Clinical Roles of Serum Autoantibody against Neuron-Specific Enolase in Glaucoma Patients

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MARUYAMA, I., IKEDA, Y., NAKAZAWA, M. and OGURO, H. Clinical Roles of Serum Autoantibody against Neuron-Specific Enolase in Glaucoma Patients. Tohoku J. Exp. Med., 2002, 197 (3), 125–132 — In our recent papers, we found the presence of serum autoantibody against neuron specific enolase (NSE) in some glaucoma patients and suggested that this antibody might have significant roles in pathogenesis of glaucomatous optic neuropathy. In order to evaluate further the clinical roles of serum autoantibody against NSE in glaucoma, serum autoantibody against NSE was examined by western blot analysis and enzyme-linked immunosorbent assay (ELISA) in 4 patients with ocular hypertension (OH) and 242 patients with glaucoma (normal tension glaucoma [NTG], 73 cases; primary open angle glaucoma [POAG], 169 cases), and the relationships between the titers of anti-NSE antibody and clinical characteristics were evaluated. The titers of anti-NSE antibody showed a regular decreasing pattern with deteriorating visual field losses and glaucoma stages in POAG, especially early and late stages. However, no systematic pattern was observed in NTG. Although maximum and mean intraocular pressures (IOP)s and progression of visual field losses showed no correlation with the levels of serum anti-NSE antibody titers in either POAG or NTG, the anti-NSE antibody titers were relatively higher in NTG with visual field deterioration than in those without it. The present observations suggest that serum autoantibody against NSE may be clinically useful for diagnosing early stages of POAG, and for monitoring glaucoma progression of NTG. ———— autoantibody; immunoreactivity; neuron specific enolase (NSE); glaucoma

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Glaucoma is a leading cause of irreversible blindness throughout the world (Shields 1997). The common denominator of all forms of glaucoma is a characteristic optic neuropathy, which drives from risk factors including increased intraocular pressure (IOP) (Van Buskirk and
Cioffi 1992). Although elevated IOP is clearly the most frequent causative risk factor for glaucomatous optic neuropathy, it is not the only factor. Nevertheless, IOP and the aqueous humor dynamics that regulate intraocular pressure are critical to our understanding of glaucoma, not only because they are the most common and best understood of the causative risk factors, but also because they are presently the only factors that can be controlled to prevent progressive optic neuropathy. As continued research expands our knowledge of the various factors leading to glaucomatous optic neuropathy, both our classifications of glaucoma and approaches to management will undoubtedly change.

So far, based upon postmortem studies of human eyes with primary open angle glaucoma (POAG) (Kerrigan et al. 1997) and neovascular glaucoma (Okisaka et al. 1997), and experimental glaucoma models with elevated IOP (Quigley et al. 1995; Garcia-Valenzuela et al. 1995), apoptotic cell death of retinal ganglion cells is known to be involved in the pathogenesis of glaucoma. In terms of the molecular mechanism triggering the apoptosis, deprivation of neurotrophic factors (Quigley et al. 1995), ischemia (Levin and Louhab 1996), chronic elevation of glutamate concentration in the vitreous (Dreyer et al. 1996), and disorganized nitric oxide (NO) metabolism (Neufeld et al. 1997) have been implicated. In addition, recently, it was reported that autoimmune responses toward rhodopsin (Romano et al. 1995), 60 kDa heat shock protein (hsp 60) (Wax et al. 1998), 27 kDa heat shock protein (hsp 27) and α-crystallin (Tezel et al. 1998) may be related with the apoptotic cell death process in some glaucoma patients, especially in normal tension glaucoma (NTG). In our previous paper (Maruyama et al. 2000), we have reported that approximately 20% of POAG patients possessed serum antibody against neuron specific enolase (NSE) and maximum IOP levels in POAG patients with anti-NSE antibody were statistically lower than those without the antibody. However, other clinical factors, including visual field defects, medications and optic nerve cupping, were comparable between the antibody positive and negative POAG patients. In addition, injection of the patient’s serum into vitreous cavity of Lewis rat caused reduction of b-wave amplitude on electroretinogram (ERG) and increase of TdT-dUTP terminal nick-end labeling (TUNEL) positive staining within the retinal ganglion cells. Clinically, we also reported that the rate and level of detection of anti-NSE antibody were significantly higher in early stages of POAG (Aulhorn-Greve’s stages 0–2) and other stages of POAG (Aulhorn-Greve’s stages 3–6) and NTG with visual field deterioration than in those without it (Ikeda et al. 2002). Therefore, based upon these findings we suggested that serum anti-NSE antibody may be one of the clinical factors responsible for glaucomatous optic neuropathy.

In this paper, to evaluate further the clinical roles of serum autoantibody against NSE in glaucoma patients, we have compared the titers of anti-NSE antibody and clinical findings in glaucoma patients.

**Patients and Methods**

The studies were performed in accordance with our institution’s guidelines and the Declaration of Helsinki on Biomedical Research Involving Human Subjects and protocols were approved by the institution’s Committee for the Protection of Human Subjects. All experimental procedures were designed to conform to both the Association for Research in Vision and Ophthalmology, Inc. (ARVO) statements for Use of Animals in Ophthalmic and Vision Research and our own institution’s guidelines.

**Patients**

Two hundred and forty-two patients with glaucoma (NTG, 73 cases; POAG, 169 cases) and 4 patients with ocular hypertension (OH)...
were used in this study. The diagnostic criteria for NTG were as follows: 1) the presence of normal open iridocorneal angles, 2) no evidence of IOP higher than 21 mmHg, 3) glaucomatous changes in visual fields and optic nerve cupping, and 4) the absence of alternative causes of optic neuropathy. For the diagnosis of POAG, the criteria were identical with that of NTG except intraocular pressures had to be higher than 21 mmHg. For the diagnosis of OH, the criteria corresponded to that of POAG except there were no glaucomatous changes in visual fields or optic nerve cupping. The IOP was measured using a Goldmann applanation tonometer. The visual field was examined by a Goldmann perimeter and/or a Humphrey visual field analyzer (Humphrey-Zeiss, San Francisco, CA, USA) using a central 30-2 program at least twice a year. Peripheral venous blood samples were immediately subjected to serum separation and stored at −80°C before use.

Criteria for progression of glaucomatous visual field loss

To study the relationship between serum anti-NSE antibody titers and glaucoma progression, clinical data of the right eyes were used. Glaucomatous visual field loss was considered to have progressed if one or more of the following occurred: (1) widening of a nasal step or other peripheral defect on kinetic perimeter by 5 degrees or more, (2) the appearance of a new scotomatous defect and (3) a change in a scotoma from relative to absolute (Werner et al. 1977; Kidd and O'Connor 1985; Shirakashi et al. 1993). To evaluate the presence of progression of glaucomatous visual field loss all patients who met the following criteria were included: (1) at least twice visual fields showing well-documented progression of defects; (2) maintenance of adequate visual acuity for reliable plotting of the fields; (3) no other ocular abnormality that would affect the visual field; and (4) at least 12 months of visual field follow-up.

Western blot analysis

Western blot analysis was carried out as described previously (Ohguro et al. 1999). For isolation of bovine retinal soluble protein fraction, 10 frozen bovine retinas were homogenized in 10 mM hepes buffer, pH 7.5, containing 100 mM NaCl, 1 mM benzamidine and 0.1 mM leupeptine and centrifuged at 50 000×g for 1 hour. The supernatant containing approximately 0.1 mg protein was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide gel. Separated proteins in a gel were electrotransferred to polyvinylidene fluoride (PVDF) membranes in 10 mM BTP buffer, pH 8.4 and 10% methanol solution. After blocking nonspecific binding by 2% skim milk in phosphate buffered saline (PBS), the membrane was probed successively with diluted patients' serum and horseradish peroxidase (HRP) labeled anti-human IgG (Funakoshi Co. Tokyo). Specific antigen/antibody binding was visualized by chemiluminescence (ECL; Amercharm Pharmacia Biotech, Buckinghamshire, UK).

ELISA

Bovine retinal soluble fraction (~3 mg/ml) was obtained as described in western blot analysis section. Bovine retinal sample was diluted 1:30 in PBS and then 50 µl was added to each well of 96-well plastic microtiter plates and stood overnight. Each well was then filled with 400 µl of PBS containing 1% skim milk and incubated at room temperature for 2 hours. Anti-NSE antibody positive patients' sera diluted from 1:100 to 1:6400 in PBS were added to wells of antigen-coated plates and incubated at 37°C for 2 hours. The primary antibody was removed by washing with PBS containing 1% skim milk three times, then incubated with secondary antibody (anti-human IgG coupled to HRP, 1:3000) at 4°C overnight. After washing with PBS containing 0.036% Tween
20° four times, the color reaction was developed with 3,3-diaminobenzidine tetrahydrochloride for about 15 minutes, and then the absorbancy at 495 nm was measured by a plate reader. Negative control wells prepared without antigen or primary antibody. For individual experiments, sera were assayed in duplicate wells, and the data presented were means of three independent experiments.

Statistical analysis

The clinical data including age, maximum and mean IOPs, and experimental data of anti-NSE antibody titers by ELISA are shown as mean±standard deviation (s.d.) and mean±standard error (S.E.), respectively. Significant differences between two groups were found using the Mann-Whitney test with a significance level of less than p<0.01. The mean level of anti-NSE antibody titers by ELISA among three different glaucoma stage groups were statistically analyzed by Scheffe’s test with a significance level of less than p<0.05.

RESULTS

As shown in Table 1, the anti-NSE antibody was recognized in about 20% of glaucoma patients (one case of OH, 24 cases of POAG and 17 cases of NTG) by western blot analysis. As compared with the analysis we previously reported (Maruyama et al. 2000; Ikeda et al. 2002) using smaller number of patients, similar figures were observed again in this study using a larger number of patients. Clinical characteristics of anti-NSE antibody positive patients are shown in Table 2.

Because western blot analysis indicated the presence of circulating antibodies against NSE

| Table 1. The positive rates of anti-NSE antibody by western blot analysis in POAG and NTG |
|-----------------------------------------------|------------------|
| Antibody positive rate                        |                  |
| POAG (n=173 cases)                            | 25 cases (14.5%) |
| NTG (n=73 cases)                              | 17 cases (23.3%) |

Four cases of OH were included in POAG.

| Table 2. Clinical characteristics of anti-NSE antibody positive patients in POAG and NTG |
|-----------------------------------------------|-------------------|
|                                          | POAG               | NTG               |
|                                          | (n=25, 50 eyes)    | (n=17, 33 eyes)   |
| Sex                                       |                    |                   |
| Male                                      | 12                 | 4                 |
| Female                                    | 13                 | 13                |
| Age                                       |                    |                   |
| Mean±s.d.                                 | 67.6±9.0           | 66.3±12.3         |
| Maximum IOP (mmHg)                        |                    |                   |
| Mean±s.d.                                 | 23.8±5.5           | 16.8±1.9          |
| Mean IOP (mmHg)                           |                    |                   |
| Mean±s.d.                                 | 17.3±4.3           | 14.2±1.22         |
| Visual field loss (Aulhorn Greve's classification) |                  |                   |
| Stage 0~1                                 | 20 (40.0%)         | 15 (45.5%)        |
| Stage 2~3                                 | 20 (40.0%)         | 15 (45.5%)        |
| Stage 4~6                                 | 10 (20.0%)         | 3 (9.0%)          |

One case of OH was included in POAG.
in approximately 20% of glaucoma patients, we performed ELISA to compare the serum immunoreactivity against NSE in 42 cases of anti-NSE antibody positive glaucoma patients and 22 cases of age-matched anti-NSE antibody negative patients by western blot analysis.

There were no statistical differences between antibody positive and negative patients. As shown in Table 3, the titers of anti-NSE antibody were statistically higher in anti-NSE antibody positive patients than those in anti-NSE antibody negative patients (Mann-Whitney's U-test; \( p < 0.0001 \)).

To elucidate the relationship between IOP and immunoreactivity against NSE, maximum and mean IOPs were examined in anti-NSE antibody positive patients with POAG (one case of OH was included) and NTG. However, no correlations were observed (data not shown).

Judging the visual field deterioration, 24 cases of POAG (one case of OH was included) and 11 cases of NTG met our criteria described

<table>
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<th>Table 3. The titers of anti-NSE antibody in anti-NSE antibody positive and negative patients</th>
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<tr>
<td>ELISA 400 dil. (( \times 10^{-4} ))</td>
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<tr>
<td>Antibody positive ( (n = 42 ) cases)</td>
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<tr>
<td>Antibody negative ( (n = 22 ) cases)</td>
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\( p < 0.0001 \) (Mann-Whitney's U-test).

![Visual Field losses progression](image)

**Fig. 1.** The progression of glaucomatous visual field losses and the titers of anti-NSE antibody in anti-NSE antibody positive patients with POAG and NTG. Anti-NSE antibody positive patients with POAG and NTG were sorted into two groups with or without progression of glaucomatous visual field losses. Serum anti-NSE antibody titers obtained by ELISA in POAG and NTG patients in each group were plotted. One case of OH was included in POAG. Long and short bars represent mean and s.d. Serum anti-NSE antibody titers of age-matched healthy control subjects are less than 100.
in the method section. Immunoreactivities against NSE were compared between glaucoma patients with and without visual field deterioration in POAG and NTG. Although there was no statistical significance in POAG or in NTG (Fig. 1), the titers of anti-NSE antibody were relatively higher in NTG with visual field deterioration than in those without it.

To examine the relationship between glaucoma stages and immunoreactivities against NSE, the titers of anti-NSE antibody were compared with their glaucoma stages and glaucomatous visual field losses in POAG (one case of OH was included) and NTG. As shown in Fig. 2, the titers of anti-NSE antibody were decreased with advancing glaucoma stages and/or deteriorating glaucomatous visual field losses in POAG (Scheff’s test; \( p < 0.05 \)). In NTG, the titers of anti-NSE antibody were almost same among those three different glaucoma stages (Fig. 3).

**Discussion**

Clearly, elevated IOP is the most frequent causative risk factor for glaucomatous optic neuropathy, but it is not the only factor. And several clinical investigators have revealed that approximately 20–30% of POAG patients show progressive deterioration of visual field losses even though their IOP levels were controlled at the lower levels (Quigley and Maumenee 1979; Katz et al. 1997). Therefore, these observations let us to suggest that some unknown factors must be involved causing glaucoma progression besides elevated IOP. We previously reported that approximately 20% of POAG patients possessed serum autoantibody against NSE and maximum IOP levels in POAG patients with anti-NSE antibody were statistically lower than those without the antibody (Maruyama et al. 2000). In addition, we also reported that injection of the purified anti-NSE antibody into vitreous cavity of Lewis rat caused apoptosis of retinal ganglion cells.

![Fig. 2. The titers of anti-NSE antibody in anti-NSE antibody positive patients with POAG. Anti-NSE antibody positive patients with POAG were sorted into three groups with different stages of glaucomatous visual field losses (determined by Aulhorn Greve’s classification): 1) stage 0–1 (n=20 eyes); 2) stage 2–3 (n=20 eyes); 3) stage 4–6 (n=10 eyes). Serum anti-NSE antibody titer obtained by ELISA were plotted. \( p < 0.05 \) (Scheff’s test).](image)
Clinical Roles of Anti-NSE Antibody in Glaucoma Patients

Fig. 3. The titers of anti-NSE antibody in anti-NSE antibody positive patients with NTG.
Anti-NSE antibody positive patients with NTG were sorted into three groups with different stages of glaucomatous visual field losses (determined by Aulhorn Greve’s classification): 1) stage 0-1 (n = 15 eyes); 2) stage 2-3 (n = 15 eyes); 3) stage 4-6 (n = 3 eyes). Serum anti-NSE antibody titers obtained by ELISA were plotted.

(Maruyama et al. 2000). Clinically, we also reported that the rates of detection of anti-NSE antibody were significantly and relatively higher in early stages of POAG (Aulhorn-Greve’s stages 0-2) and in the other stages of POAG (Aulhorn-Greve’s stages 3-6) and NTG with visual field deterioration than in those without it (Ikeda et al. 2002). Therefore, we suggested that circulating anti-NSE antibody within the blood flow reached the retina and possibly caused retinal ganglion cell damage and progression of visual field losses in addition to elevated IOP. The results of our present study also support our suggestion that the titers of anti-NSE antibody were relatively higher in NTG with visual field deterioration than in those without it. These data suggest that NTG patients with higher levels of anti-NSE antibody titers may be apt to develop visual field losses.

Presently we do not know whether the anti-NSE antibody production is secondarily associated with the destruction of retinal ganglion cells or not. However, if this is the case, the titers of anti-NSE antibody should increase as advancing glaucoma stages and/or progression of glaucomatous visual field losses. In fact, in our present study, we found that the titers of anti-NSE antibody were decreased with advancing glaucoma stages and/or progression of glaucomatous visual field losses in POAG and NTG, with the titers of anti-NSE antibody almost same among three different glaucoma stages with any systematic pattern. Therefore, we suggest that the serum anti-NSE antibody in glaucoma patients, especially in the early stages of POAG, may be one of the useful factors for diagnosing early stages of POAG, and for monitoring glaucoma progression of NTG.

References


