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TGF-β1 prevents rat retinal insult induced by amyloid-β (1–42) oligomers

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ABSTRACT

To set up a retinal degenerative model in rat that mimics pathologic conditions such as age-related macular degeneration (AMD) using amyloid-β (Aβ) oligomers, and assess the effect of TGF-β1. Sprague-Dawley male rats were used. Human $A\beta_{1-42}$ oligomers were intravitreally (ITV) injected (10 μM) in the presence or in the absence of recombinant human TGF-β1 (1 ng/μl ITV injected). After 48 h, the animals were sacrificed and the eyes removed and dissected. The apoptotic markers Bax and Bcl-2 were assessed by western blot analysis in retina lysates. Gene-pathway network analysis was carried out in order to identify pathways involved in AMD. Treatment with Aβ oligomers induced a strong increase in Bax protein level (about 4-fold; $p < 0.01$) and a significant reduction in Bcl-2 protein level (about 2-fold; $p < 0.05$). Co-injection of TGF-β1 triggered a significant reduction of Bax protein induced by Aβ oligomers. Bioinformatic analysis revealed that Bcl-2 and PI3K-Akt are the most connected nodes, for genes and pathways respectively, in the enriched gene-pathway network common to AMD and Alzheimer disease (AD). Overall, these data indicate that ITV injection of $A\beta_{1-42}$ oligomers in rat induces molecular changes associated with apoptosis in rat retina, highlighting a potential pathogenetic role of Aβ oligomers in AMD. Bioinformatics analysis confirms that apoptosis pathways can take part in AMD. Furthermore, these findings suggest that human recombinant TGF-β1 can prevent retinal damage elicited by Aβ oligomers.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss in elderly populations in developed countries. Two main forms of AMD exist, the dry and the wet one. Dry AMD is characterized by drusen bodies (cellular debris) that accumulate between choroid and retina; wet AMD includes abnormal growth of choroidal blood vessels leading to detachment of retina along with edema due to vascular leakage.

Drusen are extracellular deposits that accumulate under the basement membrane of the retinal pigmented epithelium (RPE) and the inner collagenous layer of the Bruch membrane (Fig. 1). An age-related accumulation of amyloid-β (Aβ) in the normal mouse retina and human retina has been recently demonstrated (Hoh Kam et al., 2010). Many protein and lipid constituents of drusen are similar to those found in deposits characteristic of other age-related degenerative disorders such as Alzheimer disease (AD). Several studies have led to the comprehension that prefibrillar soluble oligomers, rather than amyloid fibrils, might be the primary toxic agents in AD brain (Kayed et al., 2003; Kayed et al., 2004; Lambert et al., 1998). The presence of prefibrillar oligomers in drusen has been demonstrated (Luibl et al., 2006), suggesting that amyloid oligomers may be involved in drusen biogenesis and/or participate directly in local RPE toxicity (Isas et al., 2010).

Transforming-growth-factor-β1 (TGFβ1) is an anti-inflammatory cytokine with neurotrophic and neuroprotective properties (Caraci et al., 2011; ten Dijke and Hill, 2004). It has been proposed that TGF-β1-TGFβ receptor I (TβRI) axis plays a key role in the function of retinal vascular barrier, by promoting endothelial cell survival and homeostasis (Walshe et al., 2009).

Based on these grounds, we set up an *in vivo* model of AMD using Aβ oligomers to induce retinal damage and investigate the

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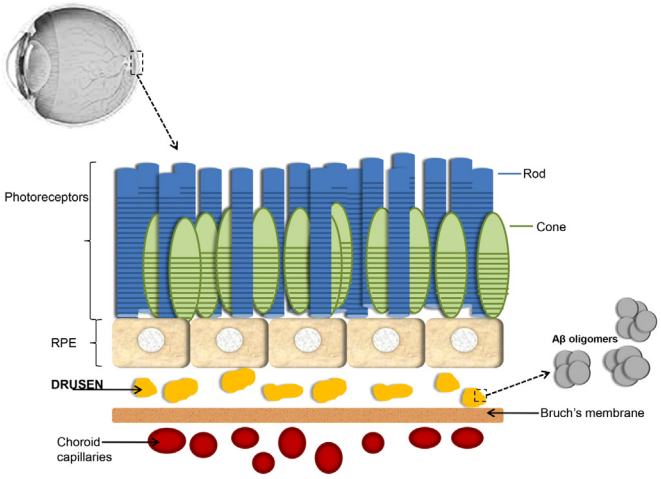

Fig. 1. Schematic diagram of the age-related macular degeneration.

potential protective role of TGF-β1. Furthermore, we carried out a bioinformatics analysis in order to sort out gene pathways commonly related to AMD and AD.

2. Material and methods

2.1. Reagents

SB431542, a selective inhibitor of TGFβ-1 receptor, and protease inhibitors cocktail were purchased from Sigma-Aldrich (St Louis, MO). Rabbit polyclonal antibody against Bax, and rabbit monoclonal antibodies against Bcl-2, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA); secondary goat anti-rabbit IRDye 680 conjugated antibody was purchased from LI-COR (Lincoln, US).

2.2. Amyloid-β oligomers

Human $A\beta_{1-42}$ oligomers were prepared according to the original protocol of Klein's group (Caraci et al., 2015b; Gong et al., 2003). Briefly, the $A\beta_{1-42}$ lyophilized peptide, purchased from Novus Biologicals (Littleton, USA), was dissolved in trifluoroacetic acid (TFA, 1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then TFA was evaporated under a gentle stream of argon and 1 ml 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added to the peptide. After 1 h incubation at 37 °C, the peptide solution was dried under a stream of argon, and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying in a lyophilizer for 1 h and $A\beta_{1-42}$ resuspended in 5 mM anhydrous dimethyl sulfoxide (DMSO), before dilution to 100 μM in ice-cold PBS. Aliquots of 100 μM $A\beta_{1-42}$ were incubated for 72 h at 4 °C and then stored at −20° until use. For *in vivo* experiments human $A\beta_{1-42}$ oligomers were diluted in sterile PBS and 1 μl intravitreally (ITV) injected at the final concentration of 10 μM.

2.3. Animal treatment

Male Sprague-Dawley rats (250–300 g) were purchased from Harlan (Udine, Italy). The animals were fed on standard laboratory food and were allowed free access to water in an air conditioned room with a 12-h light/12-h dark cycle. The animals were randomly divided in four experimental groups: 1) control; 2) ITV injected with 1 μl Aβ at a concentration of 10 μM in PBS; 3) ITV injected with 1 μl of Aβ and TGFβ1 (1 ng/μl; the dose was selected based on previous work, (Caraci et al., 2015a); 4) ITV injected with 1 μl of Aβ, TGFβ1 and the inhibitor of TβRI (SB431542; 20 μM; the dose was selected based on previous work) (Caraci et al., 2015a). Before ITV injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil (tiletamine HCl and zolazepam HCl, Virbac, Milano, Italy); and 1 drop in the eye of the local anesthetic Novesina (Novartis, Origgio, Italy). Animals were sacrificed 48 h after treatment, and retinas were dissected. Housing and treatments were in accordance to Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

2.4. Western blotting

Retinas from control and treated rats were homogenized and sonicated in RIPA buffer (Life Technologies, Monza, Italy) in the presence of protease inhibitor cocktail (Sigma P2714), serine/threonine phosphatase inhibitors (Sigma P0044) and tyrosine protein phosphatase inhibitors (Sigma P5726). Protein concentrations were determined by Bradford's method using bovine serum albumin as standard. Retina lysates (40 μg protein) were loaded into SDS-PAGE, blotted and probed for different target proteins. Membranes were incubated with primary antibodies against total Bcl-2 (Rabbit monoclonal, 1:1000 dilution), Bax (rabbit polyclonal, 1:1000 dilution), GAPDH (Rabbit, monoclonal 1:1000 dilution). The membranes were then incubated with secondary fluorescent antibodies (1:20,000 dilution) for 1 h at room temperature, and the immunocomplexes were detected by Odyssey imaging system (LI-COR, Lincoln, NE). All blots were controlled for equal loading by probing with GAPDH. Densitometric analysis was performed using Image J software.

2.5. Statistical analysis

Statistical significance between two groups was analyzed by Student's *t-test*. One-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test, was used for multiple comparisons. *P* values < 0.05 were considered as statistically significant.

2.6. Bioinformatics analysis

Gene(s) to pathway(s) information was derived from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database. AMD is a complex disease and gene association studies highlighted a series of genes involved in development of AMD: *HGS*, *TNF*, *RAD51B*, *CFH*, *CFB*, *C3*, *ARMS2*, *COL8A1*, *CX3CR1*, *FBLN5*. Analysis of pathways, and pathways interconnection, regulated by these genes was carried out with the web application KENeV-KEGG Enriched Network Visualizer (KENev) (Pilalis et al., 2015). However, output associated to this list of genes was not informative enough. Therefore, an enrichment information strategy was carried out. In a previous work (Romano et al., 2015) we have looked at pathways that are common to neurodegenerative diseases (glaucoma, AMD and AD), with particular focus on glaucoma. Results were obtained through bioinformatic prediction of microRNAs (miRNA) involved in such diseases. The enrichment analysis was carried out with the following steps, because one miRNA can regulate more than one gene:

- searching of miRNAs known to be deregulated in AMD (Romano et al., 2015);
- searching of miRNAs that putatively target genes, retrieved from gene association studies (Romano et al., 2015). This search was carried out through the web server microRNA.org (Betel et al., 2008). Only miRNAs commonly deregulated both in AMD and AD were analyzed;

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