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Serum autoantibody biomarkers for age-related macular degeneration and possible regulators of neovascularization

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ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in industrial counties. Its pathogenesis is at least partially mediated by immunological factors, including a possible autoimmune response. To date, only a few antibodies have been identified in sera from patients with AMD. In order to reveal an autoantibody profile for AMD and identify biomarkers for progression of this disease, we have performed an antigen microarray analysis of serum samples from patients with AMD and healthy controls. Sera from the AMD groups contained high levels of IgG and IgM autoantibodies to some systemic antigens when compared to the normal group. Targeted antigens included cyclic nucleotide phosphodiesterase, phosphatidylserine (PS) and proliferating cell nuclear antigen. The IgG/IgM ratio for antibodies to PS was notably elevated in the AMD group compared to the normal group, and this ratio correlated best with the stage of AMD patients with an anti-PS ratio greater than the cut-off value had a 44-fold risk for advanced AMD with choroidal neovascularization. PS immunoreactivity was also elevated in AMD retina. Moreover, IgG autoantibodies purified from sera of AMD patients induced more tube formation on choroidal–retinal endothelial cells compared to those of healthy donors. Hence, sera from patients with AMD contain specific autoantibodies which may be used as biomarkers for AMD, and the IgG/M ratio for autoantibodies to PS might allow better monitoring of AMD progression.

Introduction

Age-related macular degeneration (AMD) is currently the leading cause of irreversible vision loss in individuals over 60 years of age. This disease affects more than 1.75 million people in the United States—1.47% of the population. As the population ages, the prevalence will likely increase by 50%, to almost 3 million, by 2020 (Dineen et al., 2006). AMD is classified as either dry (atrophic) or wet (neovascular), and the dry form can progress to the wet form. The most common early signs are blurred vision for dry AMD and metamorphopsia for wet AMD. In wet AMD, damage to the macula occurs rapidly due to leakage of fluid or blood due to choroidal neovascularization (CNV), formation of fragile, new, abnormal blood vessels under the macula.

Many large-scale studies have revealed risk factors for AMD, which include age, smoking, race and family history (Ambati et al., 2003; Clemons et al., 2005). Recently, analysis of single-nucleotide polymorphisms of patients with AMD has revealed that complement factor H, which is involved in inhibiting the alternative complement cascade, is strongly associated with risk for development of AMD (Haines et al., 2005). Complement factor H is one of the components of drusen, which are small yellow deposits under the macula and the earliest clinical findings in AMD. Additional studies have suggested that the immune system also plays an important role in the pathogenesis of AMD, as inflammation promotes development of drusen and progression of AMD (Donoso et al., 2006).

Abbreviations: AEC, 3-amino-9-ethylcarbazole; AMD, age-related macular degeneration; APS, antiphospholipid syndrome; AUC, area under the ROC curve; CEP, carboxyethylpyrrole; CNPase, cyclic nucleotide phosphodiesterase; CNV, choroidal neovascularization; DM, dermatomyositis; ECM, extracellular matrix; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; HSPG, heparan sulfate proteoglycan; INL, inner nuclear layer; IPL, inner plexiform layer; LC1, liver cytosol type 1; nfi, normalized fluorescence intensity; ONL, outer nuclear layer; OPL, outer plexiform layer; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCL, photoreceptor cell layer; PM, polymyositis; PS, phosphatidylserine; RA, rheumatoid arthritis; ROC, receiver operator characteristic; RPE, retinal pigment epithelium; RPLP0, ribosomal phosphoprotein P0; SjS, Sjögren's syndrome;SLE, systemic lupus erythematosus; SSc, systemic sclerosis; VEGF, vascular endothelial growth factor.

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Several reports have indicated that autoimmunity affects the pathogenesis of AMD (Morohoshi et al., 2009). Antibodies to anti-glial fibrillary acidic protein (GFAP), expressed by retinal astrocytes, and carboxyethylpyrrole (CEP)-modified proteins, generated with oxidative stress, are significantly elevated in sera from AMD patients (Gu et al., 2003; Penfold et al., 1990). Recently, Joachim et al. have reported the presence of antibody to α-enolase, a member of the heat shock protein family, in sera of patients with wet AMD, and α-enolase has been also detected in the sera of patients with cancer-associated retinopathy (Dot et al., 2005; Joachim et al., 2006). Moreover, we have previously reported the presence of anti-retinal autoantibodies in the sera of AMD patients (Patel et al., 2005).

AMD patients clearly express specific autoantibodies, but the identity of these autoantibodies remains largely unknown. In this study, we performed a comprehensive analysis of autoantibodies in the sera of patients with AMD, using an antigen microarray technique. We found that numerous specific autoantibodies were significantly elevated in the sera of AMD patients compared to those of normal donors and identified the candidate biomarkers that best correlated with AMD.

Materials and methods

Patient recruitment and collection of sera

AMD patients were recruited from the Kings College Hospital, London, UK. Subjects included patients over 50 years of age with AMD in one or both eyes. Patients who had visual loss from other retinal disorders, in particular uveitis, were excluded from this study. Age- and sex-matched normal patients were selected from cataract clinics or were the unaffected spouses of AMD patients (Table 1). Informed consent of all study subjects was received after detailed discussion after obtaining local ethical approval and the protocols were conformed to the provisions of the Declaration of Helsinki. Stereo color photographs of both fundi were taken and graded by a retinal specialist using the International Classification System for the clinical features of AMD, as described previously (Patel et al., 2005). The blood samples were obtained through venipuncture into Vacuette™ vials containing serum clot activator and centrifuged at 4200 *g* for 10 min, after which the supernatant serum was separated, aliquoted and stored at −70 °C, samples were defrosted to room temperature for use in experiments.

Autoantigen

The antigen arrays were performed by the University of Texas Southwestern Medical Center Microarray Core Facility, with 85 kinds of antigens including negative, positive and inner control antigens. The array used in this study was a modification of one previously described (Li et al., 2007) and included the antigens for several autoimmune diseases, such as systemic lupus erythematosus (SLE), Sjogren's syndrome (SjS), rheumatoid arthritis (RA), multiple sclerosis and spondylo-arthropathy related with HLA B27. The printing concentration for all antigens was optimized at 1 μg/ml, with exception of cardiolipin, which was determined to be 0.1 μg/ml.

Table 1
Patients characteristics.

	NC (n = 20)	AMD (n = 55)			
		dry AMD (n = 35)			wet AMD (n = 20)
	Normal	Stage 1	Stage 2	Stage 3	Stage 4
Number of patients					
Male	8	3	3	6	6
Female	12	2	7	14	14
Total	20	5	10	20	20
Patient age (y)					
Mean	78.1	68.8	72.4	75.0	82.9
Range	62–90	50–83	50–85	61–88	65–91
Smoker (%)	60.0	40.0	50.0	55.0	55.0

AMD, age-related macular degeneration; NC, normal controls.

Slide manufacture and antigen microarrays

The antigen microarray was performed as previously described (Li et al., 2007; Mantel et al., 2008). Briefly, antigens diluted in phosphate-buffered saline (PBS) were robotically printed in duplicate and distributed randomly on nitrocellulose-coated 16-pad FAST™ slides (Whatman Schleicher & Scheleicher BioScience, Keene, NH, USA). The slides were incubated for 60 min with serum samples diluted 1:200 with blocking buffer (1% BSA in PBS) and washed three times for 5 min with washing buffer (PBS with 0.05% v/v Tween 20, pH 7.4). Cy3-labeled anti-human IgG and Cy5-labeled anti-human IgM secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 1:500 dilution were applied to detect binding of serum autoantibodies to specific antigens and were incubated with the autoantibodies for 60 min at room temperature. The arrays were washed as before and scanned using the Genepix 4000B scanner to generate TIFF images for analysis.

Specimen collection and preparation

Human eyes were obtained from the Georgia Eye Bank and donated to the L.F. Montgomery Laboratory, Emory University, with Institutional Review Board (IRB) exempt status. The donors were white men and women with or without AMD ranging in age from 80 to 86 years. For light microscopic processing, the eyes were fixed in 10% neutral-buffered formalin, dehydrated in increasing concentrations of alcohol, cleared in xylene and embedded in paraffin. After processing, 7-μm thick sections were obtained through the macula using a microtome (Finesse, Thermo Shandon, Astmore, UK).

Immunohistochemistry

Following fixation, sections were deparaffinized and treated with hydrogen peroxide to block the endogenous peroxidase activity. Samples were then blocked with 10% normal donkey serum in 1% BSA–PBS for 30 min at room temperature. The sections were first stained with mouse monoclonal anti-phosphatidylserine (PS) antibody (4B6, ab18005, Abcam Inc., Cambridge, MA, USA) overnight at 4 °C, washed with PBS, and incubated for 60 min with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). For immunolabeling controls, irrelevant mouse monoclonal IgG (ab37355, Abcam Inc.) was used in place of anti-PS. 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as a chromogen for visualization. The sections were mounted with aqueous mounting medium (Vector Laboratories). Finally, the sections were examined under light microscopy (Olympus America Inc., Center Valley, PA, USA).

Cell culture

Rhesus choroidal–retinal endothelial cells (RF/6A) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagles medium (DMEM, HyClone, Logan, UT, USA) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen Corp., Grand Island, NY, USA). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.