Prevalence of Neutralizing Factors Against Adeno-Associated Virus Types 2 in Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy

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Abstract: Adeno-associated virus type 2 (AAV2) mediated gene therapy providing a potential treatment in the eye. However, immune responses can limit virally mediated gene transfer and therapy. To assess preexisting AAV2 neutralizing factors (NF) titers in peripheral blood and the vitreous in patients with age-related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV). 130 subjects were enrolled: 50 with neovascular AMD, 30 with PCV, and 50 controls. The serum and the vitreous were obtained for AAV2 NF assay. We found AAV2 NF are present in all of AMD, PCV patients and controls we tested. There were no significant differences in prevalence of NAb in serum between AMD, PCV and controls (P =0.999). There was no correlation between NF in serum and in vitreous (P>0.05), and NF in vitreous was significantly less than in serum. Our results for the first time showed in Chinese population, NF against AAV2 was present in serum of all the patients with AMD or PCV and controls, and there were no significant differences among these groups. Therefore, it demonstrated there were no correlations between AAV2 NF titer and these diseases. We found NF in vitreous was considerably less than in serum in all groups. We also found no direct correlation between NF in vitreous and in serum suggesting serum antibody levels may not be used to predict their counterparts in the vitreous. Our results will provide crucial information for future clinical studies in the development of new therapies based on AAV2 mediated gene delivery in the eye.

Keywords: Neutralizing factors, adeno-associated virus types 2, age related macular degeneration, polypoidal choroidal vasculopathy.

INTRODUCTION

Age-related macular degeneration (AMD) causes irreversible central vision loss and is the leading cause of blindness in the elderly population, characterized as chronic and progressive degeneration of photoreceptors, the underlying retinal pigment epithelium (RPE), Bruch’s membrane, and possibly, the choriocapillaris in the macula [1-5]. AMD is divided clinically into dry and wet AMD. The “wet” form of the disease or neovascular, characterized by the development of choroidal neovascular (CNV) membranes, is the main cause of visual impairment in macular degeneration [6].

Polypoidal choroidal vasculopathy (PCV) is a macular disease found in the elderly that is as prevalent as neovascular AMD in the Asian population, accounting for approximately 30% to 50% of the total number of eyes with senile macular diseases in elderly Asians [7, 8]. The disease is regarded as a primary abnormality of the choroidal circulation, characterized by an inner choroidal vascular network of vessels ending in an aneurismal bulge or outward projection. The disorder is usually associated with multiple, recurrent serosanguinous detachments of retinal pigment epithelium and neurosensory retina secondary to leakage and bleeding [9].

Vascular endothelial growth factor (VEGF) has been identified as the major mediator of CNV in the eye. Several studies have shown that overexpression of VEGF in the retina resulted in retinal and choroidal NV [10, 11]. Nowadays, anti-VEGF therapy in the form of protein or factor has been proven to be effective and Lucentis/Avastin has been adapted into the standard care for neovascular AMD and PCV patients in major centers of provincial capital cities in China. However, the requirement for monthly/bi-monthly intravitreous injections presents challenges to clinicians and patients, as the vast majority of the patients in less developed areas of the nation will not have access to specialists for frequent injections. 12-17

To overcome these difficulties and achieve stable ocular anti-angiogenic therapy, gene therapy aimed at blocking the underlying stimuli for vessel growth is a potentially more effective treatment. Of the recombinant viruses used in ocular gene therapy, recombinant Adeno-associated virus type 2 (AAV2)-mediated gene delivery enables the long-term ex-
pression of transgenes, thus providing a potential solution for the long-term delivery of anti-angiogenic agents in the eye. Preclinical studies also suggest that recombinant AAV2 vectors delivered by the intravitreal route are capable of mediating efficient and prolonged transgene expression in the retina and have been used to deliver therapeutic genes for the treatment of retinal neovascularization in rodent and non-human primate models [25, 26].

However, immune responses can limit virally mediated gene transfer and persistence of therapeutic proteins. An estimated large portion of our population maintains factors to the capsid proteins of AAV2, and a significant percentage of individuals demonstrates the presence of factors against AAV2 [27-31]. In past animal studies, preimmunization with recombinant AAV2 vectors resulted in reduction or lack of transgene expression and correlated with the presence of neutralizing factor (NF) found in the serum [32]. Moreover, studies of repeated administration of AAV2 vectors indicate that immune responses generated after an initial administration may prevent or mute further vector-mediated cell transduction [33-35]. It is uncertain whether these results will be predictive of results in humans and critical to understand the difference of preexisting factors against AAV2 between normal subjects versus AMD and PCV patients that have leaking neovasculature in the retina. This study was aimed to assess preexisting factors against AAV2 in the serum and vitreous of patients with AMD and PCV in China. The results will provide important information for future clinical studies in the development of this class of new therapies.

MATERIALS AND METHODS

Human Subjects and Collection of Serum

The study protocol was approved by the Ethics Committee for Human Research of Peking University People’s Hospital and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all study subjects. AMD and PCV patients and age-matched controls, including individuals with a normal eye examination (individuals age 50 years or older with no drusen or RPE changes), were recruited in the ophthalmology clinic between August 2011 and September 2011. All participants went through a standard examination protocol as in the previous description [36, 37]. The definitions of neovascular AMD and ARM were those of the International Classification System for ARM, but grading was not performed. The diagnosis of PCV was based on indocyanine green angiography (ICGA) results, which showed a branching vascular network that terminated in aneurysmal enlargements, that is, polypoidal lesions. All cases with neovascular AMD and PCV received a general ophthalmologic examination including fluorescein angiography and ICGA with HRA2 (Heidelberg Engineering, Heidelberg, Germany). Eyes with other macular abnormalities, for example, pathologic myopia, idiopathic choroidal neovascularization (CNV), presumed ocular histoplasmosis, angioid streaks, and other secondary CNV, were excluded. In total, 50 neovascular AMD patients, 30 PCV patients and 50 matched controls were recruited. In the controls, all individuals underwent an eye exam, with idiopathic epiretinal membrane or idiopathic macular hole, no surgery history on eye, no signs of early AMD, such as soft drusen or irregular pigmentation of the RPE in the macular area, were observed. A single blood draw was performed to obtain serum for factor assays. The serum was once collected after centrifugation of peripheral blood in a sterile eppendorf microcentrifuge tube. Serum samples were stored below negative 80°C until analysis. Before performing neutralization assays, serum samples were incubated at 56°C for 30 min to inactivate complement, and were then kept at 4°C.

Collection of Vitreous

After serum collection, a standard vitreous sampling protocol was followed for 20 neovascular AMD patients (20 eyes), 16 PCV patients (16 eyes) with intravitreal injection of first anti-VEGF drugs and 20 controls (20 eyes) with surgery for epiretinal membrane or macular hole as follows.

To AMD and PCV patients, prior to injection of anti-VEGF drugs, topical anesthesia was applied, and the patients were draped completely as for any intraocular surgery. A lid speculum was inserted. The vitreous sample was obtained via a pars plana approach. A 25 gauge needle (Terumo Needle; Terumo Corporation, Elkton, MD) with a 1 ml syringe (PrecisionGlide®; Becton Dickinson) was directed into the mid-vitreous cavity and 0.10 ml of vitreous fluid was gently aspirated. After that, anti-VEGF drugs were injected immediately.

To the controls, following retrobulbar anesthesia, before the surgery for epiretinal membrane or macular hole, the controls were prepped and draped in a sterile fashion in the operating room. A sterile lid speculum was placed, an infusion cannula was placed in the inferotemporal quadrant for safety purposes. Once inserted, the infusion cannula remained in the off position until all sampling was completed. The vitreous sample was obtained via a pars plana approach. A 25 gauge needle with a 1 ml syringe was directed into the mid-vitreous cavity and 0.10 ml of vitreous fluid was gently aspirated.

After removal, the syringe was capped, transferred to ice, and immediately transferred to a negative 80°C freezer for storage. Once the vitreous sampling was completed, the infusion line was inspected for proper placement and the remainder of the case was completed.

AAV Vector

The recombinant vector AAV2- β-galactosidase, was produced at Genzyme Corporation by triple transfection of 293 cells protocol using helper plasmids p5rep-Δ-CMVcap and pHHelper (Stratagene, La Jolla, CA, USA), and purified according to the protocol using an iodixanol step gradient and HiTrap Heparin column (GE Healthcare Life Sciences, Piscataway, NJ, USA) on an ÄKTA FPLC system (GE Healthcare Life Sciences, Piscataway, NJ) [38, 39]. The AAV2- β-galactosidase viral preparation had a titer of 2.2 × 10^{12} dpks (DNase resistant particles) per ml. Viral titers were determined using a real-time TaqMan PCR assay (ABI Prism 7700; Applied Biosystems, Foster City, CA, USA) with primers that were specific for the BGH poly A sequence.

Cell Culture

The HeLa cells were obtained from the cell bank at the Cell Resource Center, IBMS, CAMS/PUMC. HeLa cells
were maintained in DMEM medium and culture media contained 10% FBS were grown in a 37°C incubator with a 5% CO₂ environment.

**Virus Neutralization Assay**

Neutralizing anti-AAV2 Ab titer in serum and vitreous was determined with a cell-based bioassay. Serial dilutions of sera were admixed with AAV2 encoding β-galactosidase and added to HeLa cells that were pre-infected with a temperature sensitive Adenovirus mutant Ad5ts149 at a multiplicity of infection (moi) of 2. The cells were lysed 48 hours later and the level of β-galactosidase was determined using a commercially available assay (Galacto-Light, Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. The titer was defined as the reciprocal of the first dilution that reduced reporter gene expression by 50% compared to cells receiving the virus encoding the reporter gene alone.

**Statistical Analysis**

Data were summarized as graphical displays and descriptive statistics (i.e., means and standard deviations for continuous variables, and counts and proportions for categorical variables). All results of NF titer value were transformed into log₁₀ and used in the statistical analysis. ANOVA followed by modified student’s t-test was used to test differences of titer of neutralizing factors in proportions among patient groups. Paired-Sample T Test was used to test difference in titer between serum and vitreous.

**RESULTS**

**Demographics (Gender, Age, etc.)**

Serum samples were obtained from 130 subjects (50 AMD, 30 PCV and 50 normal subjects). Demographic data were obtained from all subjects (Table 1). Differences in the observed genotypic distribution between the case and control groups were tested by logistic regression analysis for age and sex adjustments. The differences among different groups were not statistically significant (P>0.05).

**Prevalence of Neutralizing Factors to AAV2 in AMD, PCV and Controls in Serum**

Statistical comparisons of prevalence and factors titer of neutralizing factors activity between subgroups defined by disease status were performed (Fig. 1). We found varying amounts of AAV2 neutralizing factors in all of AMD, PCV and normal adults. The mean of Lg NF titer in serum is 1.76 in AMD, 1.76 in PCV and 1.76 in controls. There were no significant differences in prevalence of factors between AMD, PCV and controls (P =0.999).

**Comparison and Correlation of Serum and Vitreous Neutralizing Factors to AAV2 Levels in AMD, PCV and Controls**

The mean of Lg NF titer in vitreous is 0.66 in AMD, 0.68 in PCV and 0.64 in controls. Although the data were very limited, a significant difference of NF titer was observed between in the serum and in the vitreous (P=0.01) (Fig. 2). NF in vitreous were significantly less than in serum. There was no correlation of NF between in serum and in vitreous(P>0.05) (Fig. 3).

**DISCUSSION**

Adenoassociated viral (AAV) vectors have many attractive features for safe and efficient gene therapy, including their lack of pathogenesis, low toxicity, ability to efficiently infect both dividing and nondividing cells in a broad range of host tissues or organs, and long-term gene expression [40, 41]. Despite these advantageous properties of AAV vectors, preexisting immunity due to prior exposure with wild-type (wt) AAV vectors in the majority of the human population could potentially limit their therapeutic usefulness [27-30, 35, 42-45]. In animal studies, preimmunization with recombinant AAV vectors has resulted in reduction or lack of transgene expression [27, 32, 46] and correlated with the presence of neutralizing factor (NF) found in the serum.

This study was aimed to assess preexisting factors against AAV2 in the serum and vitreous of patients with AMD and PCV. To our knowledge, this is the first report on preexisting factors against AAV2 by neutralization test in patients with AMD and PCV in Chinese population. Previous estimates of AAV2 seropositivity have been high (up to 80% in adults). However, the data reported for seroprevalence of AAV2 factors were tested using enzyme immunoassay (EIA). Erles detected whether the factors detected by EIA test have neutralizing properties, and samples chosen at random were again tested by a neutralization test. It was found that out of the positive sera by EIA, 20% sera did not neutralize AAV2 [43]. Because EIA tests may detect factors directed against all viral antigens while the neutralization test

<table>
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<tr>
<th>Characteristic</th>
<th>AMD patients n=50</th>
<th>PCV patients n=30</th>
<th>Controls n=50</th>
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<tbody>
<tr>
<td>Mean age, yr(SD)</td>
<td>71.9(8.3)</td>
<td>69.3(7.0)</td>
<td>70.0(9.9)</td>
</tr>
<tr>
<td>[min,max]</td>
<td>[58,88]</td>
<td>[58,83]</td>
<td>[50,93]</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Females</td>
<td>23(46%)</td>
<td>16(53.3%)</td>
<td>24(48%)</td>
</tr>
<tr>
<td>Males</td>
<td>27(54%)</td>
<td>14(46.7%)</td>
<td>26(52%)</td>
</tr>
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Fig. (1). A comparison of mean factor titers to AAV2 between AMD, PCV and controls measured in the serum. There is no significant difference between subgroups (P>0.05) and AAV2 neutralizing factors in all of AMD, PCV and normal adults. "n" represents the number of collected serum sample from different patient. P value calculated using ANOVA followed by modified Student’s t-test.

Fig. (2). A comparison of mean factor titers to AAV2 between AMD, PCV and controls measured in the serum and in the vitreous. There is significant difference between in the serum and in the vitreous (*P<0.01). NF in vitreous were significantly less than in serum. P value calculated using Paired-Sample T Test.
detects only the factors directed against specific proteins [28, 29, 47]. Moreover, their data indicates that activation of AAV factors was related to adenovirus infection or other helper viruses HPV or CMV infection or hormonal influences. We have found varying amounts of AAV2 neutralizing factors in all of AMD, PCV and normal adults that we tested and there were no significant differences between groups. Therefore, it demonstrated there were no correlations between neutralizing AAV2 factors titer and these diseases.

It is generally assumed that previous exposure to AAV2 will not pose significant problems for the efficacy of AAV2 vectors in the retina, an immune-privileged site. This notion is supported by the observation that intravitreal or subretinal readministration of AAV2 vectors in both nonhuman primate and murine resulted in additional transduction events despite the presence of moderate levels of serum factors to AAV2 vectors [48, 49]. However, no studies have examined the impact of previous systemic immune response to AAV2 capsid on transduction and therapeutic efficacy of AAV2 vectors in different ocular tissues in patients with AMD or PCV where leaking neovascularure is present in the retina and choroid. In this study, we have detected NF levels in human vitreous. However, we found that NF levels in vitreous was significantly lower than that in serum. The results of our present study can explain that ocular tissues can be transduced efficiently by AAV vectors in spite of the presence of high titer of circulating factors to AAV capsid.

However, it remains to be determined if this level of neutralizing factors titer will affect gene transduction and impact the intended therapeutic outcome. Additional detailed studies will be needed to assess the effect of neutralizing factors on AAV-mediated gene transfer in the eye of patients. It was also noted that in our limited number of samples, we found no correlation between NF in vitreous and in serum. Our results suggest that serum factor levels may not be used to predict their counterparts in the vitreous.

**CONFLICT OF INTEREST**

The author(s) confirm that this article content has no conflicts of interest.

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e. Other Acknowledgments: none.

**PATIENT CONSENT**

Declared none.
REFERENCES


