Anti-Carbonic Anhydrase II Antibodies in End-Stage Renal Disease Patients

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Introduction

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing enzyme. Sixteen CA isoenzymes with different tissue distributions and cellular localizations have been described in mammals. Fourteen of these catalyze the reversible hydration of carbon dioxide to bicarbonate and the other two do not exhibit catalytic activity. The catalytic reaction plays important physiological roles, including CO\textsubscript{2} transport, ion secretion, pH regulation and calcification \cite{1}. The presence of certain CA isoenzymes (CA II, CA IV, CA VB and CA XII) at different cellular locations in the human kidney has been demonstrated. These are crucial for at least three physiological processes: pH regulation (by secreting and excreting protons due to the carbon dioxide hydration reaction catalyzed by the enzymes), the bicarbonate reabsorption process and ammonium ion output \cite{2}. Recent studies have shown the formation of an autoimmune response against the CA II isoenzyme in several diseases \cite{3–6} including Sjögren’s syndrome (SJS). The high urinary pH levels and renal tubular acidosis observed in this syndrome were attributed to that of the CA II autoantibodies \cite{7}. In another case, CA II antibodies were detected in patients with autoimmune pancreatitis with tubulointerstitial nephritis \cite{8}.

The mechanism responsible for autoantibody formation...
has not yet been identified, though it has been suggested that oxidative stress may be involved [9].

Patients with end-stage renal disease (ESRD) are exposed to powerful oxidative stress as a result of increased prooxidant capacity and a weakened antioxidant defense system. A chronic inflammatory state, advanced age, diabetes, uremic syndrome, leukocyte activation due to hemodialysis and the use of iron associated with anemia treatment are the main sources of oxidative stress in these patients [10, 11]. Malondialdehyde (MDA), the end product of lipid peroxidation and an important marker of in vivo oxidative status, is elevated in ESRD patients [12]. Studies have reported that oxidative stress is correlated with complications of ESRD such as atherosclerosis, dialysis-related amyloidosis, malnutrition and anemia [13]. Directly or indirectly, reactive oxygen species modify biomolecules including carbohydrates, proteins and DNA, contribute to the expansion of oxidative injury and may also trigger the initiation of the autoimmune process [14].

Ghrelin is an orexigenic peptide consisting of 28 amino acids and is mainly produced in endocrine cells in the stomach. It enhances appetite and regulates the long- and short-term energy balance. It is broken down by the kidneys and plays a significant role in the regulation of energy homeostasis, systemic inflammation and the cardiovascular system [15]. It is important in the pathogenesis of protein-energy wasting, systemic inflammation and cardiovascular complications in ESRD, all of which are significantly associated with patient outcomes including mortality [16, 17].

The existence of anti-CA II autoantibody in sera obtained from ESRD patients has not yet been reported. The objective of this study was to investigate the presence of anti-CA II antibodies in patients with ESRD and determine relationships between the autoantibody titers and other clinical parameters (ghrelin, glucose, BUN and creatinine) and discuss a possible role of anti-CA II antibody in the pathogenesis of ESRD.

Materials and Methods

Study Population

After receiving approval from the institutional ethics committee, informed consent was obtained from all patients and controls. The study enrolled 45 ESRD patients undergoing hemodialysis (3 times a week) as the study group and 45 healthy peers as the control group.

Exclusion criteria were: (1) acute ischemic diseases including acute coronary syndrome, acute ischemic cerebrovascular disease, acute peripheral arterial occlusion or acute mesenteric ischemia; (2) advanced liver or heart failure; (3) asymptomatic coronary ischemia, for which ECG testing was performed; (4) age <18 years; (5) patients starting hemodialysis for the first time, and (6) refusal to participate in the study. The healthy controls were selected according to their self-reported medical histories based on age and gender distribution.

Prehemodialysis blood samples (3–5 ml) for each patient and healthy volunteer were collected in vacutainer tubes containing separation gels. After clotting, blood samples were centrifuged at 3,000 rpm for 10 min. Serum samples were stored at −80°C until biochemical measurements were made.

Ghrelin (total ghrelin) was measured using commercial ELISA kits (Phoenix Pharmaceuticals, USA). Glucose, BUN and creatinine were measured using an autoanalyzer.

Enzyme-Linked Immunosorbent Assay for Serum Autoantibody to CA II

Human CA II, electrophoretically purified from erythrocytes, was obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Plasma anti-CA II was detected using ELISA following a previously described method [5]. Briefly, microtiter plates (high-binding, flat-bottomed plates; Deltalab, Spain) were coated with 50 μl of 10 μg/ml CA II in carbonate buffer (0.05 mM, pH 9.6) and incubated overnight at 4°C. The wells were washed 4 times with phosphate buffer (pH 7) and blocked with 2% skim milk in phosphate buffer for 2 h at room temperature (RT). After being washed 4 times with phosphate buffer containing 0.05% Tween-20, the wells were incubated with 100 μl of serum diluted with blocking buffer (1:200) for 2 h at RT. After washing, each well was incubated for 2 h at RT with 100 μl of 1:2,000 dilution of peroxidase-conjugated anti-human IgG anti-serum (Sigma) in blocking buffer. Following 5 washes with phosphate buffer containing 0.05% Tween-20, the wells were incubated with 100 μl of substrate solution for 20 min at RT. The reaction was stopped by the addition of 100 μl of 2 M H2SO4 to each well. The absorbance was read at 480 nm (VersaMax, Molecular Devices, Sunnyvale, Calif., USA). Control wells that were not coated with CA II were also used for the ELISA of each serum studied. All assays were performed in duplicate, and the specific binding of serum antibody to CA II was calculated as follows: the average absorbance of the antigen-coated wells minus the average absorbance of control wells: specific binding = A coated − A control.

Samples from an ESRD patient and a control subject were used for the determination of the intra-assay coefficient of variation. For the anti-CA II antibody assay, the intra-assay coefficient of variation was 5.8% (n = 5) in the ESRD patient and 3.9% (n = 5) in the control.

MDA Measurements

Lipid peroxidation in serum samples was determined as MDA concentration using the method described by Yagi [18]. Tetramethoxypropane was used as a standard, and MDA levels were calculated as nmol/ml [12].

Statistical Analysis

Descriptive statistical analysis was performed for all studied variables. The data were tested for normal distribution using the Kolmogorov-Smirnov test. Differences between the ESRD and control groups were investigated using the Mann-Whitney U test. The Spearman relationship coefficient analysis was used to examine the relationship between the serum anti-CA II antibody titers and other clinical parameters. p < 0.05 was considered significant.
Results

The mean values of serum anti-CA II antibody, MDA, ghrelin, BUN, glucose and creatinine as well as the age and gender of the patients and controls are summarized in table 1, and the anti-CA II antibody levels in the ESRD patients and control subjects are shown in figure 1. The mean absorbance value for the control subjects was 0.079 ± 0.032 (n = 45); absorbance values >0.175 (the mean absorbance + 3 SD of control subjects) were defined as positive. Positive results were obtained in 13 of the 45 patients with ESRD (fig. 1). The mean absorbance value of the ESRD group (0.170 ± 0.237) was significantly higher than that of the control group (p = 0.035). Serum anti-CA II antibody levels showed a positive correlation with both BUN and creatinine concentrations (r = 0.292, p = 0.034 and r = 0.269, p = 0.049, respectively; table 2).

The serum MDA levels of patients with ESRD (1.16 ± 0.89 nmol/ml) were higher than those in the control group (0.65 ± 0.59 nmol/ml; p < 0.001). There was also a positive correlation between MDA levels and anti-CA II antibodies in patients with ESRD (r = 0.287, p = 0.028; table 2).

The serum total ghrelin level in the control subjects was 6.50 ± 9.14 ng/ml while that in the ESRD patients was 13.69 ± 10.34 ng/ml, and the difference was statistically significant (p < 0.001). There was a negative correlation between the levels of ghrelin and anti-CA II (r = −0.278, p = 0.032; fig. 2).

In addition, within the ESRD group, the means of the parameters of patients with normal CA II antibody levels and those with positive CA II levels were compared. MDA, BUN and creatinine levels were higher but ghrelin and glucose levels were lower in the CA II antibody-positive group than in the normal-level CA II antibody group. However, these differences were not statistically significant (p > 0.05, not included in the table).

Discussion

This study showed an increased immune response to CA II and also an association between CA II autoantibodies and MDA (an oxidative stress marker) in ESRD patients. Antibodies have been shown to develop against

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**Table 1.** The mean values of clinical parameters and laboratory findings in the study groups

|                     | Control | ESRD    | p      |
|---------------------|---------|---------|--------|
| Number of subjects  | 45      | 45      | –      |
| Age, years          | 51.26 ± 10.7 | 53.40 ± 13.9 | 0.334 |
| Gender, M/F         | 17/28   | 27/18   | –      |
| MDA, nmol/ml        | 0.72 ± 0.84 | 1.11 ± 0.84 | <0.001 |
| Ghrelin, ng/ml      | 6.50 ± 9.14 | 13.69 ± 10.34 | <0.001 |
| Anti-CA II Ab, ABSU | 0.079 ± 0.032 | 0.170 ± 0.237 | <0.035 |
| Glucose, mmol/l     | 4.8 ± 1.26 | 6.60 ± 2.96 | <0.001 |
| BUN, mmol/l         | 5.2 ± 0.84 | 21.26 ± 4.68 | <0.001 |
| Creatinine, μmol/l  | 58 ± 13.21 | 1,044 ± 693.05 | <0.001 |

Ab = Antibodies; ABSU = mean absorbance units.

**Table 2.** Correlations between anti-CA II antibodies and other laboratory findings in ESRD patients

|                 | Anti-CA II | MDA   | Ghrelin | Glucose | BUN   | Creatinine |
|-----------------|------------|-------|---------|---------|-------|------------|
| Anti-CA II      | r = 0.287  | −0.278| −0.015  | 0.292   | 0.269 |
|                 | p = 0.028  | 0.032 | 0.465   | 0.034   | 0.049 |
| Ghrelin         | r = −0.278 | 0.169 | −0.005  | −0.055  | 0.003 |
|                 | p = 0.032  | 0.268 | 0.974   | 0.734   | 0.988 |

* p < 0.05, significant difference between the groups.
erythropoietin [19] and Tamm-Horsfall protein [20] in hemodialysis patients. Levels of CA II autoantibodies were higher in the hemodialysis patients than in the controls (p < 0.035; fig. 1). Although similar results have been reported in several inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus and SJS, the mechanism by which CA antibodies form has not yet been fully explained [3, 4]. General mechanisms of antibody formation may be responsible, and it has recently been reported that oxidative stress-related modifications may be involved [9].

We summarize here the mechanisms by which CA antibodies form in ESRD patients. (1) Lipid peroxidation end products, i.e. HNE (4-hydroxy-2-nonenal) and MDA, modify proteins and alter their antigenic properties [21]. The primary target of HNE in erythrocytes is CA II [22]. In particular, erythrocytes are exposed to both physical stress occurring during dialysis and oxidative stress caused by the factors mentioned above. CA II present in erythrocytes or damaged kidney tissue may acquire an antigenic property due to similar modifications. In agreement with this, we determined a positive correlation between CA II autoantibodies and MDA levels (p = 0.028; table 2). (2) Oxidative stress and hemodialysis are also known to induce protein catabolism and apoptosis in ESRD [23]. It has been reported that apoptotic bodies are important in the formation of antibodies against intracellular proteins [24]. (3) The CA of Helicobacter pylori may trigger an autoimmune response against human CA II as an antigen [25]. A high prevalence of H. pylori has been found in hemodialysis patients. In addition, H. pylori infection reduces ghrelin production from the gastric mucosa and therefore lowers serum ghrelin levels [26]. In our study, we also observed a negative correlation between anti-CA II antibodies and ghrelin. (4) Four different CA isoenzymes located in different cell types and in subcellular locations are present in kidney tissue [2]. CA II autoantibodies might have formed against another antigenic CA isozyme because of molecular mimicry.

It is difficult to explain the potential roles of CA II autoantibodies in the pathology of ESRD. CA II, CA IV and CA XII are present in the human kidney. Cytosolic CA II is responsible for 95% of CA activity in the kidney. CA is responsible for bicarbonate reabsorption in the kidneys, NH₄⁺ excretion and the acid-base balance [2]. The inhibition of CA II by autoantibodies has been demonstrated [27]. In addition, autoantibodies are known to be taken up into target cells via receptor-mediated (Fc receptor) and nonreceptor paths (electrostatic interactions and endocytosis) [24]. Antibodies entering into kidney tissue or filtered into the channels may cause renal tubular acidosis as a result of CA inhibition. Transporters that function with H⁺ produced by CA can also be inhibited. A similar clinical picture has been reported in patients with primary SJS [7]. Renal tubular acidosis has been observed in these patients, and a significant correlation has been found between CA II antibodies and urine pH.

Inflammation, cardiovascular complications and protein-energy wasting are the main sources of mortality in ESRD patients. Ghrelin is a peptide involved in all these different pathways. Ghrelin levels are high in ESRD, both as a decrease of breakdown in the kidney and as a defense mechanism against hunger [16, 17]. Ghrelin has been reported to be important as a potential marker of mortality, and the death rate rises as its levels decrease [17]. In this study, ghrelin levels were higher in the ESRD patients than in the controls (table 1). CA II autoantibody titers were higher in ESRD patients. Together with this, the negative correlation of CA II antibodies with ghrelin (fig. 2) and its association with oxidative stress suggest its potential use as a mortality marker in ESRD patients. In addition, CA II autoantibodies were positively correlated with BUN and creatinine (r = 0.292, p = 0.034 and r = 0.269, p = 0.049, respectively).

Conclusion

In ESRD patients, the autoimmune response against CA II, oxidative stress and the ghrelin levels increased. Further studies are required in order to reveal the pos-
Possible mechanistic link between CA II autoantibody and oxidative stress, and the role of CA II autoantibody in the pathogenesis of the ESRD as well as to explore (if it exists) the potential prognostic value of CA II in ESRD.

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