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Increased DNA breaks and up-regulation of both G_1 and G_2 checkpoint genes $p21^{WAF1/CIP1}$ and 14-3-3 σ in circulating leukocytes of glaucoma patients and vasospastic individuals

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Summary. *Objective*: Vascular disorder leading to local ischemia/reperfusion has been shown to play an important role in the glaucomatous damage. A decreased expression level of XPGC-gene has been found in circulating leukocytes of normal-tension glaucoma patients. Although decreased activity of XPGC-gene leads to insufficient DNA-repair, no leukopenia has been observed in glaucoma. Molecular mechanisms ensuring cell survival have not been elucidated yet for glaucoma with vascular disorder.

Material and methods: Using the *ex vivo* optical imaging method of alkaline "comet assay" comparative quantification of DNA breaks was performed in circulating leukocytes of non-glaucomatous non-vasospastic and vasospastic individuals as well as both normal-tension and high-tension glaucoma patients. Relative expression levels of the anti-apoptotic factors $P21^{WAF1/CIP1}$ and 14-3-3 σ were investigated in all groups tested.

Results and conclusions: The quantification of $P21^{WAF1/CIP1}$ showed the highest expression rates in high-tension glaucoma patients which were significantly higher than those in all other groups tested. The highest expression rates of 14-3-3 σ were found in both groups of glaucoma patients. These expression levels correlated well with DNA breaks measured. Since the expression of $P21^{WAF1/CIP1}$ in leukocytes was shown to be crucial for their survival under stress conditions, we suppose further that the up-regulation of this gene is the key event in the survival mechanisms of leukocytes in glaucoma accompanied with vascular disorder. The $p21^{WAF1/CIP1}$ gene should be further taken into consideration as a potential marker, the up-regulation of which in circulating leukocytes of vasospastic individuals may indicate an increased risk for the developing glaucoma.

Keywords: Glaucoma – Vascular disorder – Circulating leukocytes – Molecular imaging *ex vivo* – DNA damage – Survival factors $P21^{WAF1/CIP1}$ and 14-3-3 σ

Introduction

Glaucomatous optic neuropathy (GON) is characterised by the combination of a loss of retinal ganglion cells and their axons with a characteristic optic nerve head (ONH) excavation. This ONH excavation, in turn, results not only from axonal loss but also from a loss of glial tissue including capillaries and from a compression, stretching and rearrangement of the cribreform plates of the lamina cribrosa. Although the origin of the glaucomatous cell loss is still not clear yet, a vascular factor has been shown to play an important role in the development of the disease (Flammer et al., 2002). Vascular disorders like vasospasm lead to local ischemic events and by this way induce oxidative stress and concomitant damage of DNA. Unrepairable DNA damage or/and insufficient DNA repair lead to apoptotic cell death. These mechanisms have been considered for circulating leukocytes isolated from glaucoma patients where both a decreased expression of the XPGC-gene and an increased expression of p53 have been demonstrated (Golubnitschaja-Labudova et al., 2000). Nevertheless no leukopenia has been observed in glaucoma patients. This fact indicates an involvement of mechanisms which allow the leukocytes to survive under these conditions.

The checkpoint genes of both G_1 and G_2 phases of cell cycle are known anti-apoptotic factors which are induced by DNA damage. $p21^{WAF1/CIP1}$, the checkpoint gene of G_1 and an inhibitor of cyclin-dependent kinases, is a downstream mediator of p53 function and was shown to play a key role in survival of leukocytes under stress conditions (Gartel et al., 1996; Chen et al., 1996; Ogryzko et al., 1997; Mantel et al., 1999). The arrest of the cell cycle in the G_2 phase is controlled by 14-3-3 σ. The

induction of this gene also prevents apoptosis (Samuel et al., 2001). The activation of both $p21^{WAF1/CIP1}$ and 14-3-3 σ has been recently demonstrated in stressed cardiomyocytes *ex vivo* in the development of cardiomyopathy (Golubnitschaja et al., 2003).

In order to better understand molecular pathomechanisms of stress and defence processes of glaucoma, this study focuses on the comparative investigations of DNA damage and the expression of both anti-apoptotic factors $P21^{WAF1/CIP1}$ and 14-3-3 σ in circulating leukocytes isolated from glaucoma patients and non-glaucomatous vasospastic individuals versus non-glaucomatous non-vasospastic control subjects. Since no information about DNA damage and activation of survival factors in leukocytes of glaucoma patients is currently available, in this phase of our investigations we did not differentiate among diverse species of circulating leukocytes that will be done later.

Material and methods

Subjects

Blood samples (20 ml) were collected from six non-glaucomatous non-vasospastic individuals (later called simply "controls", group 1), six non-glaucomatous individuals with vasospasm (group 2), six patients with normal-tension glaucoma (NTG, group 3), and six patients with high-tension glaucoma (HTG, group 4, see Table 1). All glaucoma patients (groups 3 and 4) 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 NTG-patients (group 3) IOP never exceeded 21 mm Hg. The presence of vasospasm was detected by nail-fold capillaromicroscopy. After local cooling of the fingers all vasospastic patients with glaucoma as well as non-glaucomatous vasospastic individuals exhibited a stop in blood flow for more than 20 sec. In contrast, controls had an unremarkable ophthalmological 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.

Leukocytes isolation

Blood samples (20 ml) anti-coagulated with heparin were collected from patients, non-glaucomatous vasospastic individuals and controls. Leukocytes 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 volumes 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 Dulbecco's Modified Eagle Medium (DMEM, Gibco^{TM}). 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 protein isolation.

Comet assay analysis

Single-stranded DNA damage was measured as increased migration of DNA using the single-cell gel-electrophoresis technique (comet assay) under alkaline conditions. The measurement was performed in isolated leukocytes *ex vivo*. The comet assay (Trevigen Inc., USA) provides a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of cleaved DNA fragments to migrate out of the cell under the influence of an electric field. Undamaged DNA migrates slower and remains within the confines of the nucleoid (the "head" of "comet") when a current is applied. Evaluation

Table 1. Information and grouping of the tested subjects

No.	Sex	Date of birth	No.	Sex	Date of birth
Group 1: non-glaucomatos, non-vasospastic individuals (controls)			Group 2: non-glaucomatos, vasospastic individuals		
1	F	17.01.1945	7	F	08.02.1962
2	F	29.10.1944	8	F	18.08.1955
3	F	18.08.1960	9	F	03.08.1962
4	F	22.09.1954	10	M	25.12.1963
5	M	12.05.1966	11	F	17.03.1969
6	F	07.06.1932	12	F	28.05.1957
Group 3: normal-tension glaucoma with vasospasm			Group 4: high-tension glaucoma with (+VS) and without vasospasm (−VS)		
13	F	12.07.1947	19	F (−VS)	26.06.1920
14	F	16.07.1943	20	M (+VS)	18.02.1940
15	F	09.02.1957	21	M (+VS)	27.10.1956
16	F	18.08.1955	22	M (+VS)	05.09.1956
17	M	12.06.1962	23	M (−VS)	21.11.1919
18	F	14.06.1934	24	M (+VS)	07.06.1931

of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage.

In detail this method was described in literature (Ostling and Johanson, 1984; Singh et al., 1988; Malyapa et al., 1998; Lemay and Wood, 1999) and performed by us as described earlier (Golubnitschaja et al., 2003).

Western-blot analysis

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 a centrifugation step. 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 12% SDS-polyacrylamide gels and electrophoresed to resolve proteins. The proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, UK) and afterwards 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. Primary antibody incubation was performed using a 1:250 dilution of either anti-human-

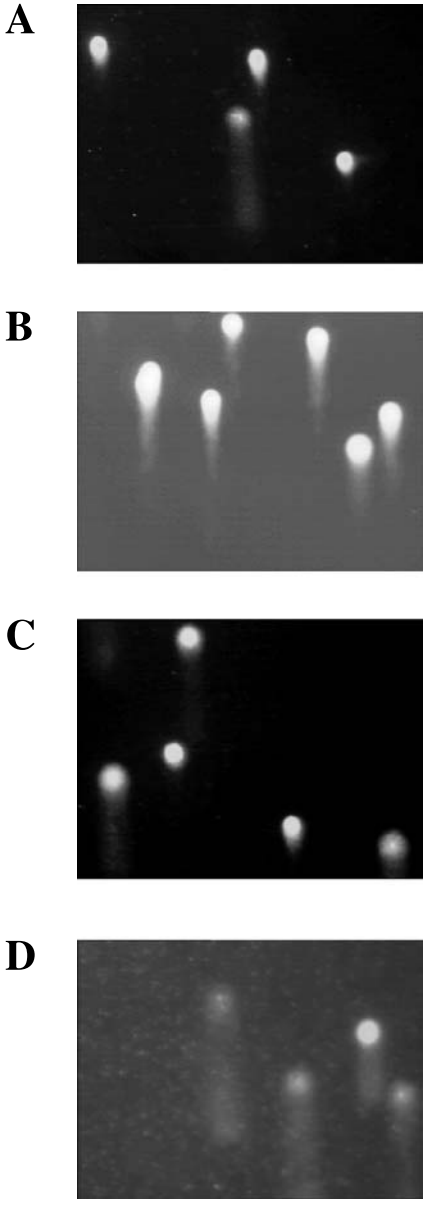

Fig. 1. "Comet assay" analysis of DNA damage: examples of the pictures typical for **A** control group, **B** group of vasospastic individuals, **C** group of normal-tension glaucoma patients, and **D** group of high-tension glaucoma patients. The "head" of each "comet" shows the intact DNA and the "tail" demonstrates a damaged DNA

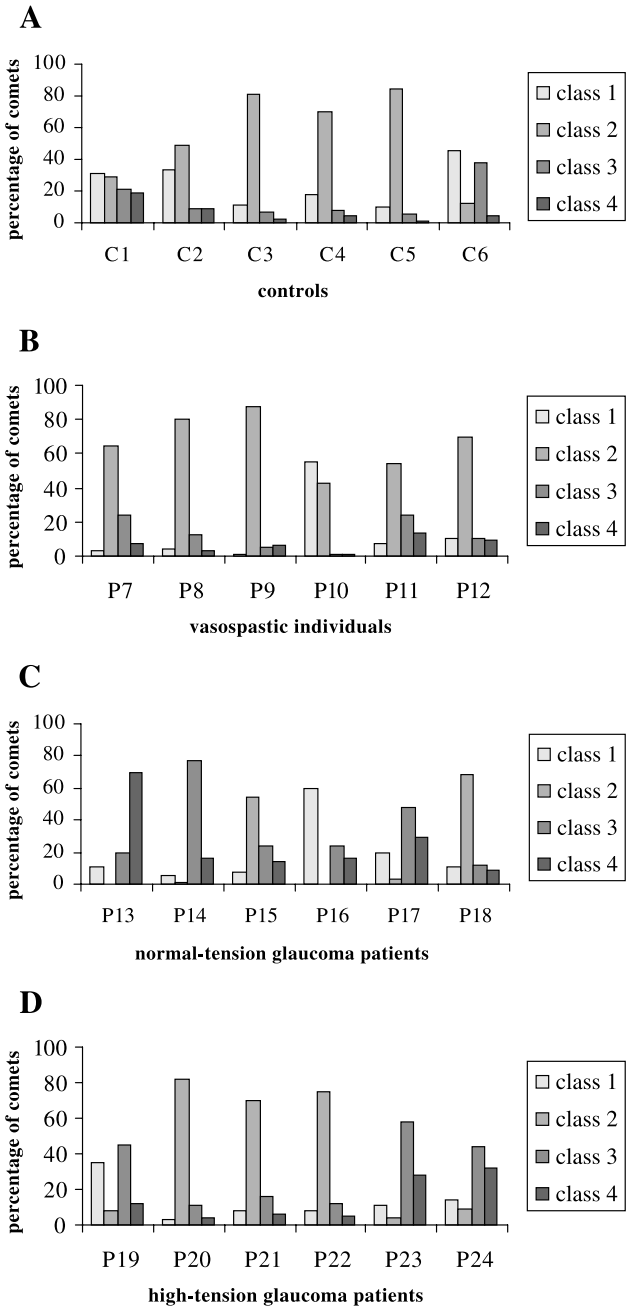

Fig. 2. Evaluation of "comet assay" individual data: **A** control group, **B** group of vasospastic individuals, **C** group of normal-tension glaucoma patients, and **D** group of high-tension glaucoma patients

$P21^{WAF1/CIP1}$ or anti-human-14-3-3 σ (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). 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 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 chemiluminescence reagent ECL plus (Detection Kit, Amersham Biosciences, UK) and processed for autoradiography. The quantification of individual signals was performed using the specialized imaging program "Quantity One" imaging system (Bio-Rad, USA).

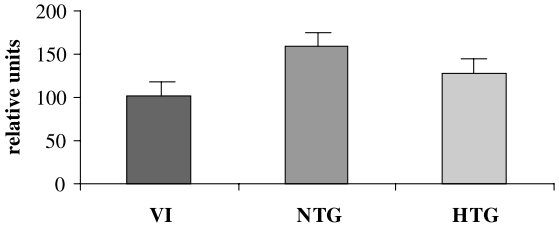

Fig. 3. Calculated mean values of relative DNA damage of groups 2 (102 ± 7%), 3 (160 ± 13%), and 4 (128 ± 10%) compared to the control group (100%). *VI*, means vasospastic individuals, *NTG*, normal-tension glaucoma, and *HTG*, high-tension glaucoma

Results

Comet assay

Compared to the control group an increased DNA damage was observed in both NTG (group 3) and HTG (group 4) as well as in the group of non-glaucomatous vasospastic individuals (group 2). The examples of "comets" typical for each group are shown as fragments of monitored pictures (Fig. 1A–D).

The ratio of the most damaged (class 4) to intact DNA (class 1) molecules is about 0.2, 0.3, 1.2, and 0.9 for groups 1, 2, 3, and 4 respectively (Fig. 2). The ratio between the amount of class 2 and class 3 comets was significantly higher in the group of non-glaucomatous vasospastic individuals compared to all other groups (3.6, 7.2, 0.7, and 2.6 for groups 1, 2, 3, and 4 respectively). The calculated relative DNA damage in groups 2, 3 and 4 compared to the group 1 is shown in Fig. 3.

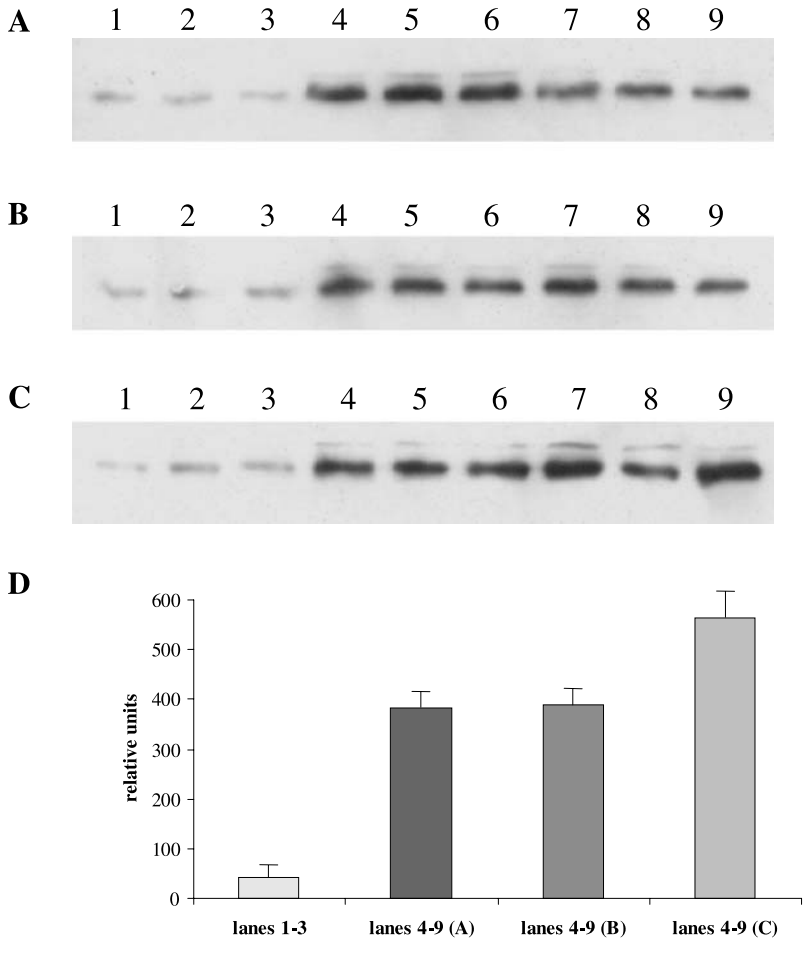

Fig. 4. Western-blot analysis of $P21^{WAF1/CIP1}$ expression in circulating leukocytes isolated from **A** vasospastic individuals (lanes 4–9), **B** patients with normal-tension glaucoma (lanes 4–9), and **C** patients with high-tension glaucoma (lanes 4–9) in comparison to the controls (lanes 1–3). **D** shows the comparative evaluation of expression rates among groups

The individual DNA damage values of the group 1 were heterogeneous in the distribution of DNA damage (Fig. 2A). Whereas group 2 showed a relatively homogeneous distribution of DNA damage with comets belonging mostly to class 2 (Fig. 2B). Compared to the group 1, the group 2 showed about 2-times more comets in class 2 and about 4-times less comets in class 1. Group 3 displayed the most heterogeneous comet profiling of all subjects analysed (Fig. 2C). Similarly to group 2, the patients with vasospastic dysregulation in group 4 demonstrated the majority of comets in class 2, whereas patients of this group without vasospastic dysregulation demonstrated the majority of comets in class 3 (Fig. 2D).

Expression of P21$^{WAF1/CIP1}$

The quantitative protein expression analysis showed a significantly increased expression of P21$^{WAF1/CIP1}$ in leukocytes of groups 2, 3 and 4 compared to group 1 (Fig. 4A–D). The highest expression was measured in group 4 which was 1.5-fold higher than in both groups 2 and 3. Compared to group 1 it was 13.2-fold increased in group 4. Groups 2 and 3 demonstrated similar expression levels to each other. There were no significant differences in the expression within the groups 3 and 4 of glaucoma patients. In contrast, we observed great divergences among individual expression rates within both groups 1 and 2.

Expression of 14-3-3 σ

We observed significantly increased expression levels of 14-3-3 σ in leukocytes isolated from both groups 3 and 4 of glaucoma patients compared to group 1 (Fig. 5A–D). Thereby, the expression rates were slightly higher in group 4 compared to group 3. Thus, the highest expression level was measured in group 4 which was approximately 1.2-fold higher than in groups 1 and 2. The expression levels measured in leukocytes of group 2 was comparable with that of group 1. There were no significant differences in the expression rates among the subjects within the groups.

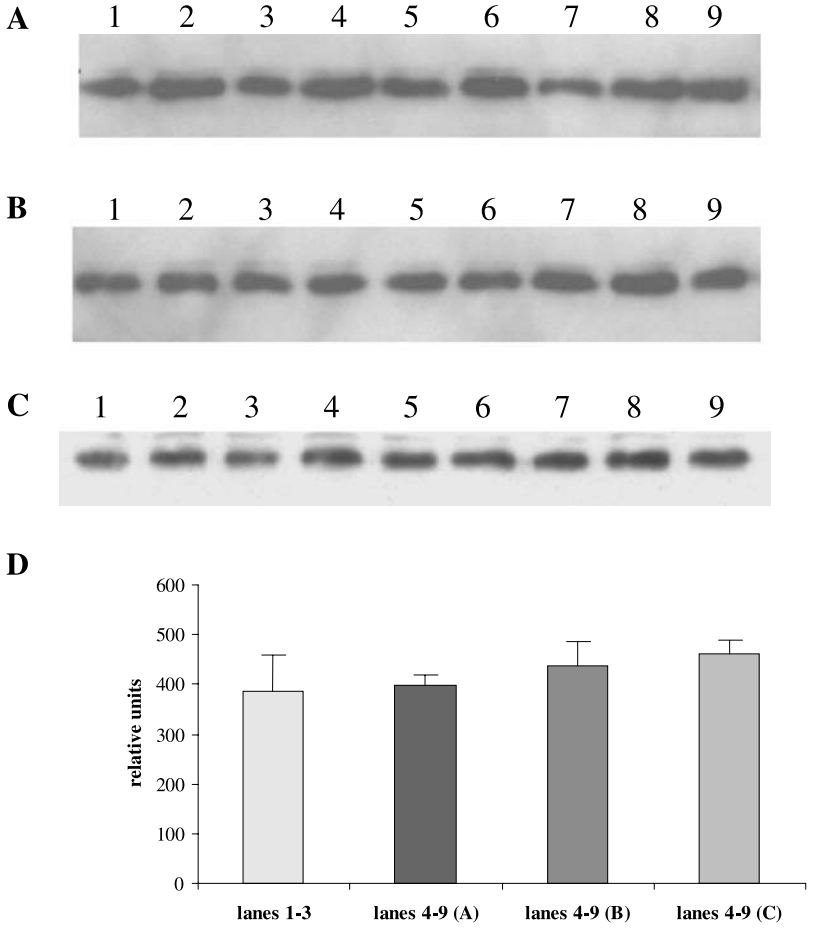

Fig. 5. Western-blot analysis of 14-3-3 σ protein expression in circulating leukocytes isolated from **A** vasospastic individuals (lanes 4–9), **B** patients with normal-tension glaucoma (lanes 4–9), and **C** patients with high-tension glaucoma (lanes 4–9) in comparison to the controls (lanes 1–3). **D** shows the comparative evaluation of expression rates among groups

Discussion

This study was focused on the *ex vivo* comparative investigations of DNA damage in circulating leukocytes isolated from patients with glaucoma and non-glaucomatous vasospastic individuals *versus* the control group with non-glaucomatous non-vasospastic subjects. Based on the results of ''comet assay'' analysis we conclude that glaucoma patients independent of the form of disease (normal-tension or high-tension) demonstrate considerably enhanced DNA damage compared to both non-glaucomatous vasospastic and non-vasospastic individuals. However, individual values measured in the group with normal-tension glaucoma were highly heterogeneous: some of the patients (patients 15 and 18) demonstrated values which were similar to those of non-glaucomatous vasospastic individuals, others, however, demonstrated values found also in the group with high-tension glaucoma. The values shown from patients with high-tension glaucoma were obviously dependent on the presence or absence of vascular dysregulation: patients with vascular dysregulation (patients 20, 21, 22) showed a comet profiling typical for the group of vasospastic individuals, who demonstrated the most homogeneous comet distribution. This typical comet profiling was found in approximately 30% of normal- and 50% of high-tension glaucoma patients. This finding confirms an important role of the vascular dysregulation in molecular pathomechanisms of glaucoma.

In this work we observed a significant activation of the $p21^{WAF1/CIP1}$ gene in groups with glaucoma patients and vasospastic *versus* non-vasospastic individuals. It is the first report clearly showing similarities between the regulation of molecular mechanisms in glaucoma accompanied with vascular disorder and vasospastic syndrome. It is important to mention that equal protein levels of $P21^{WAF1/CIP1}$ were detected in the vasospastic group and the group of normal-tension glaucoma patients, whereas the expressional level in the group of high-tension glaucoma patients was even higher.

$p21^{WAF1/CIP1}$ is the central gene for the G_1 arrest mediated by p53. Furthermore, $p21^{WAF1/CIP1}$ induction correlates with growth arrest associated with monocyte-macrophage differentiation (Zhang et al., 1995). Apoptosis induced under stress conditions was shown to be p53-dependent in T-cells (Fotedar et al., 1995). It is still unclear, whether the p53-inducible $p21^{WAF1/CIP1}$ gene acts also as a downstream effector of p53-dependent apoptosis. The experiments with p53 functional impairment and high $P21^{WAF1/CIP1}$ expression in human T-cell lymphotropic/leukemia virus type I-transformed T-cells showed, however, that expression of $p21^{WAF1/CIP1}$ can be regulated through multiple mechanisms. This suggests that strategies may be designed to restore the G_1 checkpoint which controls $p21^{WAF1/CIP1}$ gene induction *via* p53-independent mechanisms even in p53-null cells (Cereseto et al., 1996). These strategies become even more attractive taking into account the further finding that T-cells from transgenic mice with restricted expression of $p21^{WAF1/CIP1}$ are hypersensitive to radiation damage. Taken together these facts indicate that $p21^{WAF1/CIP1}$ is the central factor in anti-apoptotic molecular mechanisms, and its accumulation results in the survival of circulating leukocytes under stress conditions. Since ischemic/reperfusion events generally observed in glaucoma result in cellular damage, the activation of $p21^{WAF1/CIP1}$ in circulating leukocytes of glaucoma patients, therefore, is an adoptive response to stress conditions which ensures survival of leukocytes preventing leukopenia. The detected accumulation of $P21^{WAF1/CIP1}$ indicates further, that in vasospastic individuals and glaucoma patients the stressed leukocytes have heavily damaged DNA molecules. These findings are underlined by the detected up-regulation of 14-3-3 σ in both patient groups. In vasospastic individuals these levels were comparable to those of control subjects. The approximately equal activation of $P21^{WAF1/CIP1}$ found in our experiments in both normal-tension glaucoma patients and vasospastic groups and the even stronger activation in high-tension glaucoma patients indicates the dominating role of the vascular factor in the regulation of molecular mechanisms, and supports the hypothesis about the vascular origine of the pathophysiology of normal-tension glaucoma. Whereas the pathophysiology of high-tension glaucoma is obviously influenced by additional factors which pronounce the damaging effect of vascular dysregulation.

Taken together our results – achieved on the small patient collective – show that the combination of molecular imaging using ''comet assay'' and *ex vivo* monitoring of expression rates of $p21^{WAF1/CIP1}$ and 14-3-3 σ may be the useful imaging approach for the determination of potential risk for glaucoma and more precise diagnostics of the disease, if our findings will be confirmed on a broad patient pool.

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