Current Eye Research

The *GINKGO BILOBA* EXTRACT (EGb 761) provides neuroprotective effect on retinal ganglion cells in a rat model of chronic glaucoma

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Abbreviated title: EGb 761 protects ganglion cells

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Key Words: ganglion cell, glaucoma, glaucoma pharmacology, intraocular pressure, nitric oxide

Abstract

Purpose. To investigate the effect of *Ginkgo biloba* extract (EGb 761) against neurotoxicity of retinal ganglion cells of rats with chronic moderately elevated intraocular pressure (IOP).

Methods. Unilateral chronic moderately elevated IOP was produced in rats by cautery of three episcleral vessels. Secondary degeneration was measured with and without EGb 761 for 5 months. At 5 months, retinal ganglion cells were labeled with a fast blue tracer applied to both superior colliculi. Densities of surviving retinal ganglion cells were estimated by counting fast blue labeled cells in whole mounted retinas.

Results. When compared with their contralateral control eyes with normal IOP, in the peripheral retina, retinal ganglion cell loss in eyes with chronic, moderately elevated IOP was 29.8 ±1.5% (n = 5) at 5 months in untreated animals and 4.6 ± 4.5% (n =5) at 5 months in treated animals with EGb 761.

Conclusions. Pretreatment and early posttreatment with EGb 761 is an effective neuroprotectant in a rat model of chronic glaucoma.

In Germany and France, *Ginkgo biloba* extract EGb 761 is one of the most commonly prescribed drugs and is especially used for ameliorating peripheral vascular diseases such as intermittent claudication and cerebral insufficiency.[1] EGb 761 has been reported to prevent ischemic-induced oxidation,[2,3] improve cerebral blood flow[4] and antagonize the action of platelet-activating factor.[5] EGb 761 is a standardized mixture of active substances, including 24% flavonoid glycosides and 6% terpenoids,[6] obtained from green leaves of the *G. biloba* tree. EGb 761 is a polyvalent agent capable of scavenging free radicals such as nitric oxide (NO),[7,8] reducing Ca^{2+}-stimulated intracellular signal transduction events,[9,10] and modulating intacellular signal transduction events, including those involving phospholipases A and C[11] and PKC.[12] All of these signal transduction molecules are likely involved in neurodegenerative diseases.[13]

NO is an important mediator with diverse physiological roles and certain pathologic roles in various tissues, including the central nervous system (CNS) and the eye.[14-18] In the CNS, NO has been implicated in neurodegenerative diseases such as stroke, Alzheimer disease, multiple sclerosis, and amyotrophic lateral sclerosis. In animal models of neurodegeneration, NO can be both neuroprotective and neurodestructive. More recently, it has been hypothesized that glaucoma could be due to neurotoxic effects of NO at the optic nerve head and retina which results in optic nerve head degeneration and visual field loss.[19,20] We previously demonstrated that NO modulated retinal ganglion cell N-type Ca channels by facilitating their voltage dependent activation via a mechanism involving guanylyl cyclase/PKG dependent phosphorylation.[21]

Glaucoma, one of the world's leading causes of blindness, is characterized by progressive optic nerve damage with selective loss of retinal ganglion cells.[22,23] Since it has been reported that EGb 761 is able to dose-dependently inhibit synthesis of NO through inhibition of inducible NO synthase (NOS),[24] we investigated the possible effects of EGb 761 in a rat model of glaucoma in which there is chronic, moderately

elevated intraocular pressure (IOP) and a slow, progressive loss of retinal ganglion cells.

Materials and methods

Rat model of chronic, moderately elevated IOP

Adult, female Sprague-Dawley rats (SLC, Hamamatsu, Japan) weighing approximately 200 g at the beginning of the experiment were used. Animals were fed ad libitum and maintained in temperature-controlled rooms. Experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement. All surgical procedures were performed under general anesthesia by using Nembutal (40-50 mg/kg), given i.p.

Chronic, moderately elevated IOP was produced unilaterally in 10 rates by cautery of three episcleral vessels[25]; the contralateral eye served as the comparative control. To perform the cautery, sutures were placed in the lids to keep the eye open and in the bulbar conjunctiva to manipulate the glove. Three of the four to five major trunks formed by limbal-derived veins were exposed at the equator of the eye by incising the conjunctiva. Each vessel was lifted with small muscle hook and cauterized by direct application of an ophthalmic cautery (Tagawa, Tokyo, Japan) against the muscle hook. Immediate retraction and absence of bleeding of the cauterized end of the vessels were noted as successful cauterization. Eyes were treated topically with levofloxacin (Santen, Osaka, Japan) for a few days after the surgery to prevent infection.

EGb 761 and measurements taken during treatment

One group of five animals was treated with EGb 761 (a gift from Japan Green Wave, Tokyo, Japan) in the drinking water for 5 months; a second group of five animals was untreated. At the time of cauterization, the rats were divided randomly into two groups (drug-treated and untreated). 100 mg/kg per day EGb 761 was dissolved in their drinking water, which was made up and provided fresh twice per week. The control

group was not treated but received fresh drinking water, from the same source, on the same schedule. Once a month, the IOP measurements were made using a handheld electronic tonometer (TonoPen XL; Bio-Rad, Glendale, CA). On all given eyes, three to five tonometer readings were taken and averaged. On a given day, mean ±SD was derived for all control and surgical eyes. Significant differences between surgical and control eyes were determined by 1-way analysis-of variance (ANOVA) for each day on which measurements were performed.

Retrograde labeling of retinal ganglion cells

Five days before sacrifice, fast blue (Polysciences Inc., Warrington, PA) was injected bilaterally into the superior colliculi of anesthetized rats26. Five days after fast blue application, animals were sacrificed by overdose of Nembutal and whole, flat-mounted retinas were assayed for retinal ganglion cell density. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour. Eyes were bisected at the equator, the lens was removed, and the posterior segments were prepared for flat mounts. Retinas were dissected from the underlying sclera, flattened by six radial cuts, and mounted vitreal side up on gelatin-coated slides.

Labeled retinal ganglion cells were counted by using fluorescence microscopy. Noting the retinal topography, six fields in regional areas, approximately 4.0 (peripheral) mm from the optic disc, were counted. Cell counts were conducted by the same investigator in a masked fashion; the identity of the retinas that led to the micrographs was unknown until cell counts from different groups were complete. Changes in retinal ganglion cell densities were expressed as the percent loss of retinal ganglion cells comparing surgical and contralateral, control eyes from the same animal in the different retinal regions. All statistical data are presented as means ±SD, and analyzed using an independent Student's t test where appropriate. Statistical significance was considered with $p < 0.05$.

Results

Relationship between EGb 761 and IOP

There was no significant difference in the consumed volumes of water of the two groups. On a per day basis, the group that was not treated pharmacologically drank 23.4 ± 1.8 ml/day (n = 5), whereas the group that was treated with EGb 761 drank 22.6 ± 1.4 ml/day (n = 5) ($p = 0.71$). Given this volume of drinking, we calculate that the treated group received 30 mg EGb 761 per day.

Fig. 1 shows that IOP was elevated in all eyes for 5 months after receiving three-vessel cautery compared with the contralateral, control eyes. Comparing animals that were not treated pharmacologically with animals treated with EGb 761, the elevated IOPs in the three vessel cautery eyes were similar. The IOPs in the contralateral eyes of two groups were also similar. Thus, EGb 761 did not effect IOP. Our success rate was five out of ten animals. Animals, in which elevated IOP were not maintained for more than 4 weeks, were excluded from the subjects.

Relation between EGb 761 protection and RGC distribution in the retina

In retinas, the only cells that appeared to be labeled with fast blue were retinal ganglion cells. These were identified by the typical punctate fluorescence present in the somata as well as within the initial segments of primary dendritic processes (Fig. 2). All quadrants of all retinas were successfully labeled.

Fig. 3 shows the loss of retinal ganglion cells by comparing the eye with elevated IOP to the contralateral, control eye in animals not treated pharmacologically and in animals treated with EGb 761. In the peripheral retina, retinal ganglion cell loss in eyes with chronic, moderately elevated IOP was 29.8 ± 1.5% (n = 5) at 5 months in untreated animals and 4.6 ± 4.5% (n = 5) at 5 months in treated animals with EGb 761 (Fig. 3B) ($p < 0.05$). The number of surviving retinal ganglion cells per square millimeter was 1482 ± 85 in untreated animals and 2021 ± 214 in treated animals

with EGb 761 (Fig. 3A). The loss of retinal ganglion cells in eyes with chronic, moderately elevated IOP of animals treated with EGb 761 was not significantly different to that in normal eyes ($p = 0.33$).

Discussion

The present study indicates that EGb 761, acting principally via its flavonoid constituents, is able to protect retinal ganglion cells. Bastianetto et al. recently reported that EGb 761 protected and rescued hipocampal neuronal cells against NO-induced toxicity.[24] NO is a highly diffusible gas, synthesized from L-arginine by the enzyme NOS. The three major isoforms of NOS contain a carboxy-terminal reductase and an amino-terminal oxygenase domain.[27]

The actions of NO in the retina are still only partially understood. A low concentration of NO may play a protective role in glutamate neurotoxicity by closing the NMDA-receptor-gated ion channel.[28] However, elevated concentrations of NO, interacting with oxygen radicals, become toxic and mediate glutamate-induced neurotoxicity in the cultured retinal neurons.[28] In addition, the most consistent action of NO on many cell types is to stimulate the production of cGMP. We have previously shown that NO is transduced by a soluble guanylate cyclase to produce an increase in cGMP that in turn acts via cGMP-dependent protein kinase to enhance Ca channel activity.[21]

NOS-2 is the inducible form of the enzyme and is not usually found in cells under normal conditions. Nitric oxide synthesized by NOS-2 is implicated in neurodegeneration in several human diseases[29] and may drive progressive neuropathy such as glaucoma.[30] Recent work on acute retinal ischemia in rats has demonstrated that NOS-2 participates in retinal destruction.[31] Clusters of NOS-2-positive cells have been found in human glaucomatous optic nerve heads.[32] Shareef et al. recently reported that NOS-2 appeared in astrocytes in the optic nerve heads with chronic, moderately

elevated IOP.[33]

EGb 761 contains two major groups of active substances namely flavonoids (24%), which are nearly exclusively flavonol-*O*-glycosides, and terpenoids (6%), including ginkgolides and bilobalides.[34] It has been suggested that the combined activity and a certain interdependency of several active constituents of the extract are responsible for its beneficial effects.[35] Since Bastianetto et al. recently reported that the flavonoid fraction (CP 205) strongly inhibited both the toxicity and the free radical accumulation induced by SNP (sodium nitroprusside) and/or SIN-1 (3-morpholinosydnonimine),[36] it is most likely that the protective and rescuing effects of EGb 761 against NO-induced toxicity are attributable to its flavonoid constituents.

The doses used in the present study were chosen according to other studies showing that doses of EGb 761 between 50 and 100 mg/kg chronically administered produce inhibition of brain monoamine oxidase in the mouse,[37] protecting effect on ischemia-reperfusion damage in the rat retina.[38]

In glaucoma treatment, patients often experience progression of disease, even after maximum reduction of IOP. Also, in patients with normal-tension glaucoma, we cannot entirely depend on IOP reduction, because substantial further reduction of IOP is often difficult. As we know that the final common pathway of glaucoma is retinal ganglion cell death, an approach to protect retinal ganglion cells can widen the field of glaucoma treatment. In summary, the results of the present study suggest that EGb 761 is able to protect and rescue rat retinal ganglion cells in a rat model of chronic glaucoma. These results demonstrate that pharmacological neuroprotection, using EGb 761, may be a viable approach to treat glaucoma. This approach may be effective with or without a pharmacological agent to lower IOP. However, further studies using other models of optic nerve damage should be conducted to confirm the neuroprotective effect of EGb 761.

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Figure legends

Fig. 1 Moderately elevated IOP in rat eyes that had three episcleral vessels cauterized, unilaterally (Operated), vs. the opposite eye (Control). IOP was measured on anesthetized animals with a tonopen. In one group (n=5), EGb 761 was added to the drinking water. In the control group (n=5), nothing was added to the drinking water. Throughout the 5-month follow-up, IOP was elevated in all Operated eyes compared with Control eyes ($p < 0.05$). Elevated IOP was not different in the group treated with EGb 761 vs. the group not treated EGb 761.

Fig. 2 Retrogradely labelled retinas with normal IOP and chronic, moderately elevated IOP, with and without treatment with EGb 761 for 5 months. A: Control, B: EGb 761-treated, C: vehicle-treated. Scale bar, 30 μm.

Fig. 3 Retinal ganglion cells lost at 5 months in rat eyes with chronic, moderately elevated IOP. Ganglion cell density in retinas with chronic, moderately elevated IOP. Retinal ganglion cells (RGC) were counted in the peripheral retina, approximately 4.0 mm from the optic disc. The graph depicts the mean ± SD of five animals treated with EGb 761 and five animals treated with vehicle. A significant difference between retinal ganglion cell densities in eyes with elevated IOP that were treated EGb 761 and vehicle was evident ($p = 0.0007$). A: Density, B: % Lost.

Fig. 1

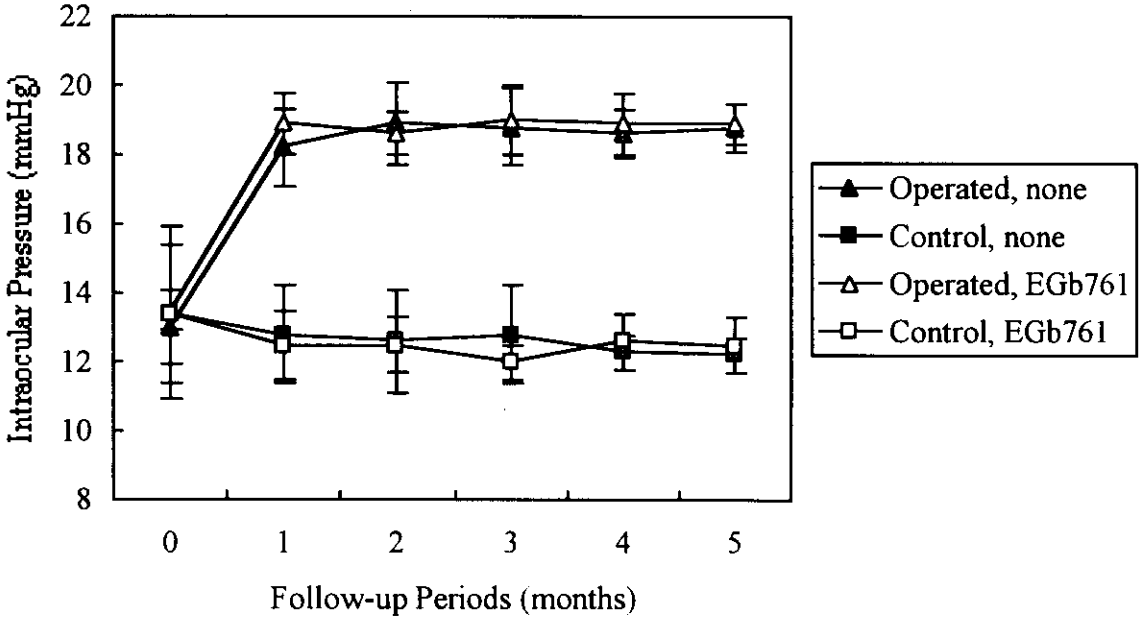
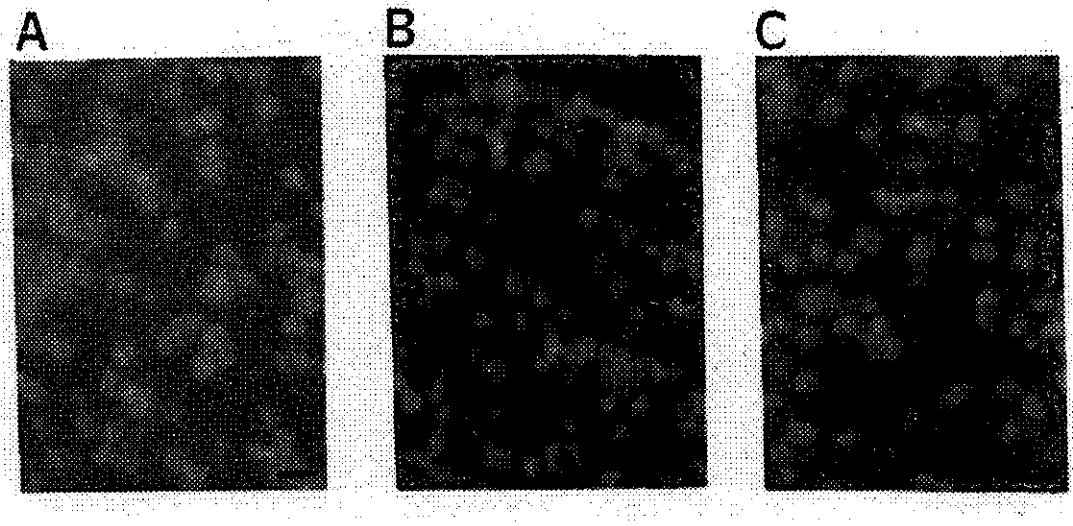
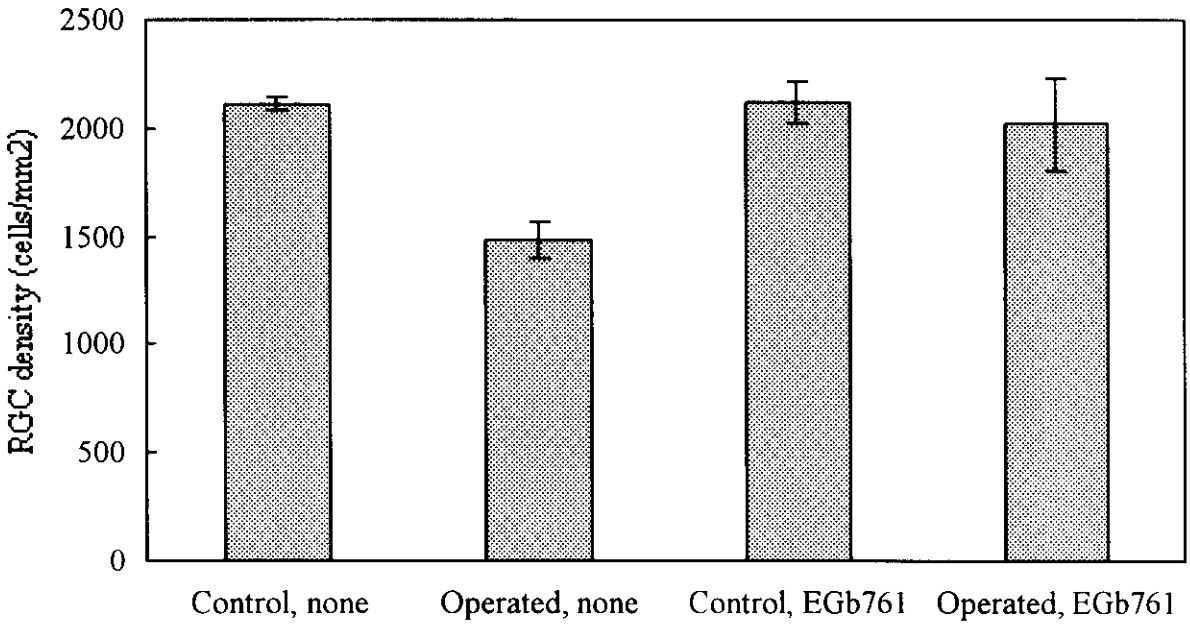

Intraocular Pressure (mmHg)
22
20
18
16
14
12
10
8
0
1
2
3
4
5
Follow-up Periods (months)
Operated, none
Control, none
Operated, EGb761
Control, EGb761

Fig 2

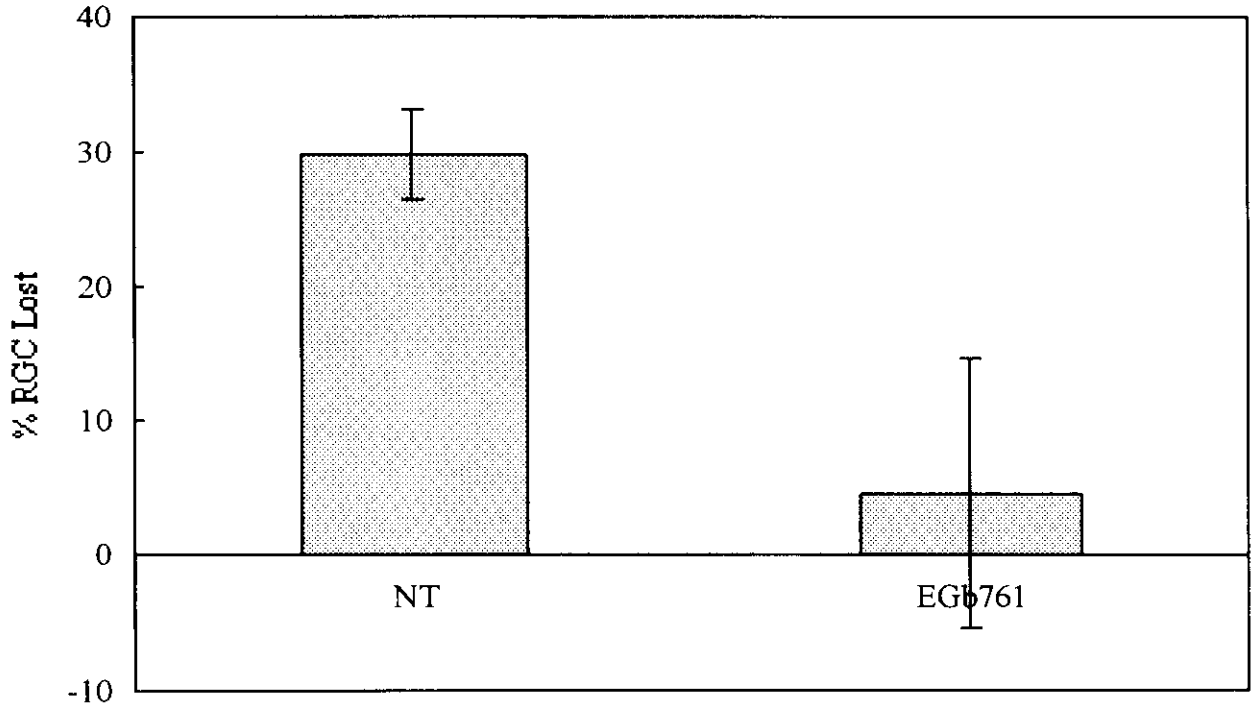


A

Fig 3


2500
2000
1500
1000
500
0
RGC density (cells/mm2)
Control, none
Operated, none
Control, EGb761
Operated, EGb761

B


40
30
20
10
0
-10
% RGC Lost
NT
EGb761

51. 視神経萎縮患者のOPA1遺伝子異常と臨床像

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研究要旨　常染色体優性視神経萎縮は両眼性の視神経萎縮を主徴とし、視力低下、中心暗点や傍中心暗点、色覚異常等を伴う。原因遺伝子は複数あると考えられるが、これまでにOPA1遺伝子が同定されている。今回我々は19家系の視神経萎縮の症例のOPA1遺伝子を検討したところ、12家系に計10種類のOPA1遺伝子の変異をヘテロ接合で検出した。家族歴から優性遺伝と考えられた7家系中6家系に変異を検出し、本邦では優性視神経萎縮の原因はOPA1遺伝子の異常によることが多いことが明らかになった。また家族歴から孤発例と考えられた11例中5例に変異を検出した。これは優性視神経萎縮では重症度が症例により著しく異なるためと考えられ、視神経萎縮では一見孤発例と思われても、遺伝形式を決定するために家族の詳細な調査が重要であると考えられた。

A. 研究目的

常染色体優性視神経萎縮は両眼性の視神経萎縮を主徴とし、神経乳頭の耳側蒼白や瀰漫性蒼白を呈する。矯正視力は中等度に低下、色覚は後天性の青黄異常を示し、視野は傍中心暗点や盲点中心暗点を示すことが多い。視機能障害の進行は遅く、重症度の程度に差があることが特徴とされ浸透率は必ずしも高くないといわれている。原因遺伝子は複数あると考えられるが、これまでにはOPA1遺伝子が同定されている。我々は2001年度の本班会議研究報告でOPA1遺伝子に変異を持つ2家系について報告したが、今回は症例を追加し計19家系の視神経萎縮の症例のOPA1遺伝子の解析を行った。

B. 研究方法

19家系の視神経萎縮の症例のOPA1遺伝子を、インフォームドコンセントを得たうえで、ダイレクトシークエンス法で検討した。7家系は常染色体優性遺伝形式を示し、11例は家族歴の聴取からは孤発例と考えられた。1家系では遺伝形式は不明だった。急性発症等の症状からレーベル病が疑われる症例や、片眼性、網膜変性を伴う症例など、視神経萎縮が認められても臨床像が常染色体優性視神経萎縮に合致しない症例は除外した。変異が認められた症例については視力、色覚、視野等の臨床所見の検討を行った。

C. 研究結果

視神経萎縮19家系のうち、12家系に計10種類のOPA1遺伝子の変異をヘテロ接合で検出した。優性遺伝の家族歴を示した7家系中6家系で変異を検出し、孤発例と考えられた11例中5例に変異を検出した。家族歴の聴取からは孤発例と考えられたが変異

が検出された症例のうち、2 例については家族の調査を行った。その結果、各々の症例の家族には、症例と同じ遺伝子異常を持ってはいる者が検出されたが、それらの臨床症状は非常に軽微で、視力も正常あるいはわずかに低下している程度だった。

検出された10種類の変異のうち８種類は新規の変異だった。10 種類の変異のうち５種類は失欠または挿入の変異でそのうち４種類はフレームシフトを伴い、３種類はナンセンス変異、１種類はスプライスサイトの変異で１種類はミスセンス変異だった。また既に海外で高頻度に認められている c.2708delTTAG の変異を３家系で認めた。

OPA1 遺伝子に変異が検出された症例では、視力は手動弁から 1.5 まで様々だった。また視神経乳頭の所見も症例により様々で、重症の症例では瀰漫性の蒼白が認められ、軽症例では蒼白があまり目立たないものもあった。パネル D-15 による色覚検査でも、正常の症例から irregular タイプまで結果は様々だったが、全体的には第３色覚異常を呈する傾向が認められた。

D. 考察

常染色体優性視神経萎縮は遺伝的に多様であり、OPA1 遺伝子以外にも、遺伝子自体はまだ同定されていないが染色体上のローカスが判明している OPA4 も同定されている。最近の海外での報告では、多くの常染色体優性視神経萎縮の原因は OPA1 遺伝子の異常によることが示唆されている。今回の結果でも、常染色体優性遺伝形式を示す視神経萎縮の７家系中６家系に OPA1 遺伝子の変異が検出され、日本人でも常染色体優性視神経萎縮は原因は OPA1 遺伝子の異常に起因する場合が多いと考えられた。

また今回の結果では、家族歴の聴取からは孤発例と考えられた11例中の５例にOPA1遺伝子の変異を検出され、これは視神経萎縮では重症度が症例により著しく異なるために起きたと考えられた。この結果から、視神経萎縮の症例では、一見孤発例と思われる症例でも、比較的高い頻度で OPA1 遺伝子の異常により発症していることが示唆された。視神経萎縮の症例では、遺伝形式を決定するために詳細な家族の調査や遺伝子検査を行うことが重要であることが確認された。

E. 結論

本邦では優性視神経萎縮の原因は OPA1 遺伝子の異常によることが多い。また孤発例と考えられた視神経萎縮症例からもしばしば OPA1 遺伝子の変異が検出され、視神経萎縮では一見孤発例と思われても、遺伝形式を決定するために家族調査を行うことが大切であると考えられた。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3．その他

なし

Ⅰ．参考文献

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52. 光干渉断層計（ＯＣＴ３）を用いた優性遺伝視神経萎縮患者の黄斑部網膜、視神経乳頭周囲神経線維層の厚みの測定

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研究要旨　2003年より本邦でも使われるようになった新型の光干渉断層計（ＯＣＴ３）は網膜の断層像を得るための検査機械で、さまざまな網膜疾患で起きる網膜の微細な変化を捉えるのに有用で臨床の場で広く使われている。一方、視神経萎縮は基本的に視神経が障害される疾患で、網膜には検眼鏡的な変化は認められないため、ＯＣＴ３はほとんど使われてこられなかった。そこで、今回、視神経萎縮のうちでもOPA1遺伝子に異常が検出された視神経萎縮患者に対しＯＣＴ３を用いた結果、有意な黄斑部網膜厚の減少、黄斑部神経線維層・視神経乳頭周囲神経線維層の著しい菲薄化など検眼鏡的には検出されない網膜の変化がＯＣＴ３では捉えられた。本研究により、さまざまな視神経疾患における視神経障害の程度の把握、網膜疾患と視神経疾患との鑑別などにＯＣＴ３が臨床的に有用である可能性が示された。

Ａ．研究目的

優性遺伝視神経萎縮の原因遺伝子であるOPA1遺伝子に異常が検出された視神経萎縮患者の、黄斑部の網膜全層・網膜神経線維層・視細胞層外節層の厚さ、および、視神経乳頭周囲の網膜神経線維層の厚さを光干渉断層計（ＯＣＴ３）を用いて調べ、本疾患における網膜の変化を調べる。

Ｂ．研究方法

ＯＣＴ３を用いて、遺伝性視神経萎縮患者の黄斑部網膜の厚さを中心窩、中心窩から１ｍｍおよび２ｍｍ、それぞれ上方、下方、耳側および鼻側の計９点で調べた。さらにそれぞれの測定点において、視細胞層外節、網膜神経線維層の厚みも測定した。次に、視神経乳頭周囲網膜に直径 3.4ｍｍの円形スキャンを行い、網膜神経線維層の厚さを内蔵された解析ソフトを用いて調べた。最後に、正常眼を同様に調べ、視神経萎縮患者の測定結果と比較、検討した。

（倫理面への配慮）

ＯＣＴ３を用いた検査は弱い光を眼底に当てるのみの非侵襲的な検査である。検査は患者に対してその検査の意義、必要性について十分に説明し同意を得た後に施行されている。

Ｃ．研究結果

OPA1遺伝子に異常を持つ視神経萎縮患者の黄斑部網膜の厚さは、正常群と比べ中心窩以外で有意に薄かった（$p<0.05$）。また視