The Role of T-Cell subsets and Natural Killer Lymphocytes in the Pathogenesis of Primary Open Angle Glaucoma

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Abstract

Background. The aim of the work is to emphasize the role of lymphocyte subsets; T-helper (Th), T-cytotoxic/suppressor (Tc/s) and natural killer (NK) and their secreted cytokines found in the peripheral blood of patients suffering from glaucoma; primary open angle glaucoma (POAG) and normal tension glaucoma (NTG) in an attempt to find out the possible change met within such patients.

Subjects and Methods. This study included 36 patients diagnosed as glaucoma of different etiologies including both intra ocular pressure (IOP) dependant glaucoma (19 patients) and IOP independent glaucoma (17 patients) and compared to 20 healthy subjects of matched age and sex as reference subjects. Lymphocyte subsets (Th, Tc/s & NK) were analyzed in the peripheral blood of POAG and NTG cases and reference subjects by flow cytometry and the results were expressed as percentage of lymphocytes that express (CD4+, CD8+ and CD16+56+). The cytokines; interleukin-2 (IL-2), interferon-gamma (IFN-gamma) and interleukin-10 (IL-10) were analyzed in the serum of POAG and NTG patients and reference subjects using ELISA technique.

Results. The results of this study revealed a significant increase of the percentage of CD4+ and CD8+ cells, significant decrease in CD4+/CD8+ ratio in both POAG and NTG patients when compared to reference subjects. There was significant increase in the mean serum level of IL-10 in POAG and NTG patients when compared to reference subjects.

Conclusion. This results support the hypothesis that the delicate intraocular balance of Th1/Th2 immune response, with the predominance of Th2, breaks down in glaucoma.

Introduction

Glaucoma, the second leading cause of worldwide blindness, is a progressive optic neuropathy characterized by loss of retinal ganglion cells (RGC) and their axons, excavated appearance of the optic nerve head and progressive loss of visual field. It represents a final common pathway for a number of conditions, of which raised intra ocular pressure (IOP) is the most important risk factor [1]. Although several studies have shown the role of IOP in the pathogenesis of glaucomatous optic neuropathy, about 20% to 25% of glaucomatous optic neuropathy develops in patients with normal IOP [2, 3].

Tazel et al., [4] reported comparable histopathological optic nerve changes, correlated with the clinical appearance of the optic nerve head, in glaucoma with high and with normal IOP. So, it is certain that an elevated IOP is not alone responsible for the disease and other possible pathogenic factors such as apoptotic processes [5], elevated nitric oxide levels [6] and an autoimmune response [7] play an important role.

Growing evidence, obtained from clinical and
experimental studies over the past decades, strongly suggests the involvement of the immune system in glaucoma [8, 9]. Therefore, further information about the pathogenesis of glaucoma especially the role of immune regulatory lymphocytes such as T-lymphocytes, and their cytokine profiles, and Natural killer (NK) lymphocytes might hopefully lead to a better understanding of the disease [8,10].

T-Lymphocytes secrete lymphokines and interact directly with receptors or determinants on target tissues or viruses. On basis of the pattern of secreted cytokines (cytokine profile), T helper (Th) lymphocytes (CD4+) are currently divided into two subsets; Th1 lymphocytes which secrete Interferon gamma (IFN-gamma) and interleukin-2 (IL-2) and Th2 lymphocytes which secrete IL-4 and IL-10 [11]. Moreover, similarly, T suppressor/cytotoxic (Ts/c) lymphocytes (CD8+) are divided into 2 types, Ts/c1 lymphocytes which secrete IFN-gamma and Ts/c2 lymphocytes which secrete IL-5 and IL-10 [12].

Natural killer cells are granular cytotoxic lymphocytes that play a crucial role of innate immunity due to their cytotoxic ability to destroy virus–infected cells and certain tumor cells without prior stimulation [13]. Aside from their cytotoxic activity, NK cells can produce and release high amounts of Th1 (IFN-gamma) and Th2 (IL-4 & IL-5) cytokines [14]. A new concept proposes classifying NK cells by their prominent cytokine pattern into NK1 and NK2 [15,16]. Moreover, natural killer cells play a crucial role in the pathogenesis of many diseases by altering the balance between Th1/Th2 lymphocytes [17, 14].

The aim of the work is to emphasize the role of lymphocyte subsets such as Th, Ts/c and NK and their secreted cytokines found in the peripheral blood of patients suffering from glaucoma (POAG and NTG) in an attempt to find out the possible change met within such patients.

Subjects and Methods

Subjects

This study included 36 patients diagnosed as glaucoma of different etiologies including both IOP dependent glaucoma (19 patients) and IOP independent glaucoma (17 patients) and compared to 20 healthy subjects of matched age and sex as reference subjects. Both patients and reference subjects were recruited from the Ophthalmology Outpatient Clinic of Research Institute of Ophthalmology after full ophthalmological examination. The selected samples were collected between 2007 and 2009. A written consent was taken from each subject in the study and approval of the ethical committee of National Research Center was obtained.

Subjects of this study were divided into:

- Group1: Included 20 healthy persons as reference subjects (controls) (9 females and 11 males). Their ages ranged between 27 and 60 years.
- Group2: Included 19 patients suffering from POAG (10 males and 9 females). Their ages range between 21 and 64 years.
- Group3: Included 17 patients suffering from NTG (8 males and 9 females). Their ages ranged between 25 and 61 years.

Sampling

The blood samples were collected under aseptic conditions by clean venipuncture, about 5 ml of blood was withdrawn into the following two portions:

- Two ml of blood were collected in sterile plain vacutainer tubes and allowed to clot at room temperature. The serum was separated by centrifugation at 2000 rpm for 10 minutes. Serum was aliquot and stored at -20°C for determination of IL-2, IL-10 and IFN-gamma.
- 100 μL of heparinized blood were collected with 10 μL for each monoclonal antibody and incubated for 15 minutes at room temperature. The erythrocytes were then analyzed using FACSLyse (Becton Dickinson Immunocytometry systems) for determination of lymphocyte subsets.

Patients and reference subjects were subjected to the following investigations:

- Accurate ophthalmic history including visual symptoms, past ocular, medical and family history, medications and allergy.
- Complete ophthalmologic examination was performed including best corrected VA, afferent papillary defect; biomicroscopy and gonioscopy for examination of cornea, anterior chamber angle, iris, lens; 90D lens to examine the OD, disk vessels, peripapillary area and the fundus. Evidence of glaucoma was considered according to:
  - raised IOP>21mmHg.
- abnormal disc: cup/disc ratio, asymmetry large C/D ratio, NR rim, disc hemorrhage, byoneting or nasal displacement of disc vessels, peripapillary atrophy zone.

- VF changes in the form of nasal step, para central scotomas, accurate scotomas or residual temporal or nasal islands of vision.

**Immunophenotyping**

Lymphocyte subsets analysis by flow cytometry [18]:

The following triple colored fluorescent monoclonal antibodies were used to determine the lymphocyte subsets:

- CD3 FITC / CD16-CD56 PE / CD45 per CP (for NK cells).

- CD4 FITC / CD8 PE / per CP (for Th and T-s/c cells).

100 μL of heparinized blood were collected with 10 μL for each monoclonal antibody and incubated for 15 minutes at room temperature. The erythrocytes were then analyzed using FACSlyse (Becton Dickinson Immunocytometry systems). The cells were suspended in 30 μL PBS and analyzed by FACS calibur flow cytometry using Multi-SET VI.0.1 software and equipped with a 15 mw argon laser and filter setting for FITC (fluorescein isothiocyanate) (530 nm), PE (phycoerythrin ) ( 585 nm) and perCP (peridinin chlorophyll protein) (>650 nm). Ungated list mode data was obtained on at least 15000 leukocytes. The percentages of cells were obtained by fluorescence control. Results were expressed as a percentage of lymphocyte expressing CD16+CD56+, CD4+, and CD8+ within the gated population.

**Cytokines analysis**

Serum levels of cytokines (IFN-gamma, IL-2 and IL-10) were quantified by sandwich ELISA (Enzyme linked immunosorbant assay) technique using the commercially available kit (Accucyte, EIA, cyt-immune Sciences Inc., USA). The concentrations of the various cytokines detected were in pg/ml, with the following minimum detection levels as determined by the manufacturers: IFN-gamma; 3·0 pg/ml, IL-2; 2 pg/ml, and IL-10; 1·5 pg/ml. Sera were calibrated against IFN-gamma [11], IL-2 [12], and IL-10 [12] standards respectively.

**Statistical interpretations**

Results were processed and statistics were carried out using SPSS program version 10. Quantitative data were tested for normality by Kolmogorov-Smirnov test and parametric tests were used. Data are expressed as mean ± SD. The percentage of total lymphocytes (NK cells, B cells, T cells) and the Th/Ts/c ratio were compared between glaucoma (POAG & NTG) patients and reference group using the paired t-test. Values are considered statistically significant at p<0.05.

### Table 1: The percentage of lymphocyte subsets in POAG patients (Mean ± SD).

<table>
<thead>
<tr>
<th>Studied Groups</th>
<th>CD 4%</th>
<th>CD 8%</th>
<th>CD4+/CD8 ratio</th>
<th>CD 16+/CD56%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=20)</td>
<td>26.6 ± 10.6</td>
<td>11.3 ± 2.6</td>
<td>2.3 ± 0.4</td>
<td>19.3 ± 1.85</td>
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<tr>
<td>POAG (n=19)</td>
<td>37.5 ± 10.7</td>
<td>23.3 ± 2.6</td>
<td>1.6 ± 0.3</td>
<td>19.6 ± 1.9</td>
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<tr>
<td>3</td>
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<tr>
<td>14.4</td>
<td>0.001</td>
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<tr>
<td>6.4</td>
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### Results

The results are represented in Tables 1, 2, 3, and 4 and Figure 1.

Table 1 and Figure 1 showed the lymphocyte subsets percentage in POAG and reference subjects. It revealed:

1- A significant increase in the percentage of Th (CD4+) cells in POAG patients when compared to those of the reference subjects (37.5 ± 10.7 Vs 26.6 ± 10.8) (P<0.01).

2- A highly significant increase in the percentage of Ts/c (CD8+) in POAG patients when compared to those of reference subjects (23.3 ± 2.6 Vs 11.26 ± 2.6) (P<0.001).

3- A highly significant decrease in CD4+/CD8+ ratio in POAG patients when compared to those of reference subjects (1.6 ± 0.3 Vs 2.3 ± 0.4) (P<0.001).

4- An insignificant increase in the percentage of NK lymphocytes (CD16+CD56+) in POAG when compared to...
pared to reference subjects (19.6 ±1.9 Vs 19.3±1.85) (P>0.1).

Table 2 and Figure 1 showed the lymphocyte subsets in NTG and in reference subjects. It revealed:

1- A significant increase in the percentage of Th (CD4+) cells in NTG when compared to reference subjects (36.4 ± 11.7 Vs 26.6 ± 10.8) (P< 0.01).

2- A significant increase in the % of Ts/c (CD8+) in NTG when compared to reference subjects (19.5 ± 2.4 Vs 11.3 ± 2.6) (P<0.001).

3- A significant decrease in CD4+/CD8+ ratio in NTG patients when compared to those of reference subjects (1.8 ± 0.4 Vs 2.3 ± 0.4) ( P<0.001).

4- An insignificant increase in the percentage of natural killer cells (CD16+CD56+) in NTG when compared to reference subjects (19.9 ± 2.11 Vs 19.3 ± 1.85) (P>0.1).

Table 3 showed the mean serum levels of different cytokines in POAG and reference subjects. It revealed:

- An insignificant increase in the mean of serum levels of IFN-gamma and IL-2 in POAG group (13.1 ± 0.8 and 7.5 ± 2.3) Vs (12.9 ± 0.5 and 6.3 ± 2.3 respectively) (P< 0.1).

- A significant increase in the mean of serum levels of IL-10 in POAG group (2.9 ± 1.1 Vs 2.6 ± 0.4), (P< 0.01).

Table 4 revealed the mean serum levels of different cytokines in NTG and reference subjects. It revealed:

- An insignificant decrease in serum levels of IFN-gamma in NTG group when compared to reference subjects (12.6 ± 0.9 Vs 12.9 ± 0.5) (P>0.1).

- An increase in serum levels of IL-2 and IL-10 in NTG group when compared to reference subjects (6.8 ± 2.9 and 3.9 ± 0.6 versus 6.3 ± 0.8 and 2.6 ± 0.4 respectively). This increase was insignificant in IL-2 (P>0.1) but was significant in IL-10 (P<0.001).

**Discussion**

Glaucome and immunity are not traditionally perceived as being causally related. Recently however, compelling observations have provided insight into a potential role for the immune system in the development of glaucomatous optic neuropathy [9].

Advances in monoclonal antibody production and flow cytometry have revolutionized the evaluation of the immune system. Flow cytometry has enabled immunophenotyping of lymphocytes to become more objective, precise, quantitative, and readily available [19]. However, it has not been possible to confirm whether these CD8+T cells have a cytotoxic or suppresser function, because FACS analysis defines phenotype and not the function of these cells. The mean blood levels of the percentage of Th, Tc/s and NK lymphocytes in this study lies within the lower range of Chng et al.,[20] this could be explained by the fact that...
It revealed also an insignificant decrease in the CD4+/CD8+ ratio in both glaucoma groups (POAG and NTG) when compared to the reference subjects. These results run parallel to other investigators who reinforce the concept that T-lymphocytes mediated immunity may be implicated in the initiation and progression of some types of glaucomatous lesions [20, 21].

Paradoxically the role of the immune system in glaucoma has been described as either neuroprotective or neurodegenerative [22]. The most important parameter for the modulation of the immune-system, from immune-protective to immune-degenerative in glaucoma is that the retina and optic nerve head are under widespread and long term stress such as high IOP, ischemia, reactive oxygen species (ROS) which provoke changes in the effect or systemic immune response that occur in response to glaucomatous injury [8, 23].

In glaucomatous eyes, the tissue stress is best represented by increased expression of stress proteins in retina and optic nerve such as heat shock proteins which function as endogenous protection of retinal neurons in response to a variety of stressors, associated with glaucoma and at the same time they also have the ability to elicit an activated immune response. Heat shock proteins are known to be highly antigenic, and the immune responses to them are implicated in the development of a number of human auto-immune consequences of molecular mimics so, a failure to properly control aberrant stress induced immune response likely converts the protective immunity to an autoimmune neurodegeneration in some patients [24]. In glaucoma, the immune system acts as an arbiter to help determine whether a neuronal cell will ultimately survive, or succumb to those stressors that are perceived as injurious [8]. A balance between beneficial immunity and harmful autoimmune neurodegeneration may ultimately determine the fate of retinal ganglion cells (RGC) in the glaucomatous eye [24]. So, targeting the cascade that leads to abnormal immune response may open a new avenue for the treatment of glaucoma.

Although there is no evidence of T-cells accumulation, in the retina or optic nerve head in glaucomatous eyes, episodic disruptions of the blood retinal barrier (BRB) may facilitate their access into these tissues [25]. However, it is unclear whether BRB breakdown is necessary before T-lymphocytes can infiltrate and enter the eye as part of the peripheral immune response or whether lymphocyte infiltration results in BRB breakdown and there are non-specific mechanisms that attract the T-cells to the eye. These recruited T-cells may initially play an important role as a protective mechanism since it allows early contact of the immune system with cellular debris and destruction of the damaged cells. Moreover, the recruited T-cells mediate the protection of neurons from degenerative conditions by providing a source of cytokines such as IFN-gamma [25].

In glaucoma, the microenvironment of Th cells detected in the peripheral blood induces the production of more cells committed to Th2 [26, 27]. This is supported by the presence of a significant increase in the serum levels of Th2 cytokine profile (IL-10) and insignificant increase of Th1 cytokine profile (IL-2 and IFN-gamma ) in POAG and NTG patients observed in this study. This suggests the predominance of Th2 cytokine profile and indicates that the increase in Th cells could be due to increased Th2 cells. It is noteworthy to mention that the Th2 cells secrete cytokines (IL-4, IL-6 and IL-10) which promote B-lymphocyte activation, proliferation and subsequent differentiation into plasma cells [28, 13].

The major targets of interest are cytokines and their functions in damage or protection of retinal ganglion cells. Recent advances in the studies of glaucoma or RGCs reveal that cytokines are a possible factor in the pathogenesis of glaucoma and may regulate RGCs survival or death [29]. This is supported also by the significant increase in CD8+ (Tc/s) cells observed in this study which contribute to immune regulation of the eye first by cells and second working as regulatory cells via their functions in damage or protection of retinal ganglion cells [30].

In this study, the observed increase in CD8+ cells could be explained by the diminished apoptosis of the CD8+ which are less susceptible to fas ligand (FasL) mediated apoptosis in glaucoma [10, 31, 32].

IL-17 is produced by Th17 cells. This cell type can be stimulated to expand by IL-2. Besides IL-17 production, Th17 cells also secrete IL-6 and TNF-gamma and now are recognized as causative agents of several
diseases previously attributed to Th1 cells, such as chronic inflammatory bowel disease. Interleukin-17 is an agent that contributes to retina damage in an EAU model [33]. Retinal damage in this model can be reduced by a neutralized by specific anti-IL-17 antibody. A significant number of studies on cytokine protection of neurons have been performed and these numbers are increasing year after year. As with the studies on neural damage, many kinds of insults have been used to test the roles of cytokines in protection of retinal neurons both in vivo and in vitro. In an EAU model, IL-27 and IFN-gamma have been shown to inhibit IL-2-induced expansion of IL-17, produced by Th17 cells, and to ameliorate retina damage by EAU [33]. This indicates that Th1 cytokines such as IFN-gamma could have protective roles on neurons under certain circumstances. Interferon-gamma inhibits the fibroblast proliferation from Tenon’s capsule in vitro [34].

The results of this study revealed no significant change in the percentage of NK-cells in the peripheral blood of glaucoma patients (POAG & NTG) when compared to reference subjects. These results run parallel to other investigators who reported the involvement of NK cells only in the pathogenesis of glaucoma associated pseudo-exfoliation disease [35, 36].

Natural killer (NK) cells actively respond to environmental changes by regulating de novo gene expression, which is strictly controlled by four-step-expression. Four-step-expression is synthesis and degradation of mRNA together with synthesis and degradation of the corresponding proteins. Fine tuning of synthesis and degradation rates is not only essential for maintaining protein levels, but also allows for fast and sensitive responses to target cells. In contrast to B and T cells, the post-transcriptional mechanisms governing NK cell activation remain poorly understood. However, there are some examples of post-transcriptional gene regulation during the trafficking, immune synapse formation, cytokine production, and cytolysis steps of NK cell responses [37]. A study showed that NK cells expressed both urokinase plasminogen activator (uPA) and its receptor (Upar) uPA and uPAR and that in vitro NK cells employ the uPA system following stimulation with IL-2 [38]. So NK cells could be triggered causing activation of T-cells receptors and secretion of cytokines that promote Th2 instead of Th1 response. This suggests that NK cells may have a role in regulating the balance between Th1/Th2 lymphocytes in glaucoma [17, 14].

The aforementioned data is no doubt, a support to the hypothesis that the delicate intra-ocular balance of Th1/Th2 immune response, with the predominance of Th2, breaks down in glaucoma [11, 26, 39].

The onset, progression and termination of tissues specific immune response are largely determined by the interactions between the tissue infiltrating T-cells, RGCs, astrocytes and Muller cells of the glaucomatous eyes and tissue macrophages (microglia). So, whether, the outcome of immune system is deleterious or beneficial for tissue integrity and function depends on complex interactions between these cells.

Continued efforts to better understanding the role of the immune system in glaucoma should allow identification of biomarkers that may signal the most advantageous time to intervene in order to minimize disease progression as well as the development of immunomodulatory strategies that could be utilized for such therapeutic gain.

References


