimmunity is a well-known complication of lymphoproliferative diseases, in particular of CLL [4]. It has been suggested that leukaemia B cells are responsible for the production of autoantibodies in patients with CLL [5]. Induction of autoimmunity is characterised by the development of autoantibodies against a large number of autoantigens associated with malignant diseases. The presence of autoantibodies has been shown in the sera of patients with solid tumour and haematological malignancies [6]. The detection of these antibodies is expected to make an important contribution to the early diagnosis of some types of cancer [7-9].

Carbonic anhydrases (CAs) are vitally important enzymes responsible for the regulation of acid-base homeostasis in both healthy and pathological conditions. Members of the CA family contain 16 isoenzymes that differ from one another in terms of tissue distribution, cell localisation, catalytic activity, and resistance to inhibitors. These perform various functions, such as transport of carbon dioxide, pH regulation, ion transport, formation of stomach acidity, bone resorption, calcification, and tumorigenesis...
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Material and methods

Study group

Informed consent was obtained from all patients and controls, and approval for the study was granted by the Local Ethics Committee under reference no. 2016/30. Thirty-seven patients with untreated CLL in the A stage of the disease as classified by Binet, and thirty-seven healthy volunteers, were enrolled [15]. Clinical staging of the patient group was also defined according to Rai et al. [16]. The subtypes of CLL according to Rai scores (0-IV) were as follows: stage 0: 1 (2.7%); stage I: 20 (54.1%); and stage II: 16 (43.2%). The study group consisted of 16 women and 21 men, with a mean age of 67.4 ±10.34 years, and the control group comprised 15 women and 21 men, with a mean age of 67.4 ±10.34 years. There was no significant difference in terms of mean age between the study and control groups (p > 0.05).

Determination of serum autoantibody to CA I and II

Serum CA I and II autoantibodies were calculated using enzyme-linked immunosorbent assay (ELISA) method as previously described elsewhere [13, 17]. Briefly, flat-bottomed plates were coated with CA I or II (10 μg/ml) (Sigma-Aldrich, St.Louis, MO, USA) in carbonate buffer (pH = 9.6). These were then incubated for 18 hours at 4°C. In the next stage, the wells were washed four times with phosphate buffer (PBS) (pH = 7) before being blocked with 3% skim milk in PBS at room temperature for 2 h. The wells were then washed again four times with PBS containing 0.05% Tween-20 before incubation with 100 μl of 1 : 200 diluted serum for 2 h. Following these washing procedures, each individual well was incubated for 2 h with 100 μl of a 1 : 2000 solution of peroxidase-conjugated anti-human IgG anti-serum (Sigma-Aldrich, St. Louis, MO, USA) in 3% skimmed milk in PBS. A further five washes were performed with PBS containing 0.05% Tween-20, and the wells were then incubated with 100 μl substrate solution for 20 min. Reactions were halted by adding 100 μl of 2 M sulphuric acid to each well. The resulting absorbance was measured at 480 nm (Molecular Devices, CA, USA).

Statistical analysis

Statistical analysis was performed on Statistical Package for the Social Sciences (Version 13.0, NY, USA) and MedCalc (Version 12.3, Mariakerke, Belgium) statistical software. Compatibility with normal distribution was determined using the Kolmogorov-Smirnov test. Data were shown as mean ±standard deviation for normal distributed and median (interquartile range-IQR) for non-normal distributed variables. Differences between the two groups were analysed using Student’s t-test for normally distributed data or Mann Whitney U test for non-normal distributed variables. Receiver operating characteristic (ROC) curves were used to determine the discriminatory dominance of CA I and II autoantibodies for the identification of CLL. Sensitivity, specificity, negative predictive values (NPV), and positive predictive values (PPV) were determined from ROC curves for autoantibodies of CA I, II, and other parameters. p < 0.05 was regarded as significant.

Results

Thirty-seven CLL patients and 37 healthy subjects were included in this study. There was no significant difference in terms of mean age between the study and control groups (p > 0.05).

The mean absorbance value of anti-CA I antibodies for healthy subjects was 0.094 ±0.048, and the absorbance was higher than 0.190. The mean absorbance +2SD of the
healthy subjects was determined as positive. Positive results were obtained in 10 of the 37 cases with CLL. The mean absorbance value of the CLL group (0.154 ±0.086) was significantly higher ($p = 0.0001$) than that of the healthy subjects (Table 1).

The mean absorbance value of anti-CA II antibodies for the healthy subjects was 0.072 ±0.030, and the absorbance was higher than 0.132. The mean absorbance +2SD of the healthy subjects was also positive. Positive results were obtained in nine of the 37 cases with CLL. Values in one of the control subjects exceeded this cut-off level. The mean absorbance value of the CLL group (0.087 ±0.074) was not significantly different to that of the healthy controls ($p = 0.278$) (Table 1).

ROC curve analysis was also used to quantify lymphocytes, and anti-CA I and II antibody levels. Values for cut-off points, AUC, sensitivity, specificity, PPV, and NPV for individual parameters are shown in Table 2 and Figure 1.

**Table 1. Clinical characteristics of the two groups**

|                      | CLL ($n = 37$) | Control ($n = 37$) | $p$  |
|----------------------|---------------|-------------------|------|
| Anti-CA I Ab (ABSU)  | 0.154 ±0.086  | 0.094 ±0.048      | 0.0001 |
| Anti-CA II Ab (ABSU) | 0.087 ±0.074  | 0.072 ±0.030      | 0.278 |
| Haemoglobin (g/dl)   | 13.1 ±2.08    | 14.7 ±0.793       | 0.0001 |
| Haematocrit (%)      | 39.1 ±6.32    | 44.6 ±1.83        | 0.0001 |
| Lymphocytes (cells/μl) |              |                   |      |
|                     | 2290 (1885-2770) | 37400 (21550-50450) | 0.0001* |
| Platelets (cells/μl) | 192865 ±84730 | 229540 ±56490     | 0.032 |
| Lactate dehydrogenase (U/l) | 192 ±22.5 | 434 ±181 | 0.0001 |
| CD5+ (%)             | 85.6 ±21.2    |                   |      |
| CD19+ (%)            | 86.8 ±12.0    |                   |      |

**Table 2. Receiver operating characteristic curve analysis of lymphocyte count, anti-CA I and II antibody levels, and their sensitivity, specificity, and positive and negative predictive values**

|                      | AUC     | Cut-off point | Sensitivity | Specificity | PPV   | NPV   |
|----------------------|---------|---------------|-------------|-------------|-------|-------|
| Anti-CA I Ab         | 0.721   | > 0.072       | 95 (79-98)  | 43 (27-61)  | 61 (47-74) | 80 (56-94) |
| Anti-CA II Ab        | 0.526   | > 0.047       | 46 (30-63)  | 84 (68-94)  | 74 (52-90) | 61 (48-75) |
| Lymphocyte count     | 0.993   | > 0.349       | 100 (91-100)| 97 (86-100)| 97 (86-100)| 100 (90-100)|

Sensitivity, specificity, PPV and NPV values were expressed as % within a 95% CI

**Discussion**

Cancer is the second most important cause of mortality, and millions of people either have or have had the
Oxidative stress has been reported to be potentially significant in the formation of these autoantibodies [12, 13, 21, 26, 27]. Oxidative stress results from acceleration of the rate of free radical formation and/or a decrease in the rate at which these are eliminated. In either condition a severe imbalance occurs between free radical formation and the antioxidant defence mechanism [28]. Increased reactive oxygen or nitrogen species (ROS/RNS) lead to tissue injury and compromise of numerous biomolecules, including proteins, nucleic acids, structural carbohydrates, and lipids. The reaction of ROS with lipids causes these molecules to undergo oxidative breakdown. Malondialdehyde (MDA) is an end-product of lipid peroxidation capable of being covalently bound to proteins, and especially to the e-amino groups of lysine residues. These oxidative disturbances may influence the immune system, resulting in the development of specific autoimmune processes [29]. The lipid peroxidation end-product 4-hydroxy-2-nonenal (HNE) and MDA are known to alter proteins and to modify their antigenic properties [30]. One study of erythrocytes proved that CA II is the first target of HNE [31]. Numerous anti-MDA-modified proteins have been detected in systemic diseases in previous studies, such as systemic lupus erythematosus, periarthritis nodosa, scleroderma, atherosclerosis, and rheumatoid arthritis. It has also been suggested that these autoantibodies may be of predictive value for systemic diseases [29, 32-35]. Studies have shown increased levels of oxidative stress parameters, such as MDA, superoxide anion, and nitric oxide, while decreased levels of the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) in the sera of patients with CLL [36, 37]. In light of these data, we anticipated that oxidative by-products, including MDA, might generate the spread of neoantigens and confirm a potential association between autoimmunity and oxidative stress.

Conclusions

In conclusion, CA I autoantibody titres were significantly higher in subjects with CLL compared to the controls. More extensive studies are now needed to reveal the entire mechanism involved.

The authors declare no conflict of interest.

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