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An enhanced expression of ABC 1 transporter in circulating leukocytes as a potential molecular marker for the diagnostics of glaucoma

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Summary. *Objective*: Glaucoma is a neurodegenerative disease. Since vascular dysregulation is supposed to be a risk factor for the development of glaucomatous damage, the preventive treatment might slow down the disease development. The efficiency of the therapeutic treatment depends particularly on a drug efflux pump regulated by ABC transporters. ABC 1 is also known to participate on the vascular regulation. This study was focused on the comparative analysis of ABC 1 expression levels in circulating leukocytes of non-glaucomatous individuals and glaucoma patients.

Results and conclusions: The expression rates of ABC 1 were significantly increased in leukocytes of glaucoma patients compared to non-glaucomatous individuals. The expression level of ABC 1 was, furthermore, highly homogeneous in glaucoma patients. In contrast, these expression levels in non-glaucomatous individuals were extremely heterogeneous. This transporter acts as the energy-dependent unidirectional transmembrane cholesterol efflux pump and can export a wide range of hydrophobic drugs. Additionally an observed enhanced ABC 1 expression in circulating leukocytes may be implicated in the vascular regulation mechanisms of glaucoma. We proposed the enhanced expression of ABC 1 in leukocytes as a potential marker for the diagnostics and *ex vivo* molecular monitoring of glaucoma.

Keywords: Glaucoma – Vascular dysregulation – ABC transporter – Circulating leukocytes – Molecular monitoring *ex vivo* – Diagnostics

Introduction

Glaucoma remains one of the leading causes of blindness with about 67 million patients world-wide. In the filtration surgery of glaucoma there is a need in strong anti-proliferative drugs that decrease post-operative fibrosis and the consequent risk of a fistula closure. A successful application of these drugs ensure, therefore, the effectiveness of trabeculectomy. Besides the cytostatic properties of the anti-proliferative drugs used, the success of their application depends on the cellular sensitivity towards the drugs. This sensitivity is regulated by multidrug resistance (MDR) proteins. MDR proteins are a subgroup of transporters that belong to adenosine triphosphate (ATP)-binding cassette (ABC) transporters, the largest gene family known that is characterized by a high structure homology among the members. While some ABC transporters translocate specifically single substances across membranes, others transport – a wide variety of structurally unrelated lipophilic compounds (Efferth, 2003). They are responsible for the regulation of many physiological processes such as amino acid transport, drug efflux, etc.

Abnormal regulation of ABC transporters has been implicated in a number of diseases. Thus, the increased expression of the lung resistance protein and multidrug resistance protein 1 has been found in surgically removed Tenon specimens from glaucoma patients (Esser et al., 2000). We found an ABC transporter down-regulated in circulating leukocytes of normal-tension glaucoma patients (Golubnitschaja-Labudova et al., 2000). Our approach of the investigation of differential expression of disease relevant genes in circulating leukocytes isolated from glaucoma patients have been positively responded from experts working actively in the research field of glaucoma (Alward, 2001). Moreover, expression profiles of trabecular meshwork and Schlemm's canal were recently found to be similar to the lymphatic expression profile in glaucoma (Tomarev et al., 2003).

Taken together these facts initiated us to investigate the differential expression of ABC transporters in circulating leukocytes of glaucoma patients. This study focuses on the ABC 1 transporter which has been shown to be expressed in macrophages and supposed to be involved in

ageing and age-related diseases (Akiyama et al., 2002; Efferth, 2003). The human ABC 1 gene is 149 kb long with 50 exons interrupted by 49 introns (Santamarina-Fojo et al., 2000). ABC 1 is expressed in a variety of human tissues with the highest levels found in the adrenal gland, liver, lung, intestine, placenta, and fetal tissues (Broccardo et al., 1999; Langmann et al., 1999). It functions as a facilitator of cholesterol and phospholipid exporter at the plasma membrane (Orso et al., 2000). ABC 1 is induced during monocyte differentiation into macrophages and is involved in vascular regulation and protection against atherosclerosis (van Eck et al., 2002; Joyce et al., 2002; Bisoendial et al, 2003). Since vascular dysregulation is supposed to play an important role in pathomechanisms of the glaucoma disease, we investigated the expression rates of ABC 1 in non-glaucomatous individuals *versus* those of glaucoma patients. ABC 1 expression rates were analysed using a non-invasive approach of protein quantification in circulating leukocytes *ex vivo*.

Material and methods

Subjects

Blood samples (20 ml) were collected from twelve non-glaucomatous individuals (later simply "controls") and twelve patients with glaucoma. All glaucoma patients had bilateral typical glaucomatous optic nerve head cupping and visual field defects with a MD greater than 7 dB in the Octopus program G1. For normal-tension glaucoma patients intra-ocular pressure (IOP) never exceeded 21 mm Hg. In contrast, high-tension glaucoma patients demonstrated IOP > 21 mm Hg. Controls had an unremarkable ophthalmologic examination and did not show any vasospastic response. No patient or control subject had received either systemic or local ocular therapy at least four weeks before the study.

All investigations conformed with the principles outlined in the Declaration of Helsinki and were performed with permission from the Ethic's Committee of the Medical Faculty, University of Basel, Switzerland.

Isolation of leukocytes

Blood samples (20 ml) anti-coagulated with heparin were collected from each recruited subject. Leukocytes (including monocytes and stem cells) were separated using Ficoll-Histopaque gradients (Histopaque 1077, Sigma, USA) as described previously (Golubnitschaja-Labudova et al., 2000). Briefly, blood samples were diluted with equal volume of physiological buffer solution (PBS, Gibco^{TM}, USA). Then, 2 ml of histopaque were placed into 10 ml sterile centrifuge tubes and 5 ml of diluted blood samples were carefully layered onto each histopaque gradient. Gradients were centrifuged at 475 g and 20°C for 15 minutes. The leukocytes bands were removed from the interface between plasma and the histopaque layers of each tube and collected into one 50 ml tube. The total volume was brought to 50 ml with cold Dubecco's Modified Eagle Medium (DMEM, Gibco^{TM}, USA). The cell suspension was washed three times with DMEM and the total number of cells was determined. Cells were finally suspended in PBS and aliquoted into Eppendorf tubes at 10^7 cells/tube. After centrifugation cell pellets were dried and stored at −80°C until isolation of individual protein pools.

Western-blot analysis

Aliquots of leukocytes were lysed by homogenisation in lysis buffer [9 M urea (Merck, Germany); 1% DTT (Sigma, USA); 2% CHAPS (Merck, Germany); 0.8% Bio-Lyte, pH 3–10, (Bio-Rad, USA); 5 mM Pefabloc, (Merck, Germany)] followed by centrifugation. The protein concentration in the supernatant was quantified by the DC-Protein Assay (Bio-Rad, USA). Fourty μg protein of each sample were loaded onto 5% SDS-polyacrylamide gels and electrophoresed to resolve proteins. The proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, UK). Thereafter, they were incubated in blocking-buffer (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 5% non-fat dry milk powder; 0.1% Tween 20) for 1 h at room temperature. Incubation with primary antibody was performed using a 1:250 dilution of ABC 1 transporter (Santa Cruz, USA) for 1 h at room temperature in washing buffer I (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% non-fat dry milk powder, 0.1% Tween 20); these antibodies display cross reactivity with the rat proteins. The membranes were then washed four times in the same solution. The horseradish peroxidase-labelled anti-goat secondary antibody was incubated with the membranes for 1 h at room temperature in washing buffer I. This was followed by three washes in washing buffer II (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% non-fat dry milk powder; 0.3% Tween 20) and three washes in washing buffer I. Then the membranes were reacted with chemiluminescent reagent ECL plus (Detection Kit,

Table 1. Information about the subjects tested

Group of controls (non-glaucomatous individuals)			Group of glaucoma patients (normal-tension (NTG) and high-tension (HTG) glaucoma)			
No	Sex	Date of birth	No	NTG/HTG	Sex	Date of birth
1	F	17-01-45	13	NTG	F	12-07-47
2	F	29-10-44	14	NTG	F	16-07-43
3	F	18-08-60	15	NTG	F	09-02-57
4	F	22-09-54	16	NTG	F	18-08-55
5	M	12-05-66	17	NTG	M	12-06-62
6	F	07-06-32	18	NTG	F	14-06-34
7	F	08-02-62	19	HTG	F	26-06-20
8	F	18-08-55	20	HTG	M	18-02-40
9	F	03-08-62	21	HTG	M	27-10-56
10	M	25-12-63	22	HTG	M	05-09-56
11	F	17-03-69	23	HTG	M	21-11-19
12	F	28-05-57	24	HTG	M	07-06-31

Amersham Biosciences, UK) and processed for auto-radiography. The quantification of individual signals was performed using the specialised imaging program "Quantity One" imaging system (Bio-Rad, USA).

Statistical evaluation

Statistical significance was calculated by the two-sided unpaired Student's t-test.

Results

Western-blot analysis demonstrated the well detectable expression of ABC 1 transporter in both normal-tension and high-tension glaucoma patients as well as controls (Fig. 1A, 1B, and 1C). The protein quantification revealed a significantly increased target expression in leukocytes of all glaucoma patients compared to the controls (Fig. 2) that was in average about 14-times higher in the group of patients. Moreover, the expression rates were extremely homogeneous among the patients. In contrast the target expression was highly heterogeneous among the non-glaucomatous individuals (Fig. 3).

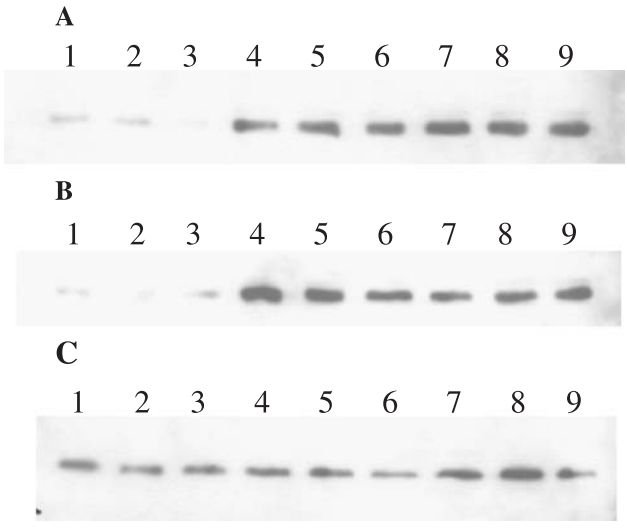

Fig. 1. Western-blot analysis of ABC 1 expression rates in: **A** normal-tension glaucoma (lanes 4–9, that correspond to numbers 10–15 respectively in Table 1), **B** high-tension glaucoma (lanes 4–9, that correspond to numbers 16–21 respectively in Table 1) *versus* controls (1–3). **C** shows ABC 1 expression in controls only (lanes 1–9 that correspond to the numbers given in Table 1)

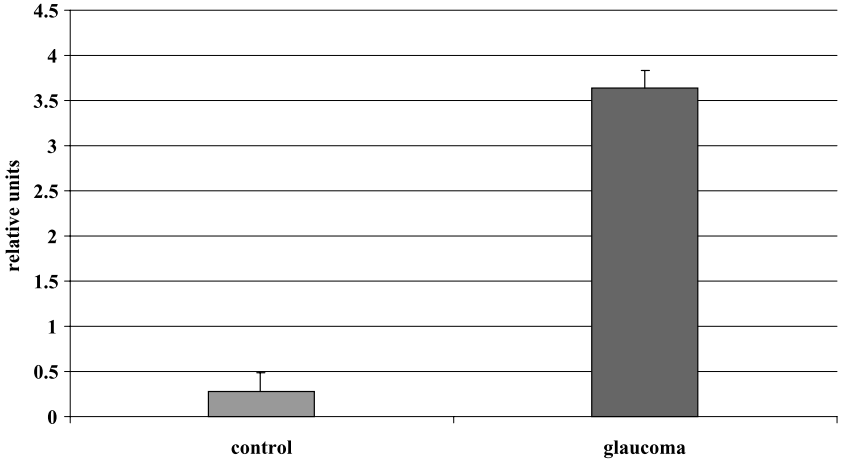

Fig. 2. Quantification of ABC 1 expression level in controls *versus* all glaucoma patients tested

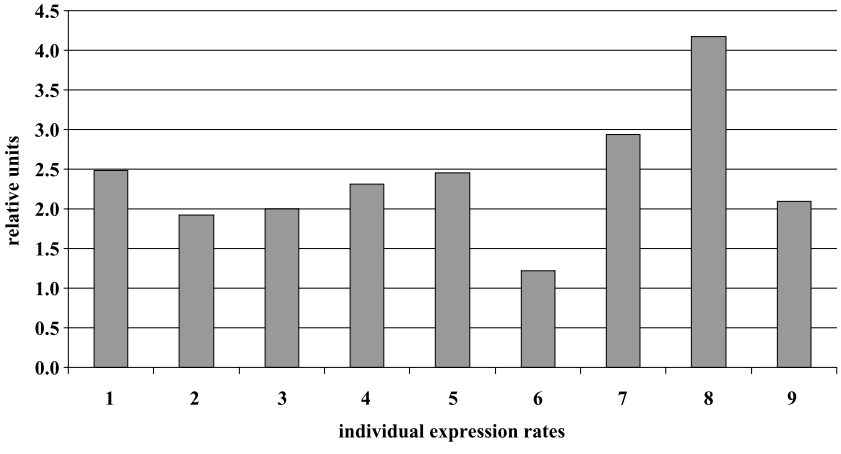

Fig. 3. Quantification of ABC 1 protein expression shows highly heterogeneous levels in controls (lanes 1–9 correspond to the numbers given in Table 1)

Discussion

Previous studies have shown that up-regulation of drug-exporter proteins (ATP-binding-cassette proteins) may be one of the key mechanisms involved in an inducible drug resistance. In this study, we demonstrated the significant up-regulation of ABC 1 transporter on the protein level in circulating leukocytes of all glaucoma patients tested. This ABC 1 up-regulation may play an important role in age-related processes of glaucoma patients, since ABC 1 has been identified as a leukocyte factor that controls the recruitment of inflammatory cells (van Eck et al., 2002; Efferth, 2003).

ABC 1 also known as ABCA 1 belongs to the ABCA subfamily which includes eleven structurally conserved members to date. Known mutations in these genes are associated with human inherited diseases: ABCA 1 – with the cholesterol transport disorders, Tangier disease, severe premature coronary heart disease, coronary artery disease and familial high-density lipoprotein deficiency (Marcil et al., 1999; Brooks-Wilson et al., 1999; Clee et al., 2001; Hayden, 2001), and ABCA 4 – with several retinal degeneration disorders (Sun et al., 2000). However, the mutation study of ABCA subfamily transporters was not the aim of this work. Recent data showed that ABC 1 expressed in leukocytes plays a critical role in the protection against atherosclerosis (Joyce et al., 2002). Furthermore, an activity of ABC 1 has been shown to have a regulating effect on the endothelial function and stimulated nitric oxide bio-activity in arterial walls (Bisoendial et al., 2003). The up-regulation of ABC 1 in leukocytes of glaucoma patients demonstrated in this study may also indicate the involvement of this gene in regulating processes induced by chronic vascular dysregulation.

Noteworthy, the target protein expression was extremely heterogeneous among the non-glaucomatous individuals: some of them demonstrated about 4-fold increase in ABC 1 expression compared to the others. These individuals with the significantly enhanced ABC 1 expression rates might be further considered as persons who suffer from chronic vascular dysregulation with an increased risk for secondary complications including glaucoma.

Our investigations were performed on a small mixed pool of patients with normal- and high-tension glaucoma. Taken together a significantly enhanced expression of ABC 1 in circulating leukocytes may be further considered as a potential marker for the non-invasive glaucoma diagnostics and even prediction of the disease, if these data will be further confirmed on a broad patient collective.

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