Matrix Metalloproteinase and Their Inhibitors Concentration and Activity Variations in Aqueous Humor and Plasma of Glaucoma Patients

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Matrix metalloproteinases (MMPs) are a group of endopeptidases with the role of reorganizing the extracellular matrix of cells through the body. In aqueous humor MMPs have an important role in turnover regulation by acting on the constant remodeling of the trabecular mesh keeping at a constant level the trabecular resistance for a good aqueous humor outflow and consequently of the intraocular pressure.In this paper, we aimed to assess the levels of MMP-2/TIMP-1 and MMP-9/TIMP-2 ratio in aqueous humor samples and in the plasma of patients with open angle glaucoma and cataract patients that serves as control, and to evaluate the gelatinolytic activity MMP-2 and MMP-9 in aqueous humor samples. Aqueous humor and plasma samples were collected from 30 patients, 14 from open angle glaucoma and 16 from cataract patients. Levels of MMP-2, -9 and TIMP -1 and -2 were determined by zymography and immunoassays, using specific kits. The data obtained suggest the presence of a direct correlation between the levels and activity of MMP influencing the accumulation of abnormal matrix and may have an impact on the pathogenesis of open angle glaucoma.

Keywords: open angle glaucoma, matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase

Glaucoma is considered an important health concern nowadays worldwide, WHO statistically attributing glaucoma the second cause of blindness, after cataract and also the first cause of irreversible blindness [1, 2]. This pathology is estimated to affect 70 million people around the world [3]. It is a progressive optical neuropathy in time leading to visual field loss, caused by multiple factors, one of the most important being intraocular pressure [4, 5].

It is generally accepted that glaucoma can be classically divided into open angle and closed angle (judging by the anatomy of the iridocorneal structures) it can also be classified as primary or secondary glaucoma.

Depending on the patient and the subjacent pathology the first step in the treatment is establishing a target IOP (the value of pressure at which the disease is not progressing) [6]. For achieving the target IOP the initial therapy preferred is topical medication using molecules that reduce the production of aqueous humor or enhancing the outflow.

Therapeutic options are represented by medical treatment or procedural treatment - surgical or laser. The purpose is maintaining the patient's visual function and the quality of life. Lowering intraocular pressure to the target IOP is regarded as the main treatment choice even though there are several studies like Parisi [7] which are trying to find new therapeutic pathways like managing ocular blood flow [8,9] and neuroprotection [10-12] as a complementary or more efficient strategy [13]. The main therapeutic target in glaucoma is lowering IOP. IOP is the equilibrium between the rate of aqueous humor production and the outflow through iridocorneal angle (trabecular meshwork, Schlemm's canal) and via uveoscleral pathway (5-15%). The extracellular matrix of the trabecular meshwork is permanently being remodeled by certainness members of the MMP group (MMP-1,-2,-3,-9 and -14) and their inhibitors TIMP-1 and TIMP-2 [14].

To obtain the target IOP the initial therapy preferred is topical medication using molecules that reduce the production of aqueous humor or enhancing the outflow.

Medical treatment is generally the first one to consider, regarding the degree of the intraocular pressure value that needs to be reduced [15]. The cost and patient compliance and tolerability are also factors that need to be considered in choosing this type of treatment. Medical treatment – local or systemic – is represented by different drug agents like beta blockers, prostaglandin analogs [16], conversion enzyme inhibitors, alfa agonisers, parasympatomimetics [17, 18].

Another treatment option is laser therapy. Argon laser trabeculoplasty may represent the first choice in glaucoma treatment, or as an alternative where medical treatment has proven inefficient [19]. The efficiency of this method is kept at a 50% rate of success in 5 years and only 30% in 10 years [20]. Other alternatives depending on the type of glaucoma, includes: NdYAG doubled in frequency, YAG laser iridotomy and laser cycloabaltion.

Surgical treatments in glaucoma are classified as penetrating or non-penetrating. Trabeculotomy is the penetrating surgical procedure considered as the gold standard in glaucoma surgery as well as artificial draining systems [21]. As for non-penetrating surgery vasco-canaloplasty and profound/deep sclerectomy represent an option [22].

Taking into consideration their efficiency, despite the multitude of the therapeutic panel and regardless of the type of treatment (medical, surgical or laser) there is a limitation in the treatment response at around 5 years. That is why glaucoma presents a high interest in research studies in the pursuit of finding new and improved treatment methods and new molecules with a response period longer than 5 years [23].

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that are involved in the

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breakdown and the reorganization of the extracellular matrix (ECM) under both normal and pathological conditions. The activity of MMPs is controlled by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) and alpha-macroglobulins are the most important [24-26]. Among the MMPs, MMP-2 and MMP-9 are also referred to as gelatinases A (72 kDa) and B (92 kDa), respectively. Both MMP-2 and -9 degrade similar substrates, such as gelatin, collagen types IV and V, elastin, laminin, fibronectin and proteoglycans.Matrix metalloproteinases have an important role in the regulation of the extracellular signal network being involved in different processes like angiogenesis, immunity, and wound healing [27]. The principal ocular cells implicated in PEX material production are those closely associated with the aqueous humor circulation (i.e., nonpigmented ciliary epithelium, iris pigment epithelium, iridal vascular cells, equatorial lens epithelial cells, and trabecular endothelial cells) and are thus influenced by the substances contained therein. The composition of the aqueous humor may therefore play an important role in influencing the matrix metabolism of adjacent tissues. Similarly, an excessive accumulation of extracellular material in the juxtacanalicular tissue of the meshwork has been postulated to cause an increased outflow resistance in eyes with primary open-angle glaucoma (POAG), and an impaired trabecular meshwork matrix turnover, which is critical to the regulation and maintenance of aqueous humor outflow, has been implicated in the development of POAG [28,29]. In aqueous humor matrix metallo-proteinases have an important role in turnover regulation by acting on the constant remodeling of the trabecula keeping at a constant level the trabecular resistance for a good aqueous humor outflow and consequently of the PIO [30,31].

In this paper, we aimed to assess the levels of Matrix Metalloproteinases-2 and -9, and of their endogenous inhibitors, TIMP- 1and TIMP-2, in aqueous humor samples and in plasma of patients with glaucoma, undergoing cataract surgery, and to evaluate the gelatinolytic activity and quantitative variety and compare the results with cataract patients.

Experimental part

Materials and methods

Patients were recruited from the outpatient service of the Department of Ophthalmology at the University Emergency Hospital of Bucharest, Romania. A total of 30 patients were included in the study, and 2 groups were formed: 14 patients with glaucoma 16 control patients with cataract. A senior ophthalmologist made the diagnosis of glaucoma and cataract after a careful evaluation that included slit-lamp examination, tonometry, gonioscopy, visual field, optic nerve fiber measure (with an optical coherence tomography), central corneal thickness measurement and optic disc assessment with a 78 diopters non-contact lens. The diagnosis of glaucoma was made according to the European Glaucoma Society guidelines

Exclusion criteria were other systemic disease (diabetes, infectious or autoimmune disease), other ocular pathology (retinal pathology, optic neuropathy, corneam distrophie, ocular inflamation).The patients were chosen to be in the same age-range (mean±SD age: 70± 7.6 years). Before surgery an informed consent regarding aqueous humor and plasma donation was obtained from the patients, to ensure that the study was in assent with the guidelines of the Declaration of Helsinki for experiments involving human tissue. The informed consent was first approved by the Ethics Committee of the University Emergency Hospital of Bucharest.

Samples

All aqueous humor samples were collected by the same experimented surgeon at the initial steps of the cataract surgery. A very strict chemoprophylaxis was made, topical use of 5% povidone iodine solution for 1 min. Anterior chamber paracentesis was made through clear cornea with a 27G tuberculin syringe and an amount of 80-100 mL aqueous humor was withdrawn[32].050568, The samples were frozen (-80°C) after prelevation, and kept for the following biochemical determinations.

The blood samples were collected after an overnight fast, before surgery. *Following the* centrifugation of blood samples at 2500*g*/ 10 min, plasma was separated, aliquoted, and stored at -80°C until use.

Zymographic Analysis of Gelatinases

The gelatinolytic activities of MMP-2 and MMP-9 were tested in aqueous humor samples prelevated from patients under study. The samples were subject to electrophoresis on 10 % SDS-PAGE co-polimerized with 1% (w/v) gelatin, under non-reducing conditions. Gels were washed twice in 2.5% Triton X-100 and incubated overnight at 37°C in developing buffer (50 mM Tris-HCl, *p*H 7.5, 5 mM $CaCl_2$, 0.2M NaCl). After staining with Coomassie Blue R250, the proteolytic activity was revealed as cleared bands against a blue background. Gelatinolytic [33] signals were quantified, and the results were reported as mean percentage of control.

ELISA procedure for human MMP-2/ TIMP-1 and MMP-9/ TIMP-2 complexes Plasma and aqueous humor levels of MMP-2/TIMP-1 and MMP9/TIMP-2 complexes were measured as per recommendations of the manufacturer (DuoSet, R&D Systems, Inc.). Goat anti-human MMP-2 and MMP-9 antibodies were coated overnight on 96 well microplates as capture antibody. Wells were washed thoroughly and plates were blocked for 1 h with block buffer (1% bovine serum albumin in PBS) and then washed thoroughly. 100 ul of samples (plasma or aqueous humor) or standard in diluent (50 mM TRIS, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij 35, *p*H 7.4) were added to each well for 2 hours. After aspiration and washing, 100 ul of biotinylated goat anti-human TIMP-1/TIMP-2 detection antibodies were added to each well and incubated for 2 h. After washing, Streptavidin-HRP reagent diluted 1:200 was added to each well for 20 minutes followed by washing and addition of Substrate solution (mixture of H_2O_2 and tetra-methylbenzidine). Stop solution (2N H_2SO_4) was added and optical density of each well was determined in a microplate reader set to 450 nm with wave length correction at 570 nm. Samples were run in duplicate.

Statistical analysis

Data are presented as the mean ± standard deviation. Differences of clinical and analyzed parameters from two independent groups were assessed using unpaired *t*- test and Fisher's exact test. Pearson correlation coefficients were computed to investigate correlations between variables. A p value less than 0.05 indicated statistical significance. All statistical analyses were conducted using SPSS version 21.0 (IBM Inc.).

Results and discusions

In this study we enrolled 30 patients, 14 patients with primary open angle glaucoma (POAG) and 16 cataract

Table 1
CLINICAL PARAMETERS OF CONTROL –CATARACT AND PRIMARY OPEN ANGLE GLAUCOMA PATIENTS

Parameters	Cataract	POAG	p-value
Age (year)	67.06±7.36	73.57±8.00	**0.028**
Gender (M/F)	9/7	5/9	0.299
IOP (mmHg)	14(10.75-21.50)	13.50(10.00-16.50)	0.322
Glycaemia (mg/dL)	95(85.00-100.00)	106.50(95.25-118.50)	0.521
LDL (mg/dL)	142.00 (110.00-162.50)	132(91.50-140.25)	0.079
HDL (mg/dL)	43 (32.00-53.00)	41.50 (36.25-49.00)	**0.0001**
TGL (mg/dL)	130 (105.00-158.00)	185 (160.25-207.50)	**0.0001**
Chol (mg/Dl)	170.00 (111.50-196.00)	185 (160.25-207.50)	0.957
VSH (mm/h)	13.00 (5.00-28.00)	13.50 (11.00-31.00)	0.915
Creatinine (mg/dL)	1 (1.00-1.05)	1.30 (0.93-1.75)	0.091
MMP-2/TIMP-1(pg/mL)	157.30 (40.87-217.85)	202.96 (190.33-222.15)	**0.056**
MMP-9/TIMP-2 (pg/mL)			0.252

Data are presented as mean ± SD for normal distributed parameters, and as median and IQR for not-normally distributed parameters. POAG: Primary open angle glaucoma; SD: standard deviation; M: male; F: female; IOP: intraocular pres-sure in mmHg; IQR: interquartile range. Statistical significance was assessed using unpaired *t* test and Fischer's exact test (gender), with $p<0.05$ considered significant.

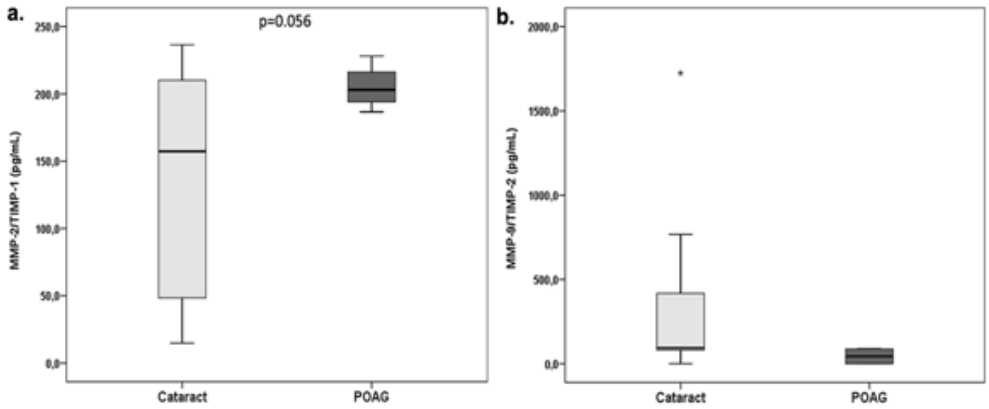

Fig. 1. Plasma concentration of MMP-2/TIMP-1 and MMP-9/TIMP-2 in cataract and POAG patients assessed with DuoSet ELISA kit. Boxplot representing the distribution (median and interquartile range) of MMP-2/TIMP-1 (a), and MMP-9/TIMP-2 levels in plasma of cataract and POAG patients Two-sided t-test with unequal variance was used to compare the analyte concentrations in control and glaucoma patients; $p<0.05$ was considered as statistically significant

patients as a control group. Clinical data including age, IOP, and biochemical parameters of the study patients are presented in table 1. All PAOG patients received medication to lower their IOP level.

Significant differences were found between the cataract control and the POAG group concerning age ($p = 0.028$), TGL ($p<0.001$), and HDL ($p<0.001$), with no differences for gender, IOP, Glycemia, LDL, Creatinine, Urea, and VSH. In the control group, we found that HDL significantly correlates with age ($r=-0.509$, $p=0.037$), LDL ($r=-0.634$, $p=0.006$), TGL($r=-0.619$, $p=0.008$), and VSH ($r=-0.464$, $p=0.038$). Also, in the POAG group, significant correlations have been also found between LDL, TGL, HDL, Total cholesterol, age and VSH.

When we analyzed the MMP/TIMP ratio in the plasma samples of the study patients, increased levels of MMP-2/TIMP-1 were found in POAG group as compared with cataract group ($p=0.056$), with no differences of MMP-9/TIMP-2 levels among the two groups (fig. 1).

Furthermore, Pearson correlation method was used to assess correlation between MMP/TIMP and clinical and biochemical parameters of patients from cataract and POAG group. Our results showed that MMP-2/TIMP-1 levels in plasma of cataract control group significantly correlates with LDL ($r=0.639$, $p=0.025$), TGL ($r=0.740$, $p=0.006$), and Chol ($r=0.696$, $p=0.012$), and with MMP-9/TIMP-2 ($r=-0.624$, $p=0.040$). In the group of glaucoma patients, correlation between plasma levels of MMP-2/TIMP-1 and glycaemia ($r=-0.735$, $p=0.06$) was observed, being near to the limit of significance.

MMP-2 levels in aqueous humor samples increase in the POAG groups compared to the cataract group

MMP-2/TIMP-1 levels in glaucomatous aqueous humor samples have been found to be significantly increased as compared to cataract-control group ($p=0.006$). Also, increased levels of MMP-9/TIMP-2 in aqueous humor samples of POAG patients have been observed when compared with controls, but due to the high dispersion of

Table 2

AQUEOUS HUMOR LEVELS OF MMP-2/TIMP-1 AND MMP-9/TIMP-2 COMPLEXES OF CATARACT AND GLAUCOMA PATIENTS

Parameters	Cataract	POAG	p-value
MMP-2/TIMP-1(pg/mL)	422.05 (328.31-517.46)	508.01 (316.87-602.76)	**0.06**
MMP-9/TIMP-2 (pg/mL)	531.00 (135.00-1029.50)	1024.5 (312.25-1919.75)	0.300

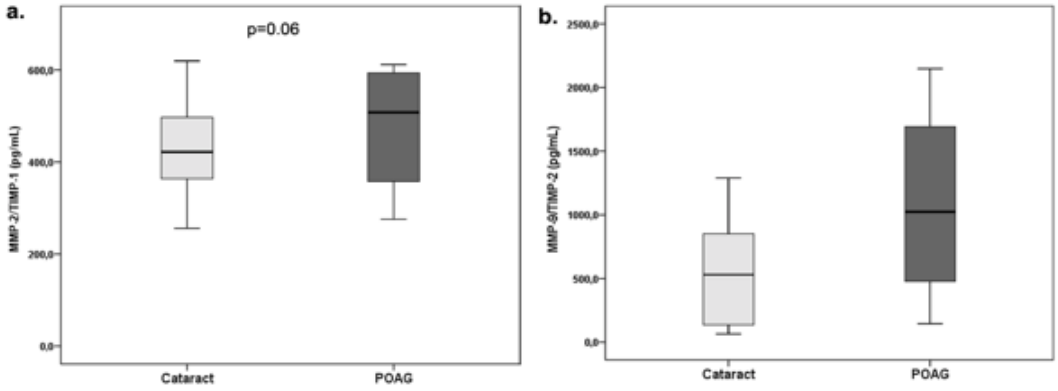

Fig. 2. Aqueous humor concentration of MMP-2/TIMP-1 and MMP-9/TIMP-2 in cataract and POAG patients assessed with DuoSet ELISA kit. Boxplot representing the distribution (median and interquartile range) of MMP-2/TIMP-1 (a), and MMP-9/TIMP-2 levels in aqueous humor samples of cataract and POAG patients Two-sided t-test with unequal variance was used to compare the analyte concentrations in control and glaucoma patients; $p<0.05$ was considered as statistically significant.

data, the difference did not reach the statistical significance (table 2, fig. 2).

When we investigate possible correlations between MMP-2/TIMP-1 in aqueous humor samples and clinical and biochemical parameters, we found that it correlate significantly only with LDL levels ($r=-0.656$, $p=0.029$) in cataract group. In the glaucoma group, significant correlations between the levels of MMP-2/TIMP-1 aqueous humor samples and TGL ($r=-0.620$, $p=0.018$), HDL ($r=0.750$, $p=0.002$), Cholesterol ($r=-0.562$, $p=0.037$) have been found. When we analyzed the correlations between MMP-9/TIMP-2 in aqueous humor samples and other parameters, only the levels of MMP-9/TIMP-2 levels measured in plasma of glaucoma patients seems to correlate with it, but did not reach a p value less than 0.05 ($r=0.849$, $p=0.069$). We thus found that concentration of MMP-2/TIMP-1 is increased in aqueous humor of patients with open angle glaucoma, and this is significantly correlated with their lipid metabolic profile.

MMP-2 gelatinolytic activity is increased in aqueous humor samples of patients with open angle glaucoma

To evaluate the enzymatic activity of MMP-2 and MMP-9, we examined their gelatinolytic activity in aqueous humor samples from cataract and POAG patients, by using zymography (fig. 3). All aqueous samples displayed MMP-9 and MMP-2 gelatinolytic activities. The levels of MMP-9 found in aqueous humor of POAG patients are slightly decreased as compared with those found at patients with cataract (16921.12±3158.48pg/mL, vs. 21666.72±8657.43pg/mL, $p=0.072$).

The mean MMP-2 level in patients with POAG (157023.09±17468.96pg/mL) was higher than mean level

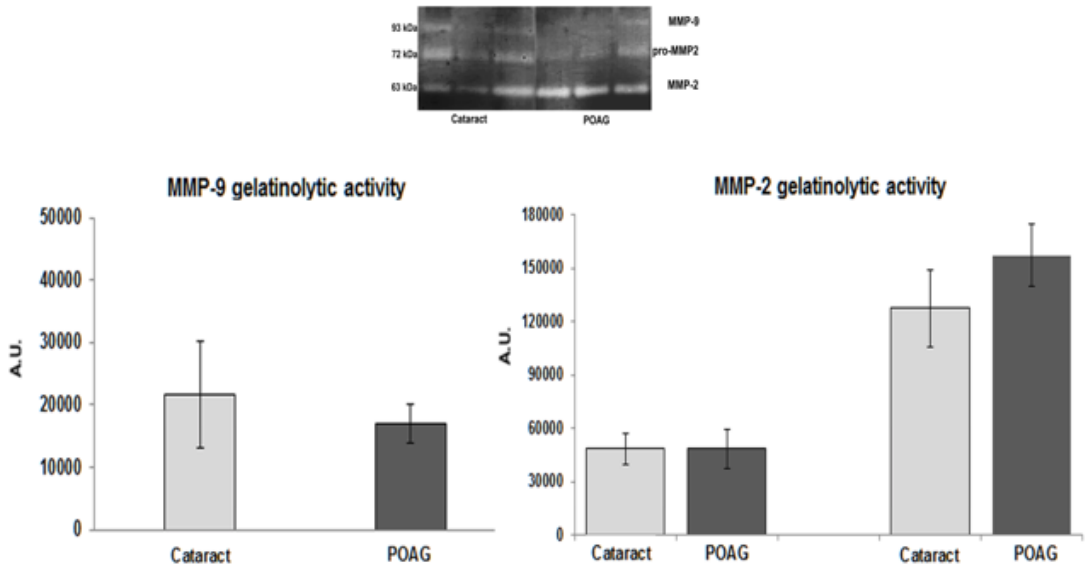

Fig. 3

in cataract control patients (127409.20±21486.94pg/mL, p=0.168).

Gelatinolytic activity of MMP-2 and MMP-9 determined in aqueous humor of open angle glaucoma patients

Thus, our results from zymography analysis showed that MMP-9 activity decrease, and in contrast MMP-2 activity increase in aqueous humor samples of patients with open angle glaucoma compared to patients from control group having cataract.

In this study we tried to emphasize the role of MMPs and TIMPs imbalance in the process of glaucoma development. By analyzing the MMP-2/TIMP-1 complex and MMP-9/TIMP-2 in plasma and aqueous humor, the most incriminated in glaucoma physiopathology, the study showed a potentially significant correlation.

The major limitation of our study is the low number of patients which affects the statistical relevance of the study. Another limitation could be regarding the local eye drops treatment with prostaglandin analogs which is believed to trigger the synthesis of MMP-2 in the ocular tissues [34].

Our results point out that plasma samples show an increased MMP-2/TIMP-1 ratio in POAG group as compared to the cataract group, while no difference was found regarding MMP-9/TIMP-2 levels among the two groups.

Both complexes MMP-2/TIMP-1 and MMP-9/TIMP-2 showed a significant correlation in plasma of the control group with different biological determinations (HLD, LDL, TGL, Chol) while for the patients with glaucoma increased glycaemia the correlation show a limited signification.

As for the aqueous humor, MMP-2/TIMP-1 ratio showed to be increased compared to the control group, and furthermore it was statistically significant.

Regarding MMP-2/TIMP-1 ratio in the glaucoma group there is a significant correlation between the levels in aqueous humor and the different biochemical parameters (HDL, LDL, TGL, Chol). We cannot say the same for the MMP-9/TIMP-1 ratio who did not show a significant statistical value.

In Angeline DC Nga et all [24] study both ratios were higher and significant compared to controls.

Most studies [14, 35-38] prove that the most significant increase concerns the MMP-2 and correlates with patients with glaucoma, just like our study indicates.

The results in literature are fairly heterogeneous due to variation of reports. Selcuk [28] did not find a significant increase in metalloproteinase and their inhibitors in patients with glaucoma.

Conclusions

Despite the limitations of this study, we found that the concentration of MMP-2/TIMP-1 is increased in the aqueous humor of patients with open angle glaucoma, being strongly correlated with their lipid metabolic profile.

These results points out the role of metalloproteinases and their inhibitors in the pathogenesis of glaucoma correlated with the oxidative stress priory developed due to other biochemical modifications.

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