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Cellular senescence in the glaucomatous outflow pathway

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Abstract

The mechanisms responsible for the progressive malfunction of the trabecular meshwork (TM)–Schlemm’s canal (SC) conventional outflow pathway tissue in primary open angle glaucoma (POAG) are still not fully understood. To determine whether POAG is characterized by an accumulation of senescent cells, similar to what has been described in other diseases, we have compared the levels of the senescence marker senescence-associated- β -galactosidase (SA- β -gal) in the outflow pathway cells of POAG and age-matched control donors. POAG donors demonstrated a statistically significant fourfold increase in the percentage of SA- β -gal positive cells. These results suggest a potential role for cellular senescence in the pathophysiology of the outflow pathway.

Keywords

Trabecular meshwork; Senescence; POAG; Glaucoma; Senescence-associated- β -galactosidase

1. Introduction

Primary open angle glaucoma (POAG) is a late-onset disease commonly accompanied by elevated intraocular pressure (IOP) that results from the progressive failure of the trabecular meshwork (TM)–Schlemm’s canal (SC) conventional outflow pathway tissue to maintain normal levels of aqueous humor outflow resistance (Moses, 1977). Although it is believed that POAG results from a combination of genetic and environmental factors (Wang et al., 2001; Klein et al., 2004), the specific causes of the malfunction of the outflow pathway in POAG have not yet been determined.

Cellular senescence has been hypothesized to constitute an antagonistic pleiotropic response that protects against cancer early in life, but has cumulative deleterious effects, contributing to aging and certain age-related diseases (for a review on the pathological effects of senescent cells see Campisi, 2005). Consistent with this hypothesis, accumulation of cells expressing cellular senescence markers has been found to be associated with several pathologic conditions, such as atherosclerosis, kidney fibrosis, hepatic cirrhosis, and osteoarthritis (Johansson, 1984; Ding et al., 2001; Minamino et al., 2002; Wiemann et al., 2002; Martin and Buckwalter, 2003; Sasaki et al., 2005).

Here, we have investigated the potential association between POAG and the expression of a well-known marker for cellular senescence, senescence-associated- β -galactosidase (SA- β -

gal) (Dimri et al., 1995; Alcorta et al., 1996; Chen et al., 2004), in the cells of the outflow pathway.

2. Methods

2.1. Human tissue procurement

Human eyes were obtained from donors within 48 h post mortem from the North Carolina Eye Bank (NCEB) and National Disease Research Interchange (NDRI). Tissues from eye donors were manipulated at all times in accordance with the Declaration of Helsinki. Six donors without a documented history of glaucoma (ages 73.2 ± 4.2) and six donors with a confirmed written history of POAG with elevated IOP (ages 74.7 ± 6.4) were used in this study.

2.2. Senescence-associated- β -galactosidase (SA- β -gal) analysis

For detection of SA- β -gal activity, anterior segments from six donors with a confirmed history of POAG and six age-matched control donors without a history of glaucoma were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, and 0.01% deoxycholate in PBS for 1 h at 4 °C. SA- β -gal activity was detected by overnight incubation at 37 °C in 2 mg/ml 5-bromo-4-chloro-3 β -D-galactoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 150 mM NaCl, and 2 mM Mg_2Cl in 40 mM citric/phosphate buffer at pH 6. After color development, the tissue was post-fixed 3 h in 4% paraformaldehyde and overnight in 30% sucrose in PBS. For microscopic analysis, tissue samples from the four different quadrants were embedded in OCT compound and frozen over dry ice for 1 h. Sections (18 μ m) were then cut in a cryostat HM505-E (Microm), mounted onto gelatin-coated slides, counter-stained with DAPI, and examined under the microscope. To quantify the percentage of SA- β -gal cells in the TM, digital images from each section were generated using light and fluorescence microscopy to visualize SA- β -gal positive cells. Images from different fields were joined using Adobe Photoshop to generate a single picture covering the entire TM/SC outflow pathway. A 50 μ m grid was superimposed, and the number of SA- β -gal positive cells for each square of the grid was counted in a blinded fashion by three independent observers. DAPI stained nuclei were counted by the same method using images of the same fields obtained by fluorescence microscopy.

2.3. Statistical analysis

All statistical analyses were carried out using the SAS software package (SAS Institute, Inc., SAS/STAT® User's Guide, Version 8, Cary, NC: SAS Institute Inc., 1999). Since it is not known whether the number of SA- β -gal positive cells in normal or POAG populations follows a normal distribution or not, we have performed two sets of analysis: a non-paired two-sided *t*-test was used to compare mean differences; a Wilcoxon signed rank test was used to compare median differences. Intra-class correlation (ICC) has been used to assess the agreement between measurements of number of cells between observers for replicate samples.

3. Results

Macroscopic analysis of SA- β -gal activity in human anterior segments from POAG and control donors demonstrated positive staining in the cornea, sclera, and outflow pathway. Although some variability was observed among the 12 pairs of eyes analyzed, increased intensity of SA- β -gal staining was apparent in the tissue from glaucoma donors, especially in the cornea and in the outflow pathway region (Fig. 1A, blue color). Microscopic analysis demonstrated granular blue SA- β -gal staining in the cytoplasm of TM and SC cells (Fig. 1B). The outflow pathway of POAG donors demonstrated a statistically significant ($P=0.01$)

fourfold increase in the percentage of SA- β -gal positive cells compared to controls (Tables 1 and 2). Overall distribution of SA- β -gal staining was preferentially found in the JCT/SC region of the outflow pathway. Specific differences between the TM/JCT and SC regions could not be accurately quantified since frozen sections do not preserve accurately tissue architecture, and were required to retain SA- β -gal intensity.

A significant decrease of 27% in the absolute number of cells (nuclei per section) was observed in the outflow pathway from POAG donors ($P=0.031$, Table 3).

4. Discussion

Acquisition of a senescent phenotype can result from either multiple rounds of cell proliferation in vitro (replicative senescence, RS) (Hayflick and Moorhead, 1961), or by exposure to different types of stress factors (stress-induced premature senescence, SIPS) (Toussaint et al., 2002). Since the proliferation rate of TM cells is very low (Rohen and Lutjen-Drecoll, 1982), it is more likely that acquisition of a senescent phenotype in the glaucomatous outflow pathway may result from SIPS rather than from the exhaustion of their replicative potential. One factor that could potentially contribute to SIPS in the TM is the constant exposure of TM cells to an oxidative environment (Garcia-Castineiras et al., 1992; Spector et al., 1998). Chronic oxidative stress has been shown to induce SIPS in vitro in several cell types (Toussaint et al., 2002), including TM cells (Caballero et al., 2003). Moreover, decreased antioxidant potential, increased expression of oxidative stress markers, and increase oxidative DNA damage have also been reported in glaucoma patients (Ferreira et al., 2004; Izzotti et al., 2003).

An additional factor that could contribute to the observed increased presence of senescent cells in the outflow pathway from POAG donors is the reported increased resistance to apoptosis associated with the acquisition of a senescence phenotype (Wang, 1995), which may favor the survival of senescent cells and lead to their accumulation in the outflow pathway over time.

The impact of cellular senescence on tissue pathophysiology is still under debate. The accumulation of senescent cells has been proposed to contribute to loss of tissue function in aging and several age-related diseases by different mechanisms (Fossel, 2002; Campisi, 2005). Senescent cells can disrupt the local tissue microenvironment by overexpression of several pro-inflammatory cytokines and production of reactive oxygen species (ROS) (Campisi, 1998; 2005; Ding et al., 2001; Kim et al., 2003). The increased production of ROS by senescent cells could potentially lead to an increase in apoptosis of the adjacent non-senescent cells, and therefore contribute to the decrease in the absolute number of cells observed in glaucoma (Alvarado et al., 1984, our own results). In addition, senescent cells can induce important changes in the extracellular matrix (ECM) composition, including the increased expression and degradation of fibronectin, which leads to the accumulation of fibronectin degradation products, that are believed to have noxious effects in tissue physiology (Robert and Labat-Robert, 2000). Accumulation of such degradation products in the TM could potentially result in increased outflow resistance and contribute to the development or progression of glaucoma.

In conclusion, POAG is associated with a significant increase in the number of senescent cells in the outflow pathway. Given the multiple potential adverse effects that these senescent cells might have on outflow pathway function, we hypothesize that cellular senescence could contribute to the increase in aqueous humor outflow resistance and IOP commonly associated with POAG.

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Fig. 1. Analysis of markers for cellular senescence in the outflow pathway of non-glaucomatous and POAG donor eyes. (A) Photographs of the anterior segments from a non-glaucomatous control and a POAG stained for SA-β-galactosidase activity. (B) Presence of SA-β-gal stained cells in cross sections from a control and POAG donor (above), and DAPI staining of the same sections (below). These results are representative of six controls and six POAG eyes analyzed.

Table 1

Donor age, absolute number of cells per section, and percentage of SA-β-gal positively stained cells

Control		POAG			
Age	Cell N ^a	SA-β-Gal% ^{a,b,c}	Age	Cell N ^a	SA-β-Gal% ^{a,b,c}
79	176	2.80	75	132	12.15
68	140	6.33	79	94	15.56
74	159	1.55	71	153	31.52
76	107	2.55	79	114	20.18
73	209	5.18	66	109	17.91
69	197	8.27	84	120	12.54

^a Average number of the four quadrants for each donor eye.
^b (Number of SA-β-gal stained cells/total DAPI stained nuclei)×100.
^c ICC>0.94.

Table 2Statistical analysis of differences in SA- β -gal positively stained cells between control and POAG tissue

	Control	POAG	<i>P</i> -value
<i>N</i>	6	6	
Mean (Std Dev)	4.45 (2.58)	18.31 (7.17)	0.001*
Median	3.99	16.73	0.004**
Min, Max	1.55, 8.27	12.15, 31.52	

*
P-value based on *t*-test,

**
P-value based on Wilcoxon rank sum test.

Table 3

Statistical analysis of differences in absolute number of cells between control and POAG tissue

	Control	POAG	<i>P</i> -value
<i>N</i>	6	6	
Mean (Std Dev)	164.92 (37.74)	120.79 (20.54)	0.031*
Median	167.63	117.50	0.055**
Min, Max	107.25, 209.50	94.25, 153.75	

*
P-value based on *t*-test,

**
P-value based on Wilcoxon rank sum test.