Experimental and Molecular Pathology 90 (2011) 231–237

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Evaluation of oxidative stress markers in pathogenesis of primary open-angle glaucoma

Ireneusz Majsterek [a,*,1], Katarzyna Malinowska [a,1], Malgorzata Stanczyk [a,1], Michal Kowalski [a,1], Jan Blaszczyk [b,1], Anna K. Kurowska [c,1], Anna Kaminska [c,1], Jerzy Szaflik [c,1], Jacek P. Szaflik [c,1]

[a] Department of Clinical Chemistry and Biochemistry, Medical University of Lodz, Poland
[b] Department of Human Physiology, Medical University of Lodz, Poland
[c] Department of Ophthalmology, Medical University of Warsaw, Poland

ARTICLE INFO

Article history:
Received 23 June 2010
and in revised form 9 January 2011
Available online 15 January 2011

Keywords:
Glaucoma
Oxidative DNA damage
Anti-oxidant enzyme
Comet assay

ABSTRACT

Primary open-angle glaucoma (POAG) is the leading cause of blindness in the industrial countries. It is reported that oxidative stress might be an important risk factor in the pathogenesis of POAG. Forty subjects including 20 patients with open-angle glaucoma (9 men and 12 women, mean age 61.8 ± 12.1 yr) and 20 controls without glaucoma symptoms (9 men and 12 women, mean age 58.1 ± 17.7 yr) were enrolled in our study. The main aim of the work was to evaluate oxidative stress markers in the pathogenesis of open-angle glaucoma. In our work the activity of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) as well as the total antioxidant status (TAS) was estimated. An alkaline comet assay was used to measure DNA damage of strand breaks (SB), oxidized purines as glicosylo-formamido-glicosylase (Fpg) sites, and oxidized pirmidines as endonuclease III (Nth) sites. We measured endogenous as well as exogenous DNA damage after 10 μM hydrogen peroxide treatment (H_2O_2). We did not observe any statistical changes of DNA strand break lesion in examined POAG patients according to healthy subjects ($P>0.05$). However, either endogenous ($P<0.01$) or exogenous ($P<0.001$) levels of oxidative DNA damage in POAG patients were found to be statistically higher than controls. A significant decrease of antioxidant enzymes: CAT ($P<0.001$), SOD ($P<0.05$), and GPX ($P<0.001$) and a non-statistical decrease of TAS status ($P>0.05$) in glaucoma patients according to controls were also indicated. In conclusion our data revealed that oxidative stress had a pathogenic role in primary open-angle glaucoma. Therefore, we suggested that the modulation of a pro-oxidant/antioxidant status might be a relevant target for glaucoma prevention and therapy.

Introduction

Glaucoma is a dangerous, chronic, progressive eye disease characterized by optic neuropathy leading to complete vision loss. One of the most frequent types of glaucoma is primary open-angle glaucoma (Kowalski et al., 2008; Nizankowska and Kaczmarek, 2005). It is estimated that over 68 million people suffer from glaucoma and a constant increase of blindness rate up to 7 million people has been reported worldwide, especially concerning open-angle glaucoma. There may be more than 750,000 people affected in Poland (Quigley and Broman, 2006). Its first symptoms are very often difficult to notice for the patient. Findings from epidemiological studies indicate that apart from high intraocular pressure and age, ethnic origin, diabetes mellitus, and familial history are associated risk factors (Quigley and Broman, 2006; Hasselwander and Young, 1998). A positive association between oxidative stress and primary open-angle glaucoma has been reported in several studies (Izzoti and Sacca, 2006; Izzoti and Sacca, 2003; Traverso et al., 2005).

Increased markers of oxidative stress including carbonyls in proteins, lipid oxidation products and oxidized DNA bases have been reported in glaucoma (Sacca and Izzoti, 2005). Reactive oxygen species (ROS) might also cause glaucoma vascular complications through the mitochondrial dysfunction, formation of advanced glycation end-products (AGEs), pseudohypoxia, altered growth factor activity or dyslipoproteinemia. Moreover, decreased levels of anti-oxidants have been associated with glaucoma (Izzoti and Sacca, 2006; Izzoti and Sacca, 2003), suggesting that oxidative stress might play a

Abbreviations: AGE, advanced glycation end-products; AP, abasic site; BER, base excision repair; CAT, catalase; DM, diabetes mellitus; Fpg, glicosylo-formamido-glicosylase; GPX, glutathione peroxidase; Nth, endonuclease III; PBS, phosphate-buffered saline; POAG, primary open-angle glaucoma; ROS, reactive oxygen species; SB, strand break; SOD, superoxide dismutase; TAS, total antioxidant status.

* Corresponding author. Department of Clinical Chemistry and Biochemistry, Medical University of Lodz, Hallera 1 square, 90-647, Lodz, Poland. Fax: +48 42 6331113.
E-mail address: ireneusz.majsterek@umed.lodz.pl (I. Majsterek).

[1] All authors participated equally in the preparation of the manuscript.

0014-4800/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2011.01.001

causal role in this disease. It has been demonstrated that primary antioxidant enzymes such as superoxide dismutase (SOD, scavenges superoxide anions), catalase (CAT, detoxifies hydrogen peroxide), and glutathione peroxidase (GPX, removes hydrogen peroxide and lipids peroxides) had an altered activity in POAG (Ghanem et al., 2010).

The purpose of this work was an evaluation of oxidative stress markers in the pathogenesis of primary open-angle glaucoma. DNA oxidative lesions were estimated in lymphocytes from peripheral blood of POAG patients and healthy control subjects by alkaline comet assay. We measured endogenous and exogenous DNA damage after hydrogen peroxide treatment (H_2O_2). An activity of antioxidant enzymes: catalase, superoxide dismutase and glutathione peroxidase as well as the total antioxidant status (TAS) was also estimated.

Materials and methods

Patients

Twenty patients with primary open-angle glaucoma, 9 men and 12 women, mean age 61.8 ± 12.1 yr were enrolled in this study (Table 1). The control group consisted of twenty subjects, 9 men and 12 women, mean age 58.1 ± 17.7 yr without glaucoma symptoms. All patients and controls were matched on age (no differences were calculated, $P>0.05$). All subjects underwent ophthalmic examination, including best-corrected visual acuity, intraocular pressure, slit-lamp examination, gonioscopy and fundus examination using non-contact and contact fundus lenses with a slit lamp. In the group of glaucomatous patients, the diagnosis of POAG was stated prior to enrolment in accordance with the guidelines of European Glaucoma Society (Terminology and Guidelines for Glaucoma IInd Edition, Dogma, Savona 2003, Italy). POAG patients at the time of enrolling in the study (and being qualified for antiglaucoma surgery) were treated with the combination of three or four topical anti-glaucoma medications including beta blockers (i.e. Timolol), prostaglandin analogs (i.e. Latanoprost) carbonic anhydrase inhibitors (i.e. Dorzolamide) and alpha2 agonists (i.e. Brimonidine).

Medical history was obtained from all subjects, and no one reported present or former cancer or any genetic disease. Patients were excluded from the study if they were subject to any of the following conditions, which could possibly interfere with the results of the study: use of eye drops other than anti-glaucoma preparations, any ocular surgeries or laser treatments performed in the past in the eye from which the specimens were to be collected, present or prior treatment with glucocorticosteroids or immunosuppressive therapy (if these treatments had not been stopped at least 1 yr before the surgery and collection of specimens), use of non-steroidal anti-inflammatory drugs (with the exception of low-dose aspirin, which had to be stopped 7 days before the surgery and collection of specimens), and prior and concurrent systemic antibiotic treatment during the last 7 days before the start of the study.

All subjects included in the study were unrelated Caucasians and resided in Warsaw District, Poland. All patients were recruited from the Department of Ophthalmology, Medical University of Warsaw. The study was reviewed and approved by the institutional ethics committee and met the tenets of the Declaration of Helsinki. Written consent was obtained from each patient before enrolment in the study.

Blood sample preparation

Peripheral blood lymphocytes from blood of healthy donors and POAG patients were isolated by centrifugation (15 min, 280 g) in a density gradient of histopaque-1077 (Sigma, Poznan, Poland). Lymphocytes accounted at about 92% of leukocytes in the obtained cell suspensions as judged by the characteristic shape of their nucleus. Erythrocytes were separated from blood plasma by centrifugation (10 min, 710 g) at 4 °C and washed 3 times with 0.9% NaCl before examination.

Comet assay

DNA damage of single and double SB levels was measured by the single-cell electrophoresis method. The final concentration of lymphocytes was adjusted to $1–3\times10^5$ cells/ml by adding RPMI-1640 medium (Sigma, Munich, Germany) to the single cell suspension. Endogenous and exogenous DNA damage after lymphocyte incubation for 10 min at 4 °C with 10 μM hydrogen peroxide at 4 °C in growth medium was investigated. The comet assay was performed under alkaline conditions according to the procedure of Singh et al. (1988), with modifications by Klaude et al. (1996). A suspension of cells in 0.75% Low Melting Point (LMP) agarose dissolved in PBS was spread onto microscope slides (Superior Marienfeld, Lauda-Königshofen, Germany) precoated with 0.5% normal-melting agarose. The cells were then lysed for 1 h at 4 °C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, and the DNA was allowed to unwind for 40 min in the electrophoretic solution consisting of 300 mM NaOH and 1 mM EDTA, pH>13. Electrophoresis was conducted at 4 °C (the temperature of the running buffer did not exceed 12 °C) for 30 min at the electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI, and covered with cover slips. To prevent additional DNA damage, all the steps described previously were conducted under dimmed light or in the dark.

Endonuclease assay

Fpg and Nth nicks DNA at sites of oxidized purines and pyrimidines respectively, giving breaks that can be detected by the alkaline comet assay (Doetsch et al., 1987). Endogenous and exogenous oxidative DNA lesions after lymphocyte incubation for 10 min at 4 °C with 10 μM hydrogen peroxide at 4 °C in growth medium were investigated. According to the standard method of comet assay, slides after lysis were washed three times in an Fpg/NthI buffer comprising 40 mM HEPES-KOH, 0.1 mM KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0, and the agarose was covered with 25 ml of buffer or NthI as well as Fpg at 1 mg/ml in buffer, sealed with a cover glass, and incubated for 30 min at 37 °C. Further steps were made as described previously.

Comet analysis

The objects were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, Czech Republic). Two parallel tests with aliquots of the same sample of cells were performed

Table 1
The characteristic of open-angle glaucoma (POAG) patients.

Patients n = 20	Age (yr)	POAG diagnosis (months)	Intraocular pressure (IOP)[a]	Visual field defect (VFD)[b]
Mean	61.8 ± 12.1	28.5 ± 2.9	13.4 ± 1.6	3.2 ± 1.3
	Gender men/women	Hypertension[c]	Low blood pressure[d]	POAG in family relatives
Number	9/12	8	5	4

[a] IOP values are expressed as mm Hg.
[b] Visual field defect (VFD) is expressed as Glaucomatous Staging System (GSS).
[c] Systolic pressure ≥ 140; diastolic pressure ≥ 90 mm Hg.
[d] Systolic pressure < 90; diastolic pressure < 60 mm Hg.