Aqueous Neuroinflammatory Cytokines in Open Angle Glaucoma

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Typically in glaucoma the injuries in the retinal ganglion cells are irreversible and mostly due to high intraocular pressure. Currently there are also accepted pathogenic theories that go beyond high intraocular pressure in the area of neuro-inflammatory molecules, autoimmunity or vascular dysfunction. Yet it is very difficult to quantify these new pathogenic aspects as easy as in the case of visual field testing or optical coherence tomography. Our study tried to identify and compare levels of inflammatory cytokines IL-1Ra (Interleukin-1 receptor antagonist), IL-1α (Interleukin-1α), IL-1β (Interleukin-1β), IL-10 (Interleukin 10) and IFN-γ(interferon-gamma) levels in open angle glaucoma and compare them to healthy subjects, matched for age and sex. The results proved an increased expression of inflammatory molecules with neurotoxicity capabilities in primary open angle glaucoma patients.

Keywords: cytokines, aqueous humor, neurotoxicity, glaucoma

In glaucoma, permanent vision loss and blindness occur when retinal ganglion cells (RGCs) are lost. Though definition is clear in glaucoma, the complete pathogenesis still remains debatable and unclear. Recently the immune imbalance and inflammatory theory in glaucoma was brought into light as an extension of other central nervous system (CNS) diseases [1-5].Yet major differences between the inflammatory model in glaucoma and other CNS degenerative pathologies are described based on the *compartmented* and non-simultaneous destruction lesions in GCL [6]. Cytokinic imbalance in glaucoma resides in two aspects: over stimulation of pro-inflammatory molecules and not enough compensatory anti-inflammatory [7-9]. As for the corresponding morphologic changes, each affected cell compartment differs in immune response type.

Methods such as ELISA and PCR are extremely useful in identifying molecules such as cytokines, though they cannot simultaneously measure small volumes and multiple parameters. Therefore alternative laboratory techniques (cytokine bead array-CBA) offer this fast, reproducible and high accuracy possibility.

Current study aimed to prove if anterior segment circulating cytokines determine focal activation of the immune system in primary open angle glaucoma patients.

Experimental part

Material and method

The Luminex Performance Assay multiplex kits are designed for use with the Luminex® 100™, Luminex 200™, or Bio-Rad® Bio-Plex® dual laser, flow-based sorting and detection analyzers.

Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin- PE), which binds to the biotinylated detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE. The microparticles are resuspended in buffer and read using the Luminex or Bio-Plex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound. The kit was stored unopened at 2-8°C. Aqueous sample collection was obtained in sterile conditions, before conventional cataract surgery. Aqueous samples required no dillution for preparation.

Preparation

All reagens were brought to the room temparature before use. Buffer solution was washed. 20 mL of of Wash Buffer Concentrate was added to deionized or distilled water to prepare 500 mL of Wash Buffer.

Standards were reconstituted from each of Standard Cocktails (1 and 2) provided by the manufacturer, aided by the Calibrator Diluent RD6-40. Standards were left to sit for a minimum of 15 mi9n with gentle agitation prior to making dilutions. This produced a 5X stock of each Standard Cocktail. 300 µLwere pipetted from the appropriate Calibrator Diluent into a tube labeled working standard 1. 200 µL of the appropriate Calibrator Diluent were pipetted further into the remaining tubes. Then 100 µL of each of the 5X reconstituted Standard Cocktail vials 1 and 2 into the working standard 1 tube. Working standard 1 was used to produce a 3-fold dilution series (below). Each tube was mixed thoroughly before the next transfer. Working standard1 served as the high standard. The appropriate Calibrator Diluent served as the blank. Standard 2, Standard 3 Standard 4, Standard 5, Standard 6, Standard 7 were also obtained (fig.1).

Diluted microparticle cocktail preparation

The Microparticle Cocktail vial was centrifuged for 30 s at 1000 x g prior to removing the cap.

The vial was gently vortexed to resuspend the microparticles, taking precautions not to invert the vial.

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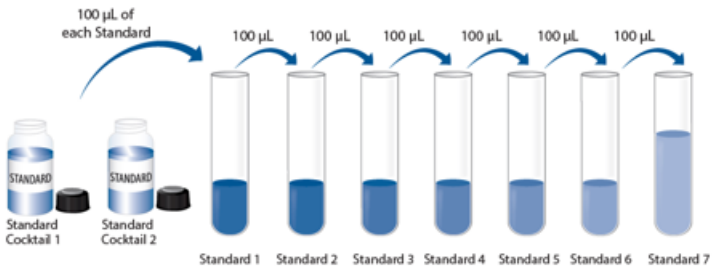

Fig. 1. Successive dilutions S1-S7 of Calibrator Diluent (RnD Systems®)

Microparticle Cocktail was diluted in the mixing bottle provided.

Diluted biotin antibody cocktail preparation

The Biotin Antibody Cocktail vial was centrifuged for 30 seconds at 1000 x g prior to removing the cap.

The vial was gently vortexed to resuspend the microparticles, taking precautions not to invert the vial.

The Biotin Antibody Cocktail was diluted in Biotin Antibody Diluent 2 and mixed gently.

Streptavidin-PE preparation

A polypropylene amber bottle or a polypropylene tube were used and wrapped with aluminum foil. Streptavidin-PE was kept away from light during handling and storage.

Streptavidin-PE vial was centrifuged for 30 s at 1000 x g prior to removing the cap.

The vial was gently vortexed to resuspend the microparticles, taking precautions not to invert the vial.

The 100X Streptavidin-PE was diluted to a 1X concentration by adding 55 µL of Streptavidin-PE to 5.5 mL of Wash Buffer.

Instruments settings

Adjustment of the probe height setting on the Luminex® analyzer was necessary to avoid puncturing the membrane. Certain steps were followed as the next sequence:

a) for each analyte being measured there was a bead region assigned

b) 50 events/bead

c) minimum events: 0

d) flow rate: 60 µL/min (fast)

e) sample size: 50 µL

f) double discriminator gates were set at approximately 7500 and 15.500

g) median Fluorescence Intensity (MFI) was collected

Assay procedure

All reagents and samples were brought to room temperature before use. According manufacturer's recommendations all samples and standards were assayed in duplicate.

All reagents, working standards, and samples were prepared as directed in the previous sections.

The filter-bottomed microplate was pre-wetted by filling each well with 100 µL of Wash Buffer; the liquid was passed through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.

The diluted Microparticle Cocktail was resuspended by inversion or vortexing. 50 µL of the microparticle mixture were added to each well of the pre-wet filter-bottomed microplate.

50µL of Standard were addedper well. The assay was pipetted within 15 min , then. securely covered with a foil plate sealer and incubated for 3 h at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout was provided to record standards and samples assayed.

Using a vacuum manifold designed to accommodate a microplate, liquid washwas performed; then each well was filled with Wash Buffer (100 ìL); liquid was removed again. All of the liquid was removed through the filter at the bottom of the microplate to avoid any loss of microparticles. The washing procedure was repeated three times.

50 µL of diluted Biotin AntibodyCocktail was added to each well and securely covered with a new foil plate sealer, then incubated for 1 h at room temperature on the shaker set at 500 ± 50 rpm.

Step 5 (washing) was repeated.

50µL of diluted Streptavidin-PE were added to each well and securely covered with a new foil plate sealer, then incubate for 30 min at room temperature on the shaker set at 500 ± 50 rpm.

Step 5 (washing) was repeated.

The microparticles were resuspended by adding 100µL of Wash Buffer to each well. Incubation for 2 min at room temperature on the shaker set at 500 ± 50 rpm.

Readings were made within 90 min using the Luminex or Bio-Rad analyzer.

Study design

This studytook place over 12 months (September 2015 - September 2016) in the St. Spiridon University Hospital – Ophthalmology Clinic, Iasi. Designed in a transversal manner, it included a total of 40 patients, distributed in two groups: 24 patients - control group (cataract), 16 patients - the primitive open angle glaucoma (POAG) group.

Ethics

All patients enrolled agreed to participation in the study and signed an informed consent. The study protocol and the informed consent were approved by the Ethics Commission of the Gr.T.Popa University of Medicine and Pharmacy Iasi, as well as by the similar department attached to the Sf. Spiridon Hospital Iasi.

Study protocol

a.The aqueous humor (AH) was obtained under sterile conditions before conventional cataract surgery. When performing the first anterior chamber paracentesis, using a tuberculin syringe, the 30G needle was inserted and 100 ìl of aqueous humor were collected. The volume obtained was immediately transferred to polyethylene sterile tubes (Eppendorf 3810X ®), which were stored at -80 ° C, within 2 h from harvesting.In the actual dosage we used 50µL of aqueous, but all determinations were made in double for better reproducibility and accuracy.

b.Analysis of cytokines in aqueous humor was possible using a Human Cytokine Premixed Kit A FCST03 LHSC000 kit (Luminex, RnD Systems®). The technique involves a cytometric bead array (CBA) variant capable of identifying and quantifying simultaneously from small volumes, different types of molecules (including cytokines) based on fluorescent antibodies. The detected cytokine

concentration depends on the degree of fluorescence produced in the analyzed well. The manufacturer supplied two cytokine mixtures (standard 1 and 2) with known concentrations of each analyte. Setting up at the beginning the standard curves implied performing successive dilutions (fig. 1) of the two standard mixtures and reading the fluorescence at each dilution. According to the obtained values, the device automatically generated a (graph) fluorescence diminution curve, depending on the concentration decrease in eachanalyte. Expression of concentration (pg /ml) was done automatically by the device, based on a logarithmic conversion and reference to the standard curve.

Quantification of cytokines was performed from undiluted and unfiltered AH samples.

The cytokine levels with values outside the normal distribution could be estimated by extrapolation, but the manufacturer's recommendation was that these data should be excluded from the analysis due to imprecision (non-linear dependence for values outside the standard chart, so low predictability). The detection limits for each analyte were 1 μg/mL (lower threshold) and 15,000 μg / mL (upper threshold), according to the manufacturer's recommendations.

Serving the purpose of this paper, we were interested in measuring: IL-1Ra (Interleukin-1 receptor antagonist), IL-1α (Interleukin-1α), IL-1β (Interleukin-1β), IL-10 (Interleukin 10) and IFN-γ (interferon-gamma) levels.

Specific glaucoma investigations

In the study, only cases with a clear diagnosis of open-angle primitive glaucoma were selected, based on: glaucomatous optic disc changes, perimetric defects or retinal nerve fibers corresponding to disc lesions, open-angle in gonioscopy angle.

For all patients were recorded: age (years), best corrected visual acuity (BCVA- decimal), preoperative intraocular pressure (IOP-mmHg) by Goldmann tonometer, perimetric examination - Humphrey automatic automated perimeter, 24-2, SITA Standard, recording specific parameters (MD and PSD in decibels, dB). The duration of glaucoma, the type and number of IOP lowering medications drugs were noted. All glaucoma patients received topical medication at the time of inclusion (beta blockers, prostaglandin analogues, carbonic anhydrase inhibitors or alpha agonists). None of the patients had a history of glaucoma surgery, including laser. For some cases, opacity of the media (advanced cataract) did not allow a complete assessment of glaucoma (Humphrey computerized perimeter). In these cases the latest values of the perimeter indexes (MD and PSD) were taken into account.

Cataract surgery was indicated by the ophthalmologist at a time when there was significant lens opacification. Patients with significant ophthalmological disorders (e.g. age-related macular degenerescence, retinal vascular occlusions) or general (autoimmune systemic tumors or diseases) that could have influenced the ocular local and systemic inflammation status were excluded.

Statistical analysis

Data analysis included qualitative and quantitative processing.

Qualitative analysis was based on *heat map* graphs (Excel, Office, Microsoft ®) showing the distribution of values from minimum (green) to maximum (red) in the studied groups without quantitative appreciation; this type of statistical processing rapidly identified data dispersion, extreme values and allowed to fit into specific (parametric or non-parametric) categories of data.

The quantitative analysis was performed using the SPSS®20.0 software. The demographic data of the two groups were analyzed. Descriptive statistics elements were used to calculate the mean and median of all numerical parameters entered in the database (age, IOP, anterior-posterior axle, duration of glaucoma, number of topical drugs used for PIO control, pro-inflammatory cytokine values of aqueous humor etc.).

The initial comparative analysis analyzed all dose parameters in the two groups (Student t test, with Bonferroni correction, $p < 0.05$). Variance analysis (ANOVA) tested the presence of a difference between several subgroups of a population. If $p < 0.05$, then the average of at least two groups was significantly different. For the numerical variables we used the one-way ANOVA test (enter method). Comparison of frequencies between two variables was performed on the Chi square test, with $p < 0.05$.

The correlations between the variables were performed by the Pearson parameter test, with the correlation coefficient (r) being reported between the variables. The correlation coefficient (r) was declared significant for a $p < 0.05$ value. For some variables, interaction tests were applied before the regression analysis was performed.

Results and discussions

In the study, data were analyzed from 40 patients distributed as it follows: 24 patients - the control group (cataract) and 16 patients - the study group (POAG). The mean age for the groups was 72.33 +/- 11.26 years for the control group and 75.69 +/- 5.54 years for the POAG group ($p > 0.05$). The mean IOP in the control group was 14.21 +/- 2.68 mmHg, whereas in the study group the mean was significantly higher 18.19 +/- 4.3 mmHg ($p = 0.000$), under 3 +/- 0.87 hypotensive drugs. Functional impairment in POAG patients quantified by perimetric indices revealed means of 13.59 +/- 9.35 dB for MD and 4.25 +/- 4.22 dB for PSD. Quantitatively, the concentrations of the cytokines / chemokines (IL-1α, IL-1β, IL-1Rα, IFNγ, IL-10) from the aqueous humor were measured in all 40 patients (table 1).

Standard curves for each parameter can be followed in the figures below (fig. 2-5).

Qualitative distribution of all parameters can be followed in the heat map representations below and show the non-parametric distribution of the measured values (fig. 6-9).

Age or sex didn't influence the current cytokine levels, but an IOP higher than 18 mmHg increased the level of IFNγ in the POAG group in a significant way, compared to the category of IOP below 18 mmHg ($p=0.003$). Current values are yet 10 times lower than other published results [10].

Same observation was made for IL-1α where an IOP higher than 18mmHg increased the cytokine level in a statistically significant manner ($p=0.000$); in this particular case younger patients (<60 years old) developed a stronger inflammatory response than those with age >60 years old. This aspect pleads for an increased immune reactivity and response in younger ages in primary open angle glaucoma patients.

Glaucoma is a neurodegenerative disease [11] characterized by GCL apoptosis and irreversible loss of vision. In POAG, the IOP remains the major risk factor in neurodegeneration, but alltogether IOP independent pathways are described counting for axonal damage, ischemia and local inflammation [4,6,8]. The

Parameter	Control group	Study group	T test (p<0.05)
IFNγ (pg/ml)			
Mean (+/- SD)	2.53+/-0.35	3.39+/-1.63	0.017
Median	2.62	2.62	
Limits	1.56-3.19	1.30-7.98	
IL-1α(pg/ml)			
Mean (+/- SD)	9.51+/- 4.83	14.73+/-5.02	0.002
Median	8.7	16.55	
Limits	0.95-22.40	5.34-21.99	
IL-1Ra(pg/ml)			
Mean(+/- SD)	656.35+/- 518.31	2276.10+/-3017.65	0.01
Median	538.21	1374	
Limits	126.94-2516.07	243.97-12729	
IL-1β			
Mean (+/- SD)	0.90+/-0.19	0.85+/-0.12	0.58
Median	0.93	0.89	
Limits	0.23-1.79	0.66-1.33	
IL10			
Medie (+/- SD)	1.55+/-0.48	1.33+/-0.29	0.44
Mediana	1.42	1.24	
Limite de variație	0.83-3.87	0.83-2.05	

Table 1
MEANS, MEDIANS AND LIMITS OF THE CYTOKINES IN THE TWO GROUPS

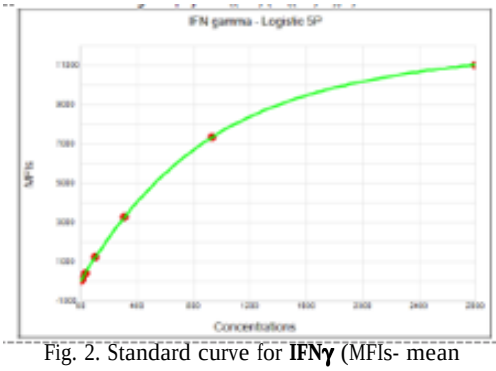

Fig. 2. Standard curve for **IFNγ** (MFIs- mean fluorescences; concentrations pg/mL)

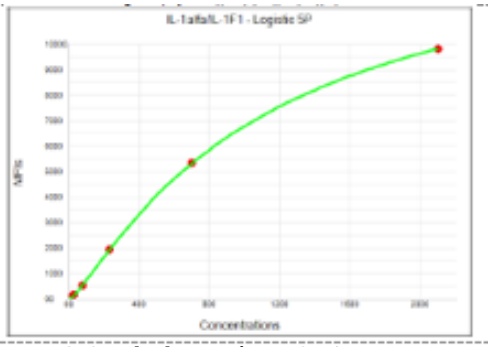

Fig. 3. Standard curve for **IL-1α** (MFIs- mean fluorescences; concentrations pg/mL)

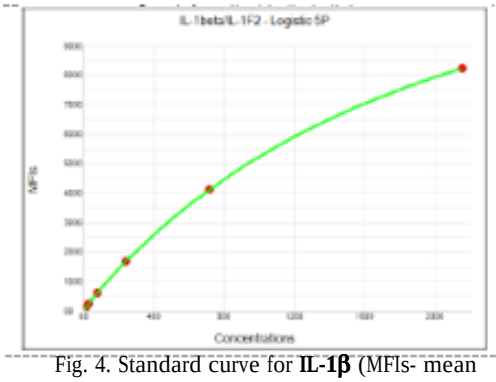

Fig. 4. Standard curve for **IL-1β** (MFIs- mean fluorescences; concentrations pg/mL)

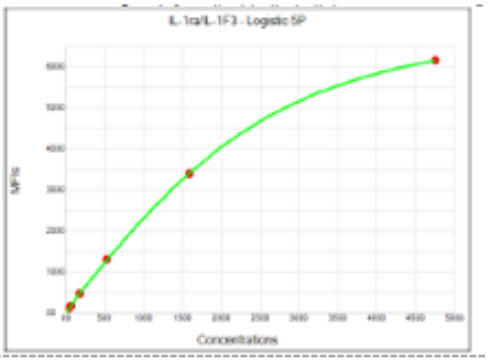

Fig. 5. Standard curve for **IL-1Ra** (MFIs- mean fluorescences; concentrations pg/mL)

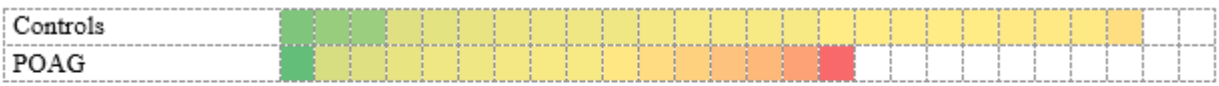

Fig. 6.IFNγ distribution values in the two groups

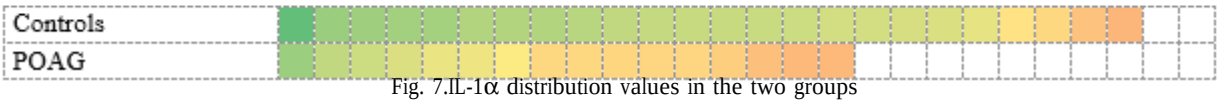

Fig. 7.IL-1α distribution values in the two groups

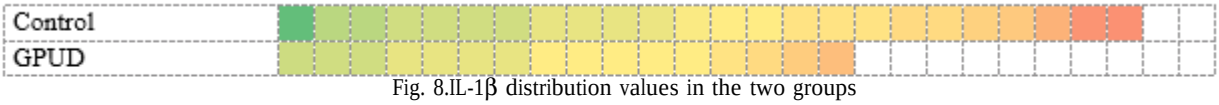

Fig. 8.IL-1β distribution values in the two groups

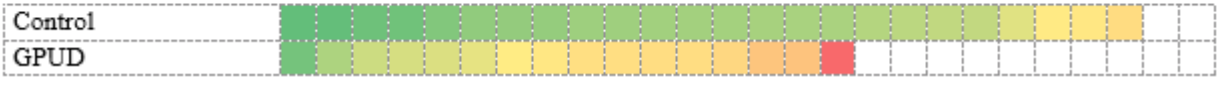

Fig 9.IL-1 Ra distribution values in the two groups

immunological and inflammatory events can be viewed in a dual way: neuroprotective vs neurodegenerative, depending on the homeostatic changes around the optic nerve and the stage of the disease. Temporary adaptive changes in the microenvironment around the optic nerve could play a physiological role in cellular remodeling. Any prolongation of the inflammatory status could trigger neurodegenerative phenomena on multiple pathways [12].

The active area of biology in glaucoma is still seeking to identify systemic and local factors in order to create a risk profile based on changes in the proteins released intraocularly under certain conditions. At the same time potential therapeutic targets and biomarkers are being studied in glaucoma, yet by now results related to cytokines in glaucoma are extremely controversial. IFNγ is a critical cytokine in both the innate and the adaptive immunity. It is primarily related to Th1 lymphocyte activity. In autoimmune neurological diseases, Th1 and IFNγ have an important function in regulating inflammatory response at the neuronal level [13], while cytokines such as IFNγ amplify cellular lesions during ischemia [14-16].

IL-1a belongs to the IL-1 superfamily; Is a chemokine primarily produced by activated macrophages, but also by neutrophils, endothelial or epithelial cells. It also has metabolic, physiological and hematopoietic properties but also participates as immune-regulatory molecule in the pro-inflammatory pathway of TNF-alpha activation [17]. The most important molecule involved in down-regulating the activity and expression of IL-1α is IL-1Rα, usually produced in excess, maybe even 10-100 higher than IL-1α level. IL-10, with anti-inflammatory effect, inhibits IL-1α synthesis [18]. TNF-alpha, in combination with IL-1α or IL-1β, induces increased metalloproteinase activity in the trabecular meshwork of POAG patients, modifies trabecular anatomy and increases flux resistance [19,20].

IL-1Ra belongs to the IL-1 molecules family. It is expressed in a multitude of tissues, including immune, epithelial, adipocytes. The primary effect is to modulate and increase the expression of IL-1α or IL-1β [21]. The latter (IL-1β) promotes the TNF-alpha activation and increased expression, therefore an increased level of this molecule in POAG patients promotes the inflammatory theory in this respect. Yet in our study we did not find significantly different means between groups.

Flow cytometry techniques are capable of detecting inflammatory cytokine concentrations in small volumes of biological samples, having the only downside of high cost. The dosages obtained by this method, corroborated with the clinical parameters of the analyzed subjects have managed to establish a certain inflammatory profile in a patient with POAG. All measured parameters point indirectly to an activation of the TNFα pathway [17]. The TNFα molecule in combination with IL-1α or IL-1β, induce increased activity in metalloproteinases found in the trabecular meshwork; thus a premise for altered trabecular anatomy and increased resistance to flow are shown [19]. During our study we proved the presence of neuroinflammatory cytokines in the aqueous of POAG patients, which corresponds to other published data [22, 23].

Conclusions

The cytokines analyzed can normally be found in aqueous humor in healthy subjects, but if their expression exceeds the level of regulation properties, toxic effects can be exerted. In this study we demonstrated increased expression of inflammatory cytokines with neurotoxicity capabilities in glaucoma patients. As such, POAG treatment options should target other pathways beyond IOP lowering strategies (ischemia, inflammation etc). Yet an ideal treatment approach in glaucoma cannot be met and validation through longitudinal clinical trials is needed in this respect.

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