Published in final edited form as:

J Autoimmun. 2009 March ; 32(2): 133–139. doi:10.1016/j.jaut.2009.02.001.

Autoimmunity against Carbonic Anhydrase II Affects Retinal Cell Functions in Autoimmune Retinopathy

Grazyna Adamus and Landon Karren

Casey Eye Institute-Department of Ophthalmology, School of Medicine, Oregon Health & Science University, Portland, Oregon

Abstract

Autoantibodies against various retinal proteins, including anti-carbonic anhydrase II (CAII) autoantibodies, have been found in patients with cancer-associated retinopathy and autoimmune retinopathy without diagnosed cancer. We studied sera from retinopathy patients that showed reactivity with a 30-kDa retinal protein, which was identified as carbonic anhydrase II (CAII), and immunolabeled cells in human retina. The goal of the study was to examine whether patients' autoantibodies induce pathogenic effects on the catalytic function of CAII, which may have metabolic consequences on cell survival. Our findings revealed that anti-CAII autoantibodies have the capacity to induce cellular damage by impairing CAII cellular function through inhibiting the catalytic activity of CAII in a dose dependent manner, decreasing intracellular pH, increasing intracellular calcium, which in effect decreases retinal cell viability. The destabilized catalytic function of CAII and alterations in cytosolic pH were found very early, suggesting that autoantibodies are the inducers of apoptosis. In summary, our study showed that anti-CAII autoantibodies provoke pathogenic effects on retinal cells by decreasing cell survival by blocking the CAII cellular functions. The current experiments may facilitate better understanding the role of the immune system in retinal degeneration and help to develop better strategies for therapy of autoimmune retinopathy.

Keywords

autoimmune retinopathy; autoantibody; carbonic anhydrase II; calcium; pH; retina

INTRODUCTION

Autoimmunity has been suggested as a possible etiologic factor in retinal degeneration. Autoantibodies against various retinal proteins, including recoverin, enolase, and carbonic anhydrase II (CAII) have been found in patients with cancer-associated retinopathy (CAR) and in autoimmune retinopathy (AR) patients without diagnosed cancer (AR) at the time of initial examination {Adamus, 2004#1111}. The presence of anti-CAII autoantibodies have also been reported in patients with retinitis pigmentosa (RP) with cystoid macular edema but rarely in the unaffected population (6% prevalence), suggesting their role in pathogenicity of disease {Heckenlively, 1999 #592}.

Corresponding author: Grazyna Adamus, Casey Eye Institute-BRB, Mail code L467AD, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239, tel. 503-418-2540, Fax 503-418-2541, email: E-mail: adamusg@ohsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The mechanism of CAII antibody generation in autoimmune diseases remains to be elucidated. We propose that autoantibodies against CAII that are detected in patients with retinopathy may be a consequence of the immune response against CAII found in other tissues. Serum autoantibodies to CAII could be a result of the cross-reactivity against other antigens that mimic CAII or were elucidated in response to cancer antigens {Chegwidden, 2000 #1722}. For autoantibodies to be effective and induce retinal disease they have to persist at high titers, reach the target autoantigen in the organ, and induce pathogenic effects on the tissue. A body of evidence from previous *in vitro* and *in vivo* studies on the role of autoantibodies in retinal degeneration indicates that anti-recoverin autoantibodies found in patients with retinopathy are cytotoxic to retinal cells and induce apoptotic death of retinal photoreceptor cells, which leads to the degeneration of the photoreceptor cell layer in our animal model of CAR {Adamus, 1998#366;Cao, 2000#486;Shiraga, 2002#962;Ren, 2004 #1055;Ohguro, 1999#467}. Previously, we showed that anti-recoverin antibody causes a significant increase in intracellular calcium level by blocking calcium binding capacity of recoverin, a calcium binding protein, leading to retinal cell mitochondrial-mediated apoptosis {Adamus, 2006#1175;Shiraga, 2002#962}. In addition, anti-enolase antibody led to apoptosis by three different pathways: by blocking the catalytic activity of enolase, which results in depletion of glycolytic ATP and disturbance of retinal cell glycolysis; by causing an increase in the intracellular calcium levels, which activates mitochondrial-mediated apoptosis; and by causing the translocation of Bax protein into the mitochondria, which will result in the release of cytochrome c into the cytoplasm, initiating apoptosis {Magrys, 2007#1343}.

CAII has been accepted as a one candidate target autoantigen recognized during the autoimmune response in retinopathy. CAII belongs to a family of physiologically important enzymes - carbonic anhydrases (CAs) that catalyze a reversible conversion of carbon dioxide to bicarbonate, participate in ion transport and pH control. CA isoenzymes differ in their tissue distribution, subcellular localization and their susceptibility to CA inhibitors. The sequence alignment suggested that the active site structure is conserved in two human cytoplasmic enzymes CAI and CAII. However, the amino acid sequence identity of human CA XIV relative to the other membrane-associated isozymes (CAIV, CAIX, and CAXII) is only 34–46% {Whittingtons, 2004#1352}. Multiple CA isozymes are expressed in the retina, including membrane-associated CAIV and CAXIV, which have been implicated in facilitating the removal of CO₂ from the neuroretina and in photoreceptor function {Yang, 2005#1210;Nagelhus, 2005#1363}. Although systemic CA inhibition using acetazolamide increases retinal and cerebral blood flow, oxygen concentration in the optic nerve and blood CO₂ levels but these inhibitors can also impair photoreceptor function {Ilies, 2003#1738}.

The goal of the studies was to examine whether anti-CAII autoantibodies isolated from patients with AR induce pathogenic effects on the catalytic function of CAII, which may have metabolic consequences on cell survival. The *in vitro* studies were done using E1A-NR3 retinal cell line that represents immortalized undifferentiated retinal cells, expressing abundant retinal and neuronal markers of photoreceptor, Müller, and ganglion cell phenotypes {Seigel, 1999#1198}. The cells possess the functional capacity to respond to specific neurotransmitters and apoptotic insults {Seigel, 2004#1197;Adamus, 1997#168}. Our studies demonstrated that autoantibodies against CAII showed their pathogenic potential through the inhibition of the enzymatic activity, which in effect led to decrease the intracellular pH and increase the intracellular Ca²⁺ in retinal cells. These cellular alternations had detrimental effects on retinal cells viability leading to cell death.

METHODS

Autoantibodies

Sera were obtained from patients with progressive visual loss identified through the Ocular Immunology Laboratory - Oregon Health & Science University (OHSU). The study has been approved by the OHSU Institutional Review Board. Patients' sera were affinity-purified on a Protein G column (Pierce) and then dialyzed, concentrated, and checked for purity by SDS-gel electrophoresis, as described previously {Adamus, 1998#359}.

Carbonic anhydrase and Antibodies

Human CAII was purchased from Chemicon International and then purified on Ultralink Protein-G (Pierce) before use to remove impurities. Anti-human CAII II (Erythrocytes) polyclonal antibodies were also purchased from Chemicon International and used as control.

Cell Culture

E1A-NR3 retinal cell line was maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% MEM nonessential amino acids, 1% MEM vitamins, and 50 mg/mL gentamycin {Adamus, 1993#31}.

Western Blot Analysis

Initial screening of patients' sera was performed using human retinal proteins as described previously {Adamus, 2004#1111} to select the sera that reacted with 30-kDa on the blot. Then, the reactivity of sera with 30-kDa retinal protein was confirmed on blots containing 1 µg pure CAII.

Immunocytochemistry

Human retina sections from a donor eye were fixed in fresh 4% paraformaldehyde in PBS, pH 7.4, for 10 minutes at room temperature followed by washing and incubation with 0.3% H₂O₂ in PBS for 10 min. Sections were then blocked in 10% normal goat serum, 1% BSA, 0.2% Tween 20 in PBS for 1 hour. Then, patients' purified IgG at 50 µg/ml in 1% BSA, 0.2% Tween 20 in PBS were added for overnight incubation at 4°C; negative controls contain no antibodies. Sections were then washed as before, incubated in with biotinylated anti-human secondary antibody (Invitrogen) for 1 hour at room temperature, and washed again. Immunoreactivity was revealed by incubating the tissue sections in streptavidin-HRP conjugate (Invitrogen) for 30 minutes, washed, and incubated for 5 minutes with peroxidase DAB substrate (Pierce).

MTT Cytotoxicity Assay

Cells were allowed to attach to 96-well, flat-bottomed microtiter plates for four hours at a density of 2×10^4 cells/well in DMEM medium at a final volume of 100 µl per well. Doses of 75 µg, 150 µg, and 300 µg purified IgG were added to the culture for 72 hours. Three hours before the end of the antibody incubation, 25 µL of a 0.5% MTT (Thiazolyl blue, Sigma) solution was added to each well and then 100 µL of cell lysing buffer (10% SDS in 50% DMF) following by overnight incubation at 37°C. The color reaction was read in a BioRad Microplate Reader at 570 nm. The data are presented as a percentage of cell survival, calculated as follows: % cell survival = $\{1 - ((A_{\text{total}} - A_{\text{sample}})/A_{\text{total}})\} \times 100$, where A_{total} = OD of untreated cells and A_{sample} = OD of the cells treated with antibodies.

Titration of Antibodies

Microtiter plates were coated with 0.5 µg/well of CAII in 0.1 M Tris-HCl buffer, pH 9.0, overnight at room temperature. After blocking with 1% BSA in PBS for 1 hour and washing, 0.1 mg/ml of double serially diluted autoantibodies were added to each well and allowed to incubate for 1 hour, washed, and followed by 1 h incubation with anti-human IgG conjugated to peroxidase (Invitrogen.) Color reaction was developed for 30 minutes by incubation with peroxidase substrate (2–2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer, pH 4.5, containing 3% H₂O₂ and measured at 405 nm using a Bio-Rad Microplate Reader.

Isotyping of Antibodies

Microtiter plates coated with 0.5 µg/well of CAII in 0.1 M Tris-HCL buffer, pH 9.0, were blocked with 1% BSA, 5% normal goat serum (NGS) in PBS for 1 hour at room temperature. Patient serum (1:1000) was added and incubated for 1 hour followed by incubation with biotinylated Ig representing IgG1, IgG2, IgG3, IgG4, IgM, IgA, IgG (H+L) (Invitrogen) at 1:2000. Then, streptavidin conjugated to peroxidase (1:5000) was added for 30 min. Color reaction was developed for 30 minutes by incubation with peroxidase substrate (2–2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer, pH 4.5, containing 3% H₂O₂ and measured at 405 nm using a Bio-Rad Microplate Reader.

Measurement of Intracellular Ca²⁺

Changes in concentrations of free intracellular Ca²⁺ were measured using the Ca²⁺-sensitive fluorescent dye Fluo-4 NW (Molecular Probes). Cells were allowed to attach to 96-well, flat-bottomed, half area, black walled microtiter plates overnight at a density of 5×10³ cells/well in DMEM medium at a final volume of 100 µl per well. The medium was then removed and the cells were washed once with PBS containing 2.5 mM probenecid (Molecular Probes). Fluo-4 NW dye in 2.5 mM of probenecid acid was then added and the plate was incubated in the dark at 37°C for 30 minutes. The dye was then removed and the cells were washed once in Hank's Balanced Salt Solution (HBSS) containing 2.5 mM of probenecid acid followed by 30 minutes of incubation at 37°C in the dark in the same solution. Then, a 5 minute baseline measurement of each well was read using an FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments) with excitation 485/20, emission 528/20, and sensitivity 80 settings. Following baseline measurements purified patients' purified autoantibodies and positive controls were added at a final concentration of 1 mg/ml and immediately read using the same settings as the baseline at 15 minute intervals. The results are presented as a fold of increase in intracellular [Ca²⁺] from baseline.

Measurement of Intracellular pH

Changes in intracellular pH ([pH]_i) were measured using the pH-sensitive fluorescent dye BCECE-AM (Molecular Probes.) Cells were allowed to attach to 96-well, flat-bottomed, black walled microtiter plates overnight at a density of 2×10⁴ cells/well in DMEM medium at a final volume of 100 µl per well. Purified IgG were added at a final concentration of 1 mg/ml for 8 hr incubation. After the incubation, the medium was removed and the cells were washed with HBSS and 100 µL of HBSS containing 2.5µL/well of 200 µM BCECE-AM was added to all wells. The plate was incubated for 20 min at 37°C protected from light in a tissue culture incubator and shaken periodically. Then, the dye was then removed from the wells and 100 µL of HBSS for 30 min incubation at 37°C and then read using an FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments) with excitation 485/20, emission 530/20, and sensitivity 80 settings. Standard curve solutions were made from mixtures of 130 mM KH₂PO₄ or 110 mM K₂HPO₄ containing 20 mM NaCl in ddH₂O. In wells designated for pH

standards, 100 μ L of standard pH solutions were added. The pH values were then calculated using the regression equation from the pH standard curve.

Antibody Inhibition of Carbonic Anhydrase II Activity

Carbonic anhydrase activity was assayed by the colorimetric method using bromothymol blue {Rickli, 1964#1387}. The reaction mixture contained 100 μ L of 25 mM Tris-HCl, pH 8.2, with 1 mg/100 ml of bromothymol blue (Sigma) and was kept on ice. CA II was added to the reaction mixture to a final concentration of 100 ng. Various molar ratios of CAII II to anti-CA II antibodies were pre-incubated together for 1 hr at 4°C with rotational mixing. After incubation, 100 μ L of 25 mM Tris-HCl, pH 8.2, bubbled 30 minutes with CO₂ on ice was added to the reaction mixture and the colorimetric change was timed from the moment of injection until the endpoint and measured at 430 nm using a spectrophotometer (Beckman). Activity units (U) are calculated according to the formula: $U = 10 * ((T_b/T_c) - 1) / \text{mg protein}$ where T_b is the time of uncatalyzed reaction, T_c is the time of enzyme-catalyzed reaction endpoint, and mg protein is the amount of carbonic anhydrase II present.

Statistical analysis

Significant differences between groups were assessed using analysis of variance (ANOVA), the nonparametric Mann-Whitney U test and Chi-square test. A p value <0.05 was considered significant.

RESULTS

Based on sample availability in quantities needed to perform experiments, we selected six retinopathy sera designated P1–P6 that reacted with a protein of molecular weight 30-kDa on the blot containing retinal proteins. As shown in Figure 1A, these sera reacted with purified human CAII on the blot. Thus, the antigen was confirmed to be carbonic anhydrase II. Immunostaining of human retina with patients' purified autoantibodies revealed the cytoplasmic labeling of the cells in the ganglion cell layer, possible feet of the Muller cells, some cell bodies in the inner nuclear layer and the cytoplasm of inner and outer segments of photoreceptor cells (Fig. 1B–E).

Fig. 2A shows titration curves for patients' affinity purified IgG against pure human CAII determined by ELISA. These autoantibodies show high activity against CAII at titers up to 1:10,000. We also determined subclass distribution for immunoglobulins specific for CAII. As is shown in Fig. 2B, IgG subclass was IgG4 and IgG1 dominant.

CAII is present in the cytoplasm of the cells and is responsible for controlling intracellular pH. To examine whether autoantibodies affect the catalytic function of CAII enzyme the purified anti-CAII autoantibodies P2 were incubated with CAII at increasing ratios of IgG and then the enzymatic activity was measured. Fig. 3A shows that P2 inhibited the catalytic activity in a dose dependent manner. The activity dropped from 90% with 10-fold excess of P2 IgG to 48% with 40-fold excess of IgG of the initial CAII activity. Autoantibodies without specificity to CAII (normal subject) had no effect on the enzymatic activity but other anti-CAII autoantibodies (P1, P4, P5) inhibited the CAII activity at the level similar to that of the control - specific goat anti-human CAII antibodies (Fig. 3B). These results demonstrated that binding anti-CA autoantibodies to CAII was associated with decreased enzymatic activity, suggesting that the site of the immunologic reaction at least partially involved the active catalytic site of the enzyme. Because anti-CAII autoantibodies impact the CAII catalytic function thus we hypothesized that inhibition of the CAII catalytic function will affect ($[pH]_i$) pH changes in the cell.

To test this hypothesis we determined $[pH]_i$ of retinal cells grown in the presence of purified patients' autoantibodies for 8 hr. After incubation with autoantibody the cells were loaded with the pH-sensitive fluorescent dye BCECE-AM followed by the $[pH]_i$ measurement. The $[pH]_i$ of untreated retinal cell was estimated to be 7.6. Within 1 hr after the addition of IgG the pH in antibody-treated cells started to decrease (not shown). Fig. 4A shows that P1 – P6 autoantibodies caused a drop in $[pH]_i$ from 0.5 to 0.8 pH units after 8 hr-treatment, depending on anti-CAII antibody titers (Fig. 2). The lowering of $[pH]_i$ to 6.8 corresponds to the threshold for activation of acid endonuclease and caspase 3 and correlates with the induction of apoptosis {Gottlieb, 1996#1195;Reber, 2002#1733}. Normal IgG did not alter the $[pH]_i$ in retinal cells with the same amount of IgG added and time of incubation with cells. These findings suggest that the drop in $[pH]_i$ for a longer period of time will lead to intracellular cellular acidification, which may affect intracellular calcium levels {Barnes, 1991#1737;DeSantiago, 2007#1751;Masuda, 2007#1752}.

To examine whether anti-CAII autoantibodies have effect on free intracellular calcium $[Ca^{2+}]_i$, we investigated changes in $[Ca^{2+}]_i$ in retinal cells grown in the presence of purified autoantibodies for a period of 120 min and measured at 15 min intervals. Figure 4A shows a time response curve for P3 IgG. The maximum Ca^{2+} release was observed at 15 min and was significantly higher from normal control antibodies ($p<0.05$). Figure 4B shows that, at 15 min, other anti-CAII autoantibodies triggered a small but significant increase in $[Ca^{2+}]_i$ from about 3 fold to 8 fold compared to untreated cells. An ANOVA test revealed statistically significant differences between groups for different autoantibodies (all $p=0.02$). In summary, these results imply that changes in intracellular calcium may be linked to the inhibition of catalytic activity and acidification, which possibly causes a rise in $[Ca^{2+}]_i$.

To determine whether anti-CAII autoantibodies affect on retinal cell viability, cells were grown at different concentrations of purified autoantibodies (75 μ g, 150 μ g and 300 μ g/ml) and viability was examined by a MTT assay. Fig. 5 shows decreased cell survival after 72-hr antibody treatment in a dose dependent manner. At the highest antibody dose, the cell survival dropped and ranged from 30% (P1) to 70% (P5) compared to untreated control. In conclusion, anti-CAII autoantibodies, depending on specific their titers, were cytotoxic to retinal cells and had a devastating effect on retinal cell survival.

DISCUSSION

Our studies were designed to understand the pathogenic effects of anti-CAII autoantibodies on retinal cells to determine their role in retinal degeneration. The major conclusions from our studies revealed that anti-CAII autoantibodies have the capacity to induce cellular damage by impairing its cellular function through (1) inhibiting the catalytic activity of CAII, (2) decreasing intracellular pH, (3) increasing intracellular calcium, (4) which in effect influence cell survival. The destabilized catalytic function of CAII and alterations in cytosolic pH were found very early after autoantibodies were added to the living cells and before apoptosis occurs; usually after 24 hr {Shiraga, 2002#962}, further suggesting that autoantibodies are inducers of apoptosis. The decrease of $[pH]_i$ related to the function of the enzyme in the cell could cause acidification by changes in CO_2/H_2CO_3 metabolism and in proton pump activity, the Na^+/H^+ exchanger {Kniep, 2006#1362;Reber, 2002#1733}. Some anti-CAII autoantibodies caused a decrease of pH below pH 6.8, which could lead to caspase activation in those retinal cells. The efficiency of activation of caspase-3 by cytochrome *c* has been found to be pH sensitive, with an optimum pH of 6.6 to 6.8 {Gottlieb, 1996#1195;Reber, 2002#1733}. The earlier studies showed that acidification in Jurkat cells caused by anti-Fas antibody also preceded apoptosis, suggesting acidification is an early event in programmed cell death and may be essential for DNA destruction {Gottlieb, 1996#1195}. Hence, one can envision that antibody entry into the cell and binding to CAII will disrupt metabolic functions, which, in effect, increase the

propensity toward apoptosis (Fig. 6). We believe that autoantibodies not only block the CAII catalytic function but also can block the CAII binding to the Na^+/H^+ exchanger {Li, 2002 #1732}. Blocking of both CAII functions may lead to acidification, which are consistent with increases in intracellular calcium {Nicotera, 1998#1063}. Our previous studies showed that the increase in $[\text{Ca}^{2+}]_i$ induced by autoantibodies specific to recoverin or enolase led to apoptosis and cell death {Magrys, 2007#1343; Adamus, 2006#1175}. Even though these autoantibodies found in patients with retinopathy have different antigenic specificities, which affect different metabolic pathways, including phototransduction for recoverin, glycolysis for enolase, and pH control for CAII, they all induce an increase in the $[\text{Ca}^{2+}]_i$. Thus, we propose that the marked elevations of $[\text{Ca}^{2+}]_i$ in retinal cells triggered by autoantibodies ultimately result in cell death. Such calcium-induced cytotoxicity may play a major role in autoimmune retinopathy and several other diseases {Mattson, 2007#1703}.

Anti-CAII autoantibodies are also found in autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, autoimmune cholangitis, chronic pancreatitis, primary biliary cirrhosis, endometriosis, systemic sclerosis, and type I diabetes {Taniguchi, 2003 #1371; Bataller, 2004#1348; Frulloni, 2000#1359; Itoh, 1992#1360; Alessandri, 2003#1372; D'Cruz, 1996 #1374; Invernizzi, 1998#380}. In all of these conditions, antibody levels of anti-CA II were higher in patients with disease than in the control group as reported from 3.8 – 10% in normal subjects. Such a broad occurrence of anti-CAII led to questions concerning the role of CA II in the pathogenesis of autoimmune retinopathy. Autoimmune response against CAII in CAR was present months before diagnosis of cancer in some retinopathy patients {Adamus #1871}. The dominant IgG subclass against CAII is IgG4. Interestingly, IgG4 is predominantly expressed under conditions of chronic antigen exposure {Aalberse, 2002#1736}, which may be a case during cancer growth and turnover.

It is possible that serum autoantibodies to CAII detected in patients with CAR/AR are a consequence of the cross-reactivity of autoantibody against another antigen that mimics CAII or were elucidated in response to cancer antigens {Chegwidden, 2000#1722}. In addition, during oxidative stress, including the continuous destruction of oxidized erythrocytes appears to induce the formation of autoantibodies against certain erythrocyte components together with CAII {Iuchi, 2007#1369; Kurien, 2006#1749}. An insult by environmental factors, such as viruses, affects organs and triggers autoimmunity against CAII. However, it is unlikely that these autoantibodies are simply the result of organ degeneration because autoantibodies were not detected in patients with alcoholic or gall stone-related pancreatitis, during which degeneration of exocrine pancreatic tissues occurs {Nishimori, 2004#1361}.

There is evidence that CAII is a potent autoantigen. Cellular immune responses to CA II measured by PBMC proliferation assay were detected in idiopathic chronic pancreatitis and in Sjögren's syndrome {Nishimori, 2004#1361}. More interestingly, active immunization of susceptible mice with purified CA II resulted in inflammation of submandibular glands {Nishimori, 1995#1356} and bile ducts and cholangiocytes in autoimmune cholangitis mouse model {Ueno, 1998#1358}, suggesting a possible pathogenic role of anti-CA II in immune-mediated diseases. The target organs were inflamed in those animals but the eyes were not evaluated. The authors speculated that the disease may start with anti-CA II antibody formation in predispose mice (or humans), which bind to and impair the function of CA II, resulting in secondary effects that lead to inflammation at organ site where CAII function is critical. Some anti-CA II antibodies are able to clear the CAII antigen without mounting an additional immune response. Both effects may be mediated by antibody internalized but not degrade by the affected cells.

In summary, our study showed that anti-CAII autoantibodies induce pathogenic effects on retinal cells by decreasing cell survival through distressing CAII cellular function. It is therefore

possible that once such autoantibodies reach the retina they will provoke pathological changes in retinal physiology and cell death leading to retinal degenerating (Figure 6). The current experiments may facilitate better understanding the role of the immune system in retinal degeneration and help to develop better strategies for therapy of autoimmune retinopathy.

Acknowledgments

This study was supported by grants from the NIH (EY13053 to GA) and unrestricted departmental funds from the Research to Prevent Blindness.

References

1. Adamus G, Ren G, Weleber RG. Autoantibodies against retinal proteins in paraneoplastic and autoimmune retinopathy. *BMC Ophthalmol* 2004;4:5. [PubMed: 15180904]
2. Heckenlively JR, Jordan BL, Aptsiauri N. Association of antiretinal antibodies and cystoid macular edema in patients with retinitis pigmentosa. *Am J Ophthalmol* 1999;127:565–73. [PubMed: 10334350]
3. Chegwidden, WR.; Dodgson, SJ.; Spencer, IM. The Carbonic Anhydrases New Horizons. Chegwidden, WR.; Carter, ND.; Edwards, YH., editors. Birkhauser Verlag Basel; Basel, Switzerland: 2000. p. 343–363.
4. Adamus G, Machnicki M, Elerding H, Sugden B, Blocker YS, Fox DA. Antibodies to recoverin induce apoptosis of photoreceptor and bipolar cells in vivo. *J Autoimmun* 1998;11:523–33. [PubMed: 9802939]
5. Cao W, Chen W, Elias R, McGinnis JF. Recoverin negative photoreceptor cells. *J Neurosci Res* 2000;60:195–201. [PubMed: 10740224]
6. Shiraga S, Adamus G. Mechanism of CAR syndrome: anti-recoverin antibodies are the inducers of retinal cell apoptotic death via the caspase 9- and caspase 3-dependent pathway. *J Neuroimmunol* 2002;132:72–82. [PubMed: 12417436]
7. Ren G, Adamus G. Cellular Targets Of Anti-alpha-Enolase Autoantibodies Of Patients With Autoimmune Retinopathy. *J Autoimmun* 2004;23:161–7. [PubMed: 15324934]
8. Ohguro H, Ogawa K, Maeda T, Maeda A, Maruyama I. Cancer-associated retinopathy induced by both anti-recoverin and anti- hsc70 antibodies in vivo. *Invest Ophthalmol Vis Sci* 1999;40:3160–7. [PubMed: 10586938]
9. Adamus G, Webb S, Shiraga S, Duvoisin RM. Anti-Recoverin Antibodies Induce an Increase in Intracellular Calcium in Retinal Cells. *J Autoimmun* 2006;26:146–153. [PubMed: 16426815]
10. Magrys A, Anekonda T, Ren G, Adamus G. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol* 2007;27:181–192. [PubMed: 17235687]
11. Whittingtons DA, Grubb JH, Waheed A, Shah GN, Sly WS, Christianson DW. Expression, Assay, and Structure of the Extracellular Domain of Murine Carbonic Anhydrase XIV: implications for selective inhibition of membrane-associated isozymes. *J Biol Chem* 2004;279:7223–7228. [PubMed: 14660577]
12. Yang Z, Alvarez BV, Chakarova C, Jiang L, Karan G, Frederick JM, Zhao Y, Sauve Y, Li X, Zrenner E, Wissinger B, Hollander AID, Katz B, Baehr W, Cremers FP, Casey JR, Bhattacharya SS, Zhang K. Mutant carbonic anhydrase 4 impairs pH regulation and causes retinal photoreceptor degeneration. *Hum Mol Genet* 2005;14:255–265. [PubMed: 15563508]
13. Nagelhus EA, Mathiisen TM, Bateman AC, Haug FM, Ottersen OP, Grubb JH, Waheed A, Sly WS. Carbonic anhydrase XIV is enriched in specific membrane domains of retinal pigment epithelium, Muller cells, and astrocytes. *Proc Natl Acad Sci U S A* 2005;102:8030–5. [PubMed: 15901897]
14. Ilies MA, Vullo D, Pastorek J, Scozzafava A, Ilies M, Caproiu MT, Pastorekova S, Supuran CT. Carbonic anhydrase inhibitors. Inhibition of tumor-associated isozyme IX by halogenosulfanilamide and halogenophenylaminobenzolamide derivatives. *J Med Chem* 2003;46:2187–96. [PubMed: 12747790]
15. Seigel GM. The golden age of retinal cell culture. *Mol Vis* 1999;5:4. [PubMed: 10209197]

16. Seigel GM, Sun W, Wang J, Hershberger DH, Campbell LM, Salvi RJ. Neuronal gene expression and function in the growth-stimulated R28 retinal precursor cell line. *Curr Eye Res* 2004;28:257–69. [PubMed: 15259295]
17. Adamus G, Machnicki M, Seigel GM. Apoptotic retinal cell death induced by autoantibodies of cancer associated retinopathy. *Inves Ophthal Vis Sci* 1997;38:283–291.
18. Adamus G, Amundson D, Seigel GM, Machnicki M. Anti-enolase alpha autoantibodies in cancer-associated retinopathy: epitope mapping and cytotoxicity on retinal cells. *J Autoimmun* 1998;11:671–677. [PubMed: 9878089]
19. Adamus G, Guy J, Schmied JL, Arendt A, Hargrave PA. Role of anti-recoverin autoantibodies in cancer-associated retinopathy. *Invest Ophthalmol Vis Sci* 1993;34:2626–2633. [PubMed: 7688357]
20. Rickli EE, Ghazanfar SAS, Gibbons BH, Edsall JT. Carbonic Anhydrases from Human Erythrocytes. Preparation and properties of two enzymes. *J Biol Chem* 1964;239:1065–1078. [PubMed: 14165909]
21. Gottlieb RA, Nordberg J, Skowronski E, Babior BM. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. *Proc Natl Acad Sci U S A* 1996;93:654–8. [PubMed: 8570610]
22. Reber F, Kasper M, Siegner A, Kniep E, Seigel G, Funk R. Alteration of the intracellular pH and apoptosis induction in a retinal cell line by the AGE-inducing agent glyoxal. *Graefes Arch for Clin Exp Ophthalmol* 2002;240:1022–1032.
23. Barnes S, Bui Q. Modulation of calcium-activated chloride current via pH-induced changes of calcium channel properties in cone photoreceptors. *J Neurosci* 1991;11:4015–23. [PubMed: 1660538]
24. DeSantiago J, Battle D, Khilnani M, Dedhia S, Kulczyk J, Duque R, Ruiz J, Pena-Rasgado C, Rasgado-Flores H. $\text{Ca}^{2+}/\text{H}^{+}$ exchange via the plasma membrane Ca^{2+} ATPase in skeletal muscle. *Front Biosci* 2007;12:4641–60. [PubMed: 17485401]
25. Masuda Y, Aiuchi T, Mihara S, Nakajo S, Nakaya K. Increase in intracellular Ca^{2+} concentrations and the corresponding intracellular acidification are early steps for induction of apoptosis by geranylgeraniol in HL60 cells. *Biol Pharm Bull* 2007;30:880–4. [PubMed: 17473429]
26. Kniep EM, Roehlecke C, Ozkucur N, Steinberg A, Reber F, Knels L, Funk RHW. Inhibition of Apoptosis and Reduction of Intracellular pH Decrease in Retinal Neural Cell Cultures by a Blocker of Carbonic Anhydrase. *Invest Ophthalmol Vis Sci* 2006;47:1185–1192. [PubMed: 16505057]
27. Li X, Alvarez B, Casey JR, Reithmeier RAF, Fliegel L. Carbonic Anhydrase II Binds to and Enhances Activity of the $\text{Na}^{+}/\text{H}^{+}$ Exchanger. *J Biol Chem* 2002;277:36085–36091. [PubMed: 12138085]
28. Nicotera P, Orrenius S. The role of calcium in apoptosis. *Cell Calcium* 1998;23:173–80. [PubMed: 9601613]
29. Mattson MP. Calcium and neurodegeneration. *Aging Cell* 2007;6:337–350. [PubMed: 17328689]
30. Taniguchi T, Okazaki K, Okamoto M, Seko S, Tanaka J, Uchida K, Nagashima K, Kurose T, Yamada Y, Chiba T, Seino Y. High prevalence of autoantibodies against carbonic anhydrase II and lactoferrin in type 1 diabetes: concept of autoimmune exocrinopathy and endocrinopathy of the pancreas. *Pancreas* 2003;27:26–30. [PubMed: 12826902]
31. Bataller L, Sabater L, Saiz A, Serra C, Claramonte B, Francesc Graus. Carbonic anhydrase-related protein VIII: Autoantigen in paraneoplastic cerebellar degeneration. *Ann Neurology* 2004;56:575–579.
32. Frulloni L, Bovo P, Brunelli S, Vaona B, Di Francesco V, Nishimori I, Cavallini G. Elevated serum levels of antibodies to carbonic anhydrase I and II in patients with chronic pancreatitis. *Pancreas* 2000;20:382–8. [PubMed: 10824693]
33. Itoh Y, Reichlin M. Antibodies to carbonic anhydrase in systemic lupus erythematosus and other rheumatic diseases. *Arthritis Rheum* 1992;35:73–82. [PubMed: 1531015]
34. Alessandri C, Bombardieri M, Scrivo R, Viganego F, Conti F, de Luca N, Ricciari V, Valesini G. Anti-carbonic anhydrase II antibodies in systemic sclerosis: association with lung involvement. *Autoimmunity* 2003;36:85–9. [PubMed: 12820690]
35. D'Cruz OJ, Wild RA, Haas GG Jr, Reichlin M. Antibodies to carbonic anhydrase in endometriosis: prevalence, specificity, and relationship to clinical and laboratory parameters. *Fertil Steril* 1996;66:547–56. [PubMed: 8816615]

36. Invernizzi P, Battezzati PM, Crosignani A, Zermiani P, Bignotto M, Papa ND, Zuin M, Podda M. Antibody to carbonic anhydrase II is present in primary biliary cirrhosis (PBC) irrespective of antimitochondrial antibody status. *Clin Exp Immunol* 1998;114:448–54. [PubMed: 9844056]
37. Adamus G. Autoantibody targets and their cancer relationship in the pathogenicity of paraneoplastic retinopathy. *Autoimm Rev.* 2009In Press, Corrected Proof
38. Aalberse RC, Janine Schuurman. IgG4 breaking the rules. *Immunology* 2002;105:9–19. [PubMed: 11849310]
39. Iuchi Y, Okada F, Onuma K, Onoda T, Asao H, Kobayashi M, Fujii J. Elevated oxidative stress in erythrocytes due to a SOD1 deficiency causes anaemia and triggers autoantibody production. *Biochem J* 2007;402:219–27. [PubMed: 17059387]
40. Kurien BT, Hensley K, Bachmann M, Scofield RH. Oxidatively modified autoantigens in autoimmune diseases. *Free Radical Biology and Medicine* 2006;41:549–556. [PubMed: 16863987]
41. Nishimori I, Miyaji E, Morimoto K, Kohsaki T, Okamoto N, Onishi S. Diminished cellular immune response to carbonic anhydrase II in patients with Sjogren's syndrome and idiopathic chronic pancreatitis. *Jop* 2004;5:186–92. [PubMed: 15254347]
42. Nishimori I, Bratanova T, Toshkov I, Caffrey T, Mogaki M, Shibata Y, Hollingsworth MA. Induction of experimental autoimmune sialoadenitis by immunization of PL/J mice with carbonic anhydrase II. *J Immunol* 1995;154:4865–73. [PubMed: 7722336]
43. Ueno Y, Ishii M, Takahashi S, Igarashi T, Toyota T, LaRusso NF. Different susceptibility of mice to immune-mediated cholangitis induced by immunization with carbonic anhydrase II. *Lab Invest* 1998;78:629–37. [PubMed: 9605187]

Figure 1.

Immunoblotting showing 6 patients' autoantibodies reacting with purified human CAII (A). Specific antibodies against CAII serve as positive control (+) and no primary antibody as negative control (-). Immunolabeling of human retinal cells by autoantibodies against CAII by immunocytochemistry: (B) no antibody - negative control, autoantibodies P6 (C), P1 (D), P3 (E); patients' purified IgG were incubated with thin cryosections of a donor human retina at 50 µg/ml concentration. Arrows indicate intracellular staining of the outer segments in photoreceptor cells (Ph), and cells in the inner nuclear (INL) and ganglion cell (GCL) layers.

Figure 2.

Immunological analysis of patients' autoantibodies: (A) Titration of autoantibodies by ELISA using human CAII on the plate. Serum from normal subject served as a control. (B) Distribution of anti-CAII autoantibodies subclasses by ELISA using human CAII on the plate. Secondary antibodies specific to different IgG isotypes, IgM and IgA were used. Note that all antibodies are dominated by IgG4 and IgG1 subclass.

Inhibition of CAII Catalytic Activity

Figure 3.

Inhibition of CAII catalytic function by affinity purified anti-CAII autoantibodies: (A) Dose dependent inhibition of CAII catalytic activity with autoantibodies P2 at the increasing ratios of IgG from 1:10 to 1:40. (B) Inhibition of CAII activity with patients' autoantibodies P1, P2, P4, P5, normal subject IgG and specific antibodies against CAII were incubated at the ratio 1:20.

Figure 4.

(A) Decrease in intracellular pH induced by autoantibodies P1, P2, P3, P4, P5, P6, and normal subject IgG in retinal cells after 8 hr incubation. Normal pH in those cells was measured 7.6. Bars $\pm$ SD represent results for 2 independent experiments done in triplicate. (B) Increase in intracellular calcium $[Ca^{2+}]_i$ induced by autoantibodies P2, P3, P4, P5, P6 and normal subject IgG measured over 90 min. Plot represents results for 2 independent experiments done in triplicate.

Figure 5.

Cytotoxic effect of anti-CAII autoantibodies on E1A. NR3 retinal cells. Living cells were grown with 75 μ g, 150 μ g, or 300 μ g of purified autoantibodies for 72 hr. Cell survival was measured by MTT assay and is presented as a percentage of surviving cells. Bars $\pm$ SD represent results for 2 independent experiments done in triplicate.

Figure 6.

Diagram representing a proposed mechanism of anti-CAII autoantibody action on retinal cell physiology. Carbonic anhydrase II is a cytoplasmic enzyme that catalyzes a reversible conversion of carbon dioxide to bicarbonate (a) and participates in ion transport (b) and pH control. After antibody enters the cell it binds to CAII, which leads to the direct inhibition of CAII catalytic activity (a), which in effect lead to decrease in intracellular pH and increase in intracellular calcium. Antibodies will also prevent CAII binding to the Na⁺/H⁺ exchanger (b), and successful blocking of both CAII functions will lead to cell acidification. All those events will activate apoptosis followed by the cell death.