Article

Serum anti-carbonic anhydrase I and II antibodies and idiopathic recurrent pregnancy loss



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Abstract

In up to 50% of recurrent pregnancy losses (RPL), the underlying pathophysiological mechanisms remain undetermined. Immunological factors may be involved. The objective of this study was to compare serum concentrations of autoantibodies to carbonic anhydrase (CA) I and II in women with and without RPL. Serum anti-CA-I and anti-CA-II concentrations in 29 women with RPL and 39 women without RPL on menstrual cycle day 3 were assessed and compared for this prospective age-matched controlled study. The mean serum anti-CA-I (0.287 ± 0.177 versus 0.184 ± 0.093, $P = 0.003$) and anti-CA-II (0.496 ± 0.240 versus 0.344 ± 0.108, $P = 0.001$) antibody concentrations were significantly higher in women with RPL, compared with controls. For serum anti-CA-I, an absorbance higher than 0.463 was taken as positive (mean + 3 SD of controls) and was detected in four of 29 patients with RPL (13.79%, 0.287 ± 0.177). For serum anti-CA-II, an absorbance higher than 0.668 was taken as positive (mean + 3SD of controls) and was detected in seven of 29 patients with RPL (24.14%, 0.496 ± 0.240). All patients with positive anti-CA-I antibody also had positive anti-CA-II antibody. Antibodies specifically reactive to CA-I and CA-II were found to be present at a higher frequency in the serum of subjects with RPL.

Keywords: *antibody, carbonic anhydrase I and II, recurrent pregnancy loss*

Introduction

Recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses prior to week 20 of gestation (Practice Committee of the American Society for Reproductive Medicine, 2008). It is a common clinical problem in reproduction affecting 1–2% of women of reproductive-age (Hussein, 2003; Daher *et al.*, 2004). A series of aetiological factors, including parental chromosomal abnormalities, uterine abnormalities, hereditary thrombophilias, endocrinological disorders, immunological factors, infections and nutritional and environmental factors have been identified for this condition. In up to 50% of cases, however, the exact underlying pathophysiological mechanisms remain undetermined (Papazoglou *et al.*, 2005). It has been suggested that these unexplained RPL might be due to immunological factors and several have been shown to be involved in RPL (Darmochwal-Kolarz *et al.*, 2002). Autoimmune factors represent the immunological response of the mother to a pregnancy that can cause fetal rejection in 30% of women with RPL. Various autoantibodies that may be related with abortion in women with RPL are antiphospholipid

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antibodies, antinuclear antibodies, antithyroid antibodies and anti-endothelial cell antibodies (Pandey *et al.*, 2005; Baek *et al.*, 2007).

Carbonic anhydrases (CA; EC 4.2.1.1) are zinc-containing enzymes, widely distributed and present in at least 16 different isoforms in mammals. These enzymes catalyse a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification, tumourigenicity and many other physiological or pathological processes including the reproductive tract (Supuran *et al.*, 2003; Supuran and Scozzafava, 2007).

Anti-CA-I and anti-CA-II antibodies have recently been isolated from patients with systemic lupus erythematosus, endometriosis, Sjögren syndrome, idiopathic chronic pancreatitis, ulcerative colitis, Hashimoto's thyroiditis (mean anti-CA-I, 0.242 ± 0.143; mean anti-CA-II, 0.412 ± 0.144) and Graves' disease (mean anti-CA-I, 0.361 ± 0.407; mean anti-CA-II. 0.543 ± 0.242), for some of which their presence can be a reliable diagnostic indicator (Itoh and Reichlin, 1992; D'Cruz *et al.*, 1996; Andoh *et al.*, 2002; Aparisi *et al.*, 2005; Alver *et al.*, 2007). The existence of anti-CA antibodies in sera obtained from the subjects with RPL has not been reported until now.

The main objective of this study was to compare the serum concentrations of serum autoantibodies to CA-I and CA-II in women with and without RPL.

Materials and methods

Patient selection

Following the allocation of women with RPL to the study group ($n = 29$), women who were age-matched with the study group were selected for the control group ($n = 39$). The control group comprised of healthy, regularly menstruating women attending the gynaecology unit for routine gynaecological examination. The women in the control group had no history of pregnancy losses and had no abnormalities in serum hepatic, renal function tests and had had at least one successful pregnancy.

RPL was diagnosed according to the American Society for Reproductive Medicine definition criteria as three or more consecutive pregnancy losses. Pregnancy was defined by multiple positive urine β-human chorionic gonadotrophin and/or ultrasound documentation. Pregnancy loss was defined as any natural abortion occurring at or before 20 weeks of gestation or with a fetal weight of less than 500 g prior to the 20th week of gestation (Practice Committee of the American Society for Reproductive Medicine, 2008). Patients whose losses all occurred after 20 weeks were excluded. Molar pregnancies, chemical pregnancies, ectopic pregnancies and pregnancy terminations were also excluded from analysis. For each woman, a thorough medical and obstetric history was obtained and a physical examination was performed by one of the authors. As part of an ongoing investigation of the causes of RPL, all patients had an evaluation consisting of: karyotypes on both partners; hysterosalpingogram; blood tests for PRL, thyroid-stimulating hormone, fasting glucose and midluteal progesterone; tests of lupus anticoagulant activity (dilute Russell viper venom test and partial thromboplastin time-lupus anticoagulant and immunoglobulin G (IgG) and IgM anticardiolipin and antiphosphatidyl serine antibodies; and cervical cultures for chlamydia, mycoplasma and ureaplasma. As a part of a research protocol, a thrombophilic workup was added that included testing for protein C activity, protein S activity, antithrombin activity, activated protein C resistance, factor V Leiden mutation, factor II mutation and fasting homocysteine. All testing was performed when women were not pregnant and at least 6 weeks after a miscarriage. The women with positive laboratory findings were not included; only women with unexplained recurrent pregnancy losses were allocated for this prospective case controlled study.

Exclusion criteria were as follows: (i) endocrinopathies (including diabetes mellitus, hyperprolactinaemia, Cushing's disease and congenital adrenal hyperplasia), a systemic disease (e.g., asthma), a collagen disorder, hypercholesterolaemia, sickle cell anaemia, a history of neoplasm; (ii) patients with hypertension, a family history of coronary artery disease and electrocardiographic changes suggestive of coronary artery disease, a history of angina or myocardial infarction, or a history of any known vascular, infectious, or inflammatory diseases; (iii) use of any medication (e.g., insulin-sensitizing drugs, oral contraceptives, antiandrogens, statins, aspirin, corticosteroids and GnRH agonists and antagonists) in the preceding 3 months; (iv) current smoker; (v) abnormal renal, hepatic and thyroid function test results; and (vi) refusal to participate in the study. The protocol of this investigation was approved by the Medical Ethics Committee of the study institution and informed consent to participate in the study was obtained from all subjects.

A total of 5 ml blood sample for each subject was collected in serum separator gel containing vacutainer tube. After clotting, blood samples were centrifuged at 900 *g* for 10 min. Serum samples were stored at −80°C until anti-CA-I and anti-CA-II antibody measurements.

Enzyme-linked immunosorbent assay (ELISA) for serum antibodies to CA-I and CA-II

Human CA-I and CA-II, electrophoretically purified from erythrocytes, were purchased from Sigma Chemical (St. Louis, MO, USA). Serum anti- CA-I and CA II were detected by ELISA according to a previously described method (Kino-Ohsaki *et al.*, 1996) with minor modifications. Briefly, microtitre plates (high binding, flat-bottomed plates; BD Biosciences, USA) were coated with 50 µl of 10 µg/ml CA-I and CA-II in carbonate buffer (0.05 mmol/l, pH 9.6)

and incubated overnight at 4°C. The wells were washed four times with phosphate buffer (pH 7) and blocked with 2% skimmed milk in phosphate buffer for 2 h at room temperature (RT). After being washed four times with phosphate buffer containing 0.05% Tween 20, wells were incubated with 100 µl of serum diluted with dilution buffer (1:200) for 2 h at RT. After washing, each well was incubated for 2 h at RT with 100 µl of 1:2000 dilution of peroxidase-conjugated anti-human IgG anti-serum (Sigma) in dilution buffer. Following five washes with phosphate buffer containing 0.05% Tween 20, wells were incubated with 100 µl substrate solution for 20 min at RT. The reaction was stopped by the addition of 100 µl of 2 mol/l H_2SO_4 to each well. The absorbance was read at 480 nm. Control wells that were not coated with CA-II were also used for ELISA of each serum studied.

All assays were performed in duplicate and the specific binding of serum antibody to CA-I or 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).

Statistical analysis

Statistical analyses were performed by using Statistcial Package for Social Sciences version 13.0.1 (SPSS Inc., USA). Results are expressed as means ± SD. The data were assessed for normal distribution by using Kolmogorov–Smirnov test. Mann–Whitney *U*-test was used to compare the variables obtained from patient and control groups. The relationship between serum concentrations of anti-CA antibodies was assessed using Pearson's correlation coefficient analysis. A value of $P < 0.05$ was accepted as significant.

Results

The mean ages of the women in RPL and control groups were 30.6 ± 4.9 years (range 23–38) and 30.9 ± 3.8 years (range 19–39), respectively. There was no significant difference in age between the groups (**Table 1**).

The mean serum anti-CA-I (0.287 ± 0.177 versus 0.184 ± 0.093, $P = 0.003$) and anti-CA-II (0.496 ± 0.240 versus 0.344 ± 0.108, $P = 0.001$) antibody concentrations were significantly higher in women with RPL, compared with control subjects.

The anti-CA-I antibody concentrations in patients with RPL and control subjects were quantified by ELISA at a serum dilution of 1:200 (**Figure 1**). The mean absorbance

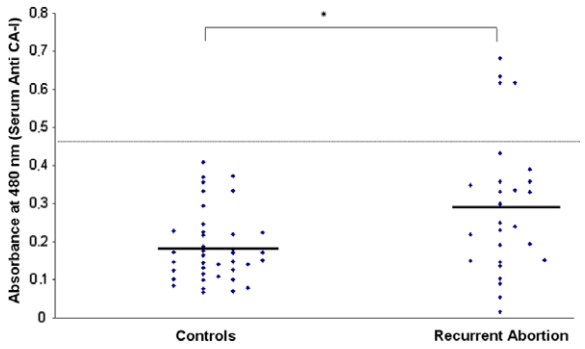


Figure 1. Anti-carbonic anhydrase I (anti-CA-I) antibodies in sera from women with recurrent pregnancy loss (RPL) and healthy controls. The dotted line indicates the mean value plus 3 SD of healthy control sera ($A_{480} = 0.463$). * = significant difference in comparison of mean ± SD value between women with RPL and healthy controls ($P < 0.05$).

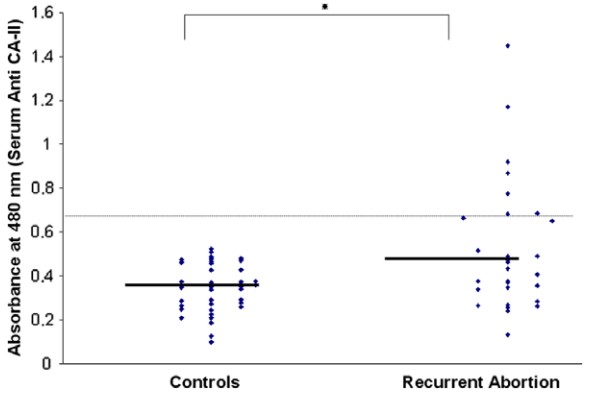


Figure 2. Anti-carbonic anhydrase II (anti-CA-II) antibodies in sera from women with recurrent pregnancy loss (RPL) and healthy controls. The dotted line indicates the mean value plus 3 SD of healthy control sera ($A_{480} = 0.668$). * = significant difference in comparison of mean ± SD value between women with RPL and healthy controls ($P < 0.05$).

value for the control subjects was 0.184 ± 0.093 ($n = 39$) and the absorbance higher than 0.463 was taken as positive (mean + 3 SD of control subjects). Anti-CA-I antibody was detected in four of 29 patients with recurrent pregnancy losses (13.79%, 0.287 ± 0.177).

The anti-CA-II antibody concentrations in patients with recurrent pregnancy loss diagnosis and control subjects

Table 1. Demographic characteristics of women with recurrent pregnancy loss and controls.

	Recurrent pregnancy loss group (n = *29*)	*Control group* (n = *39*)	P-*value*
Age (years)	30.6 ± 4.9	30.9 ± 3.8	NS
Gravida (*n*)	3.2 ± 1.2	2.6 ± 1.3	<0.01
Parity (*n*)	0.4 ± 0.9	1.7 ± 0.7	<0.001
Abortion (*n*)	3.1 ± 1.1	0.0 ± 0.0	<0.01

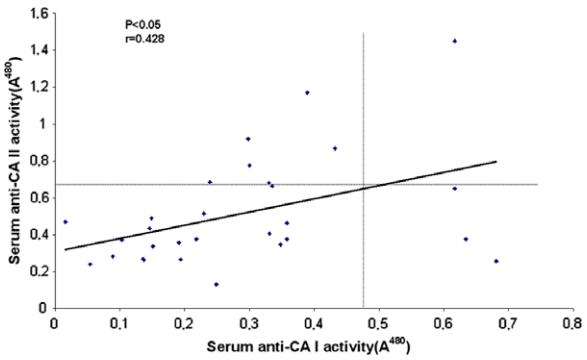


Figure 3. Correlation between anti-CA-I antibody titre and anti-CA-II antibody titre in patients with recurrent pregnancy loss. Dotted lines indicate the mean value + 3 SD of healthy control sera absorbance (A_{480}) in both antibodies.

were quantified by ELISA at serum dilution of 1:200 (**Figure 2**). The mean absorbance value for the control subjects was 0.344 ± 0.108 ($n = 39$) and the absorbance higher than 0.668 was taken as positive (mean + 3 SD of control subjects). Anti-CA-II antibody was detected in seven of 29 patients with recurrent abortion (24.14%, 0.496 ± 0.240).

All patients that were positive for anti-CA-I antibody were also positive for anti-CA-II antibody. A significant positive correlation was found between anti-CA-II antibody and anti-CA-I antibody titres in patients with RPL ($r = 0.428$, $P < 0.05$) (**Figure 3**).

Discussion

The present study showed that serum anti-CA-I and anti-CA-II antibodies have a higher concentration in women with RPL than in those with women without RPL. As far as is known, this is the first report describing significant increases in serum anti-CA-I and anti-CA-II antibody concentrations in patients with RPL.

RPL represents the most common complication of reproductive failure but, despite years of research, information is still lacking on all the aetiologies of this event. Chromosomal defects, anatomical anomalies, endocrine disorders and infections are among the causes of RPL. However, in about 50% of cases, abortion remains unexplained and this has generated great interest in the investigation of possible immunulogical aetiologies of pregnancy failure (Raghupathy *et al.*, 1999; Darmochwal-Kolarz *et al.*, 2002). Autoantibodies are more common in patients with recurrent miscarriage including antiphospholipid (14%) and antinuclear antibodies (7%). Thyroid antibodies have also been shown to be increased in women with recurrent miscarriage (Horne and Alexander, 2005; Baek *et al.*, 2007).

Carbonic anhydrases, which reversibly catalyse the hydration of carbon dioxide to bicarbonate and hydrogen ions, are widely distributed in mammalian tissues and have an important role in gas transport, acid/base regulation and various secretory functions in tissues (Chiang *et al.*, 2004). Since CA-I and CA-II are the most common isoenzymes in human tissues, immunoglobulin G autoantibody against CA-I and CA-II has been detected in the sera of patients with a variety of autoimmune diseases (Hosoda *et al.*, 2004; Kita, 2004). Similarly, in the present study, serum concentrations of anti-CA-I and anti-CA-II antibodies were significantly increased in women with RPL. The uterine endometrium and placenta are known as sources of CA-I, CA-II and the other CA isoenzymes involved in many aspects of reproduction in human (Aliakbar *et al.*, 1990; Karhumaa *et al.*, 2000). The placenta functions as does the lung, liver, kidney and other endocrine organs to guide the exchange of all factors necessary for a successful embryonic development and, later, fetal development and growth. In the placenta, the enzyme CA may provide ions for exchange with Na^+, K^+ and Cl^- in the transepithelial movement of ions and fluid, as well as facilitate carbon dioxide diffusion. It can also be active in intermediary metabolism, such as gluconeogenesis, urea and fatty acid synthesis (Ridderstrale *et al.*, 1997).

Decidualized uterine tissues are maternal in origin and functionally important not only for implantation, but also for the maintenance of pregnancy. The uterine endometrium in humans has been reported as a source of CA and the activity of the endometrial CA doubles during the luteal phase of the menstrual cycle (Ali Akbar *et al.*, 1998). In summary, the expression of CA isoenzymes in decidual tissues, placental tissues and in a variety of other fetal tissues during the early stages of intrauterine life suggests that the enzyme has an important role in the processes of fertilization, implantation and feto–placental development.

Pincus and Bialy (1963) showed that Diamox, a highly specific inhibitor of CA, terminated pregnancy in rats when administrated directly into the uterine lumen. Also, direct distillation of acetazolamide, an efficient CA inhibitor, into the rabbit uterine lumen prevented pregnancy, by preventing secretion of bicarbonate by the endometrial epithelium.

It has been ascertained experimentally that anti-CA-II antibody specifically inhibits the enzyme activity of CA, suggesting that the site of the immunological reaction may involve the active site of the enzyme (Botre *et al.*, 2003; Hosoda *et al.*, 2004). Rojas and Apodaca (2002) reported that IgA, IgG and IgM are transported across epithelial cells during the receptor-mediated transcytosis process and it is possible that anti-CA antibodies, which are capable of inhibiting CA catalytic activity, may similarly contribute to the destruction of biliary epithelial cells during transcytosis in primary biliary cirrhosis. It may be speculated from these findings that antibodies against CA-I and CA-II enzymes are capable of inhibiting CA catalytic activity and may contribute to the pathogenesis of RPL by disturbing placental and endometrial functions.

In the current study, detectable serum concentrations of anti-CA-I and CA-II were observed in normal subjects. This may be attributed to the assay used, since there is no available gold-standard method for measuring serum concentrations of anti-CA-I and CA-II antibodies. This point is one of the limitations of this study. Another limitation is that serum concentrations of anti-CA-I and CA-II antibodies were not measured in women with a single

miscarriage. In order to confirm the association with recurrent pregnant loss, it is necessary to show that patients with a single miscarriage are comparable to the general population and not to the recurrent pregnancy loss population. This will be investigated in a future study.

Although RPL may result from a variety of factors, there is an increase in the amount of evidence supporting an immunological mechanism for the losses in some women. In conclusion, antibodies specifically reactive to CA-I and CA-II were found to be present at a higher frequency in the serum of subjects with RPL in the present study. The results suggest that autoimmune responses against CA isozymes may be involved in the pathogenesis of unexplained RPL. Furthermore autoantibodies to CA-I and CA-II may also be a possible epiphenomena of RPL. Continued investigation may further the understanding of the pathophysiology of the RPL will lead to more successful treatment and improved outcome in women with recurrent miscarriage.

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