Elastin Layer in Bruch's Membrane as a Target for Immunization or Tolerization to Modulate Pathology in the Mouse Model of Smoke-Induced Ocular Injury

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Abstract

Age-related macular degeneration (AMD) is associated with an overactive complement system and an increase in circulating antibodies. Our search for potential neoantigens that can trigger complement activation in disease has led us to investigate elastin. A loss of the elastin layer (EL) of Bruch's membrane (BrM) has been reported in aging and AMD together with an increase of serum elastin-derived peptides and α-elastin antibodies. In the mouse model of cigarette smoke exposure (CSE), damage in BrM, loss of the EL, and vision loss are dependent on complement activation. We have examined the hypothesis that CSE generates immunogenic elastin neoepitopes that trigger an increase in α-elastin IgG and IgM antibodies, which can then bind to the neoepitopes in the target cells or membranes, triggering complement activation. Specifically, we showed that immunization with elastin peptide oxidatively modified by cigarette smoke (ox-elastin) exacerbated ocular pathology and vision loss in CSE mice. In contrast, mice receiving peptide immunotherapy (PIT) with ox-elastin did not lose vision over the smoking period and exhibited a more preserved BrM. Immunization and PIT correlated with humoral immunity and complement activation and IgG/IgM deposition in the RPE/BrM/choroid. Finally, PIT modulated immune markers IFNγ and IL-4. The data further support the hypothesis that complement activation, triggered by immune complex formation in target tissues, plays a role in ocular damage in the CSE model. As PIT with ox-elastin peptides reduces damage, we discuss the possibility that AMD progression might be preventable.

Keywords

Age-related macular degeneration · Elastin · Immunization · Tolerance · Complement · Smoking

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1 Introduction

AMD is a multifactorial, complex disease, which occurs in two forms, wet and dry. Patients experience a loss of central vision, and clinical underpinnings include drusen and retinal pigment epithelium (RPE) disturbances, damage to macular photoreceptors, and pathology at the RPE/choroid interface. Among the risk factors associated with disease, we wish to highlight factors relevant for the hypothesis presented here, which include aging [1], cigarette smoking [2], increased susceptibility of elastic fibers to photic degenerative stimuli [3], and genetic variants in genes regulating the complement cascade [4] and extracellular matrix turnover [5].

The complement system is the humoral backbone of the innate immune system. Its main role is to participate in antimicrobial defense, clearance of immune complexes, and tissue regeneration, but excessive complement activation can be involved in the pathogenesis of disease states, including AMD [6]. The complement system can be activated by three distinct pathways: the classical (CP), lectin (LP), and alternative pathway (AP), with IgG and natural IgM antibodies binding to ligands generated by injury, immunity, and infection participating in CP and LP activation. This begs the question as to what might be their ligands.

The potential role of BrM in initiation and progression of disease has been investigated based on its changes with aging and disease [7], including lipid buildup and calcifications [8], changes in diffusion characteristics [9], and a reduction of the cross-linked linear elastin fibers of the EL [7]. Additional alterations in elastin metabolism in aging and AMD have been reported. Blumenkranz et al. published on a generalized increased susceptibility of elastic fibers to photic or other degenerative stimuli and suggested that this might be a new and important risk factor for choroidal neovascularization [3]. Serum-elastin-derived peptides (s-EDPs) were found to be significantly higher in AMD patients than in controls [10], and serum IgG and IgM antibodies against elastin are elevated in AMD [11]. However, for both the s-EDPs and the elastin epitopes the antibodies recognize, their modification status is unknown. Finally, elevating serum elastin fragments in mouse increased expression and deposition of collagen IV in the RPE/choroid complex [12]. Based on these observations, we have previously postulated that abnormalities in elastin homeostasis together with antibody production may contribute to an inflammatory feedback loop, ultimately leading to AMD pathology [13].

2 Cigarette Smoke Exposure (CSE) as a Model for Dry AMD

We consider CSE, which is a passive inhalation model lasting for 6 months, a model for dry AMD, as it shows structural alteration and complement deposition in the RPE/BrM/choriocapillaris (CC) complex similar to that seen in patients [14]. Smoke exposure leads to the formation of deposits in the RPE/BrM that contain proteins also found in human drusen and basal laminar deposits such as C3a, C5, MAC, and CFH [15]. Also, we have shown that long-term smoke in C57BL/6J mice reduces scotopic and photopic ERG amplitudes, in addition to contrast sensitivity. Structural changes include mitochondrial swelling in the RPE, a loss of CC fenestrations, and thickening of BrM due to the deposition of material in the outer collagenous layer. These structural alterations were not observed in $CFB^{-/-}$ mice, in which the AP is eliminated [14] or can be repaired by treatment with an AP inhibitor [16].

3 Neoepitopes Trigger Chronic Inflammation in Smoke-Induced Pathology

Based on our premise that breakdown of the EL in the presence of aging or oxidative stress generates elastin neoepitopes, triggering an immune response that leads to complement activation and pathology, we tested the hypothesis that immunization with smoke elastin neoepitopes augments pathology. Mice were immunized with 10 μg of mouse lung elastin peptides or 10 μg of cigarette

smoke-modified elastin peptides mixed with adjuvant followed by a booster shot and exposed to 6 months of smoke. After 6 months, mice were examined for anti-elastin antibody production, contrast sensitivity, structural changes in BrM, as well as IgG and IgM deposition and the presence of complement activation fragments in the RPE/choroid [13]. The amount of antibody generated was analyzed in ELISA assays, using the corresponding mouse serum as the antibody source, and demonstrated that ox-elastin (neo-epitope) immunization generated more IgM and IgG antibodies when compared to control elastin (self-epitope). While room air-raised mice exhibited stable contrast sensitivity over the 6-month analysis period, smoke-exposed mice immunized with ox-elastin exhibited a greater loss in contrast sensitivity than the elastin-immunized mice. This loss in vision was correlated with a thicker BrM and more damaged RPE mitochondria when compared to nonimmunized mice or those immunized with a control elastin peptide. Finally, structural and functional alterations were correlated with increased levels of IgM, IgG3, and IgG2b in the RPE/choroid fraction, and the increase in antibody binding was correlated with an increase in complement activation as assayed by Western blotting, probing for C3 breakdown products. Based on these results, we would like to propose that, in CSE, antibodies, generated either de novo against ox-elastin (IgG) or natural antibodies amplified and selected (IgM), bind to ox-elastin generated by smoke in BrM or other extracellular matrices containing elastin, to trigger complement activation. Antibodies might be activating complement via either the CP or LP, which is then amplified by the AP [14] leading to the observed pathology.

4 Is Pathology in the CSE Model Preventable with Peptide Immunotherapy?

Given this data on immunization, albeit with only indirect evidence of ox-elastin-induced pathology, we argued that if pathology can be increased by increasing antibody production, pathology should be decreased by immunotherapy. PIT has been studied for the treatment of various autoimmune diseases, allergy, and cancer (e.g., [17]). Overall, in laboratory animals, peripheral tolerance toward a particular antigen is generated by repeated exposure, and while the exact mechanism of action has not yet been fully defined [18], it is thought to involve deletion of reactive T cells, the generation of a regulatory T (Treg)-cell response, or an altered macrophage response [19]. Hence, if the generation of modified s-EDPs and their corresponding antibodies were to trigger pathology in eyes of CSE mice, this vicious cycle of inflammation should be preventable by PIT or tolerization. This hypothesis was tested by providing PIT consisting of 1 or 10 μg of cigarette smoke-modified mouse lung elastin peptides weekly during the smoking period. After 6 months, the mice were examined for anti-elastin antibody production, contrast sensitivity, structural changes in BrM, as well as IgG/IgM and complement activation in the RPE/choroid. PIT at the 1 μg dose was efficacious in reducing the humoral immune response, resulting in the suppression of ox-elastin-specific IgG and IgM antibodies when compared to control smoke-exposed mice, almost reducing levels to those seen in animals raised in room air. Low dose PIT reduced the amount of IgG and IgM deposited in the RPE/choroid, and this decrease in IgG/IgM deposition was correlated with reduced complement activation. Finally, structurally, low dose PIT resulted in preservation of BrM, and, functionally, improved contrast could be observed. Finally, we asked whether low dose PIT induces tolerance. Tolerance to antigen (neo-antigen or against self-antigen) engages both central and peripheral mechanisms. Central clonal deletion providing tolerance to self-antigen occurs following negative selection and deletion during thymic development, and peripheral tolerance is a constant surveillance to prevent immune activation of T cells escaping clonal deletion and to potentially neoantigens. Peripheral tolerance is mediated largely via natural and inducible Trgs, T helper 3 cells, and T regulatory type 1 cells that orchestrate regulation via immunoregulatory cytokines such as TGFβ, IL-10, and IL-4 [20]. As an induction of tolerance requires, in part,

decreasing Th1/Th17 activation and increasing Th2 activation or enhancing Treg activity (either antigen or non-antigen specific), we examined Th1 versus Th2 activation by performing a partial representative cytokine analysis. Cytokine analysis in the RPE/choroid fraction in response to smoke suggests an induction of tolerance in PIT animals, as a decrease IFN-y expression (surrogate of reduction of non-self-activation) and an increase in IL-4 expression (promotion of self-tolerance) were observed. Overall, these data support the hypothesis by Nussenblatt et al. that AMD might be suitable for tolerance therapy [21]. It would be of great interest to determine the presence of ox-elastin versus control elastin antibodies in AMD, as the modification status of the s-EDPs and the epitope recognition sites of the anti-elastin antibodies are unknown. Other neoepitopes that might also be involved in the immune response identified here might include malondialdehyde (MDA) or carboxyethylpyrrole (CEP) adducts. Antibodies against MDA have been shown to be involved in complement-dependent mouse choroidal neovascularization and to recognize neoepitopes on RPE cells exposed to smoke extract [22], and immunization of mice with CEP-modified mouse serum albumin results in complement deposition in BrM, the accumulation of drusen-like deposits with aging, and decreased retinal function [23].

We acknowledge that our studies have a number of limitations, which have been addressed in primary publications [13, 24], and include, among others, that the effects of smoke exposure and treatment on the eye cannot be distinguished from those of the effects of the two on other organs, as long-term smoke exposure leads to emphysema and other organ damage in mice [25]. In addition, we recognize that the gold standard to test the hypothesis of the role of T cells, their role in tolerance, and thereby elucidate pathogenic pathways is the use of adoptive and passive transfer of T cells as well as specific ox-elastin antibodies. However, as our model requires 4–6 months to propagate pathology, systemic T-cell responses were not established. Also, the sources of the cytokines identified would further illuminate mechanisms. Nevertheless, as AMD pathogenesis has been linked to smoking, complement activation, and pathogenic T- and B-cell immunity, our hypothesis that AMD pathology is increased in the presence of neoantigens and decreased by peptide or antigen immunotherapy to suppress immunity appears justified. Our results may open a novel avenue for immunotherapies in dry AMD.

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