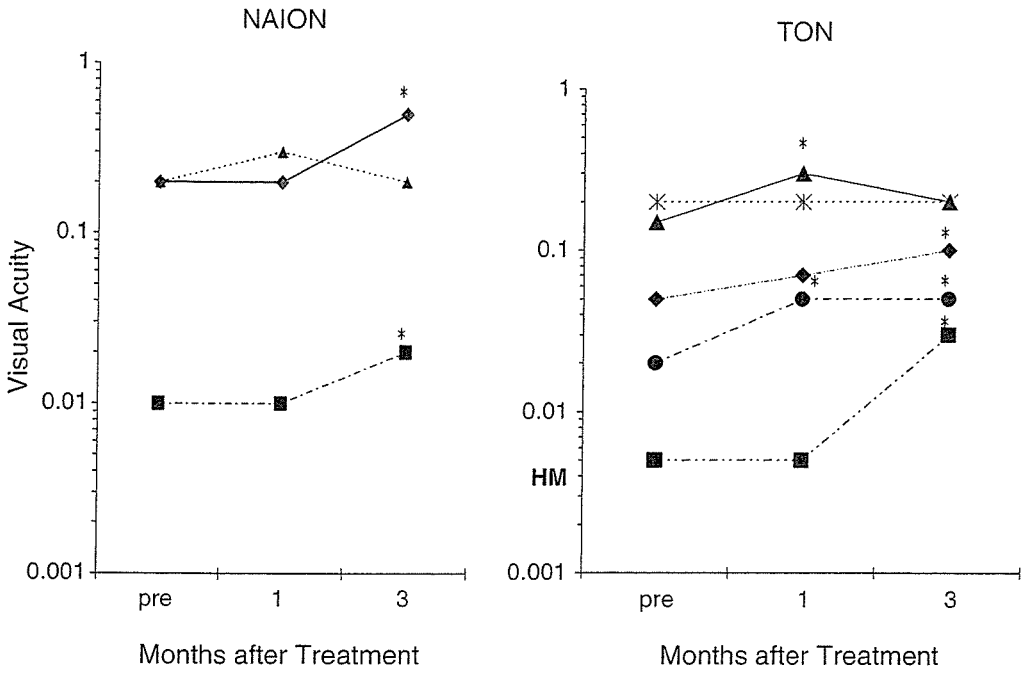

Figure 2. Effect of TES on visual acuity. Change in visual acuity after TES in eyes with nonarteritic ischemic optic neuropathy (*NAION*) (*left*) or traumatic optic neuropathy (*TON*) (*right*). *, improvement of vision by ≥0.3 log minimum angle of resolution(logMAR) units compared with the pretreatment value; *pre*, denotes pretreatment value; *HM*, hand motion. —/◆, Case 1; –·–/■, Case 2; ·····/▲, Case 3; —·—·—/◆, Case 4; —/▲, Case 5; ·····/✳, Case 6; – · –/●, Case 7; —·—/■, Case 8

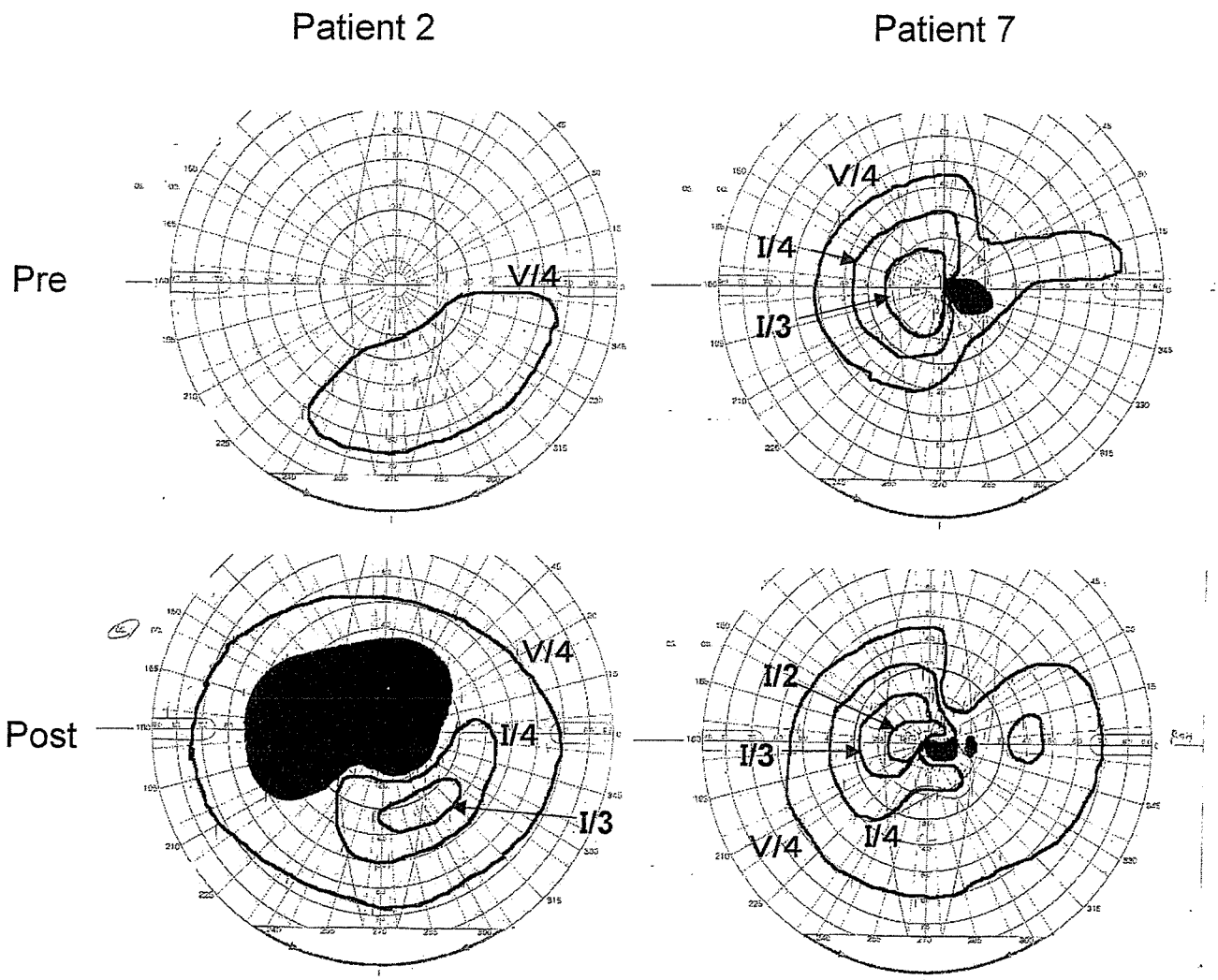

Figure 3. Visual fields. The Goldmann visual fields of patient 2 (NAION, *left*) and patient 7 (TON, *right*) before (*top*) and after (*bottom*) treatment. After treatment, the isopter of the peripheral visual field (V/4) enlarged, and more sensitive isopters (I/4 and I/3 in patient 2 and I/2 in patient 7) were detected.

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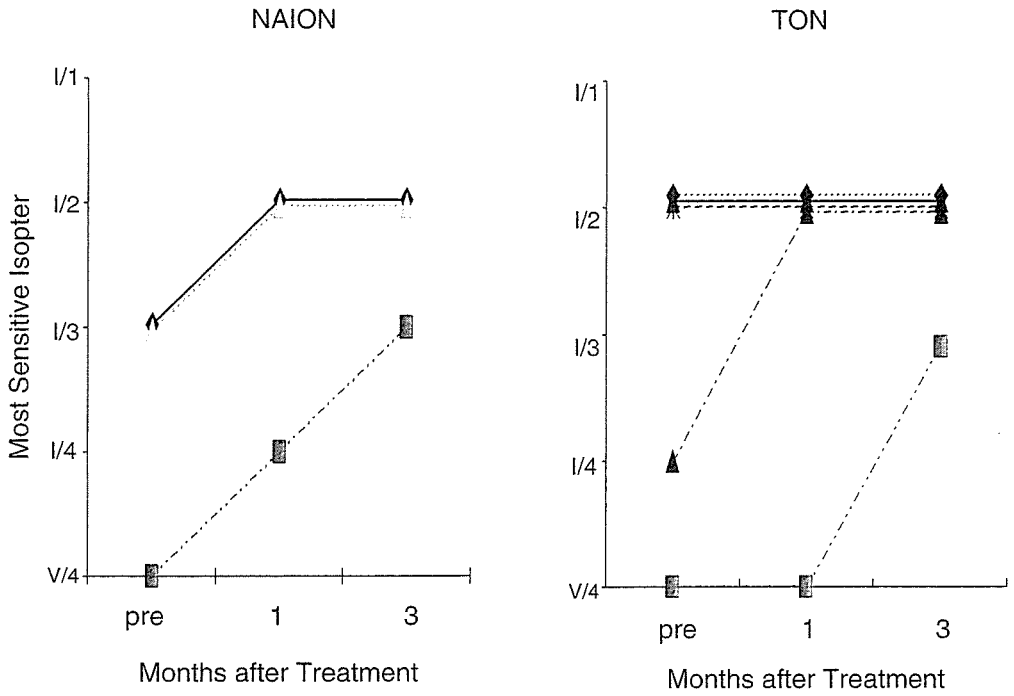

Figure 4. Appearance of more sensitive isopters in the NAION group (*left*) and the TON group (*right*). In all three patients with NAION and in two of the five patients with TON, more sensitive isopters appeared 1 to 3 months after treatment. *I/1* denotes the most sensitive isopter, followed by *I/2*, *I/3*, and *I/4*. *V/4* denotes the isopter of the peripheral visual field. —/◆, Case 1; —/■, Case 2; ·····/△, Case 3; –·–/◆, Case 4; – –/▲, Case 5; —/✳, Case 6; – · –/▲, Case 7; –··–/■, Case 8

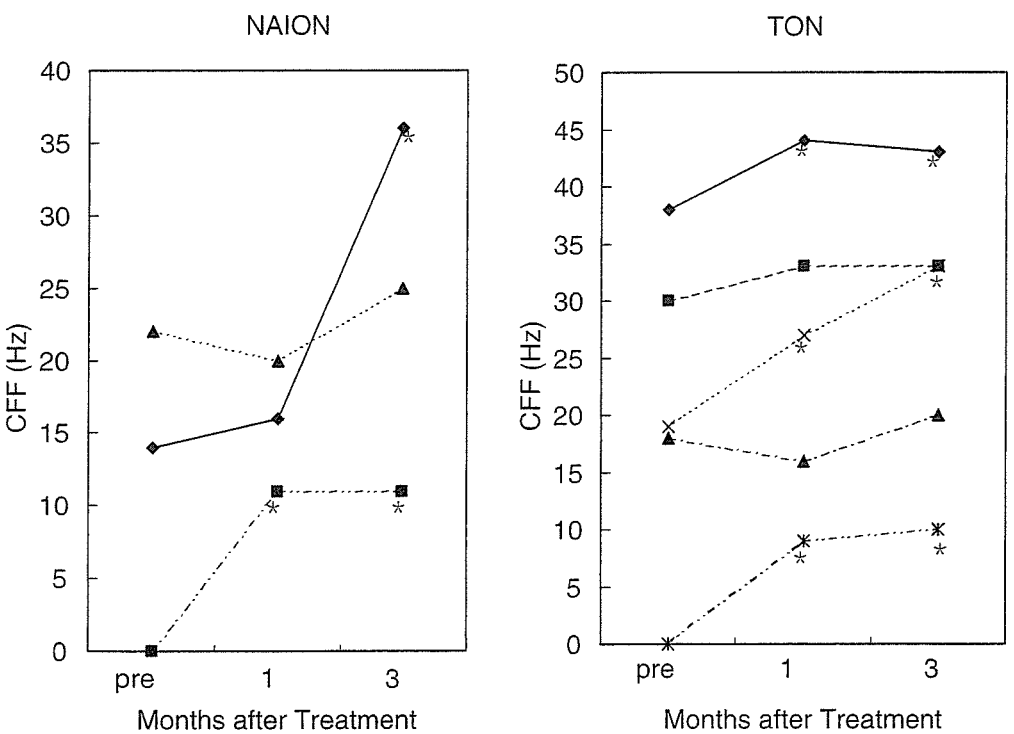

Figure 5. Effect of TES on critical flicker fusion frequency (*CFF*) Change of CFF after TES in eyes with NAION (*left*) and *TON* (*right*). *denotes an improvement of CFF by 15% or more compared with the pretreatment value. —/◆, Case 1; –··–/■, Case 2; ·····/▲, Case 3; —/◆, Case 4; ----/■, Case 5; –··–/▲, Case 6; – · –/×, Case 7; –··–/✳, Case 8

unchanged in the remaining three eyes (one NAION and two TON; Fig. 5).

None of the patients reported experiencing pain during the TES treatment. A mild superficial punctuate keratopathy was observed in all eyes after the treatment, which healed by the next day in all eyes (Fig. 6). The number of corneal endothelial cells had not changed significantly in any of the eyes at 3 months, compared with the number before treatment.

The size and shape of the pupil was unchanged in all eyes, and no cells or flare was observed in the anterior chamber of any eyes just after the treatment or on the next

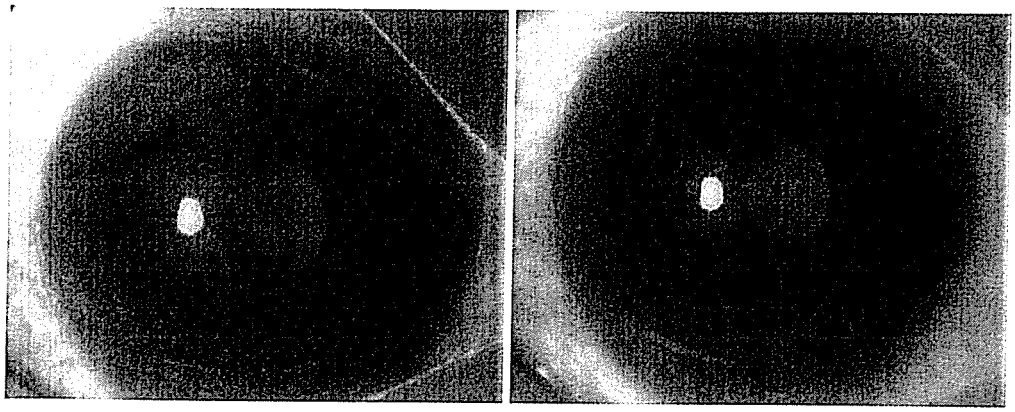

Figure 6. Photographs of the cornea of patient 1 stained with fluorescein dye, taken just after treatment (*left*) and on the following day (*right*). A superficial punctuate keratopathy was observed just after treatment, but was not present the next day.

day. The IOP did not change, and fundus examinations showed no change in the retina or optic disc on the day following treatment.

Case Report

A 64-year-old woman (patient 1) noticed a sudden loss of the inferior visual field in her left eye on 14 November 2002. She was referred to us the next day, and her BCVA was RE, 1.0 and LE, 0.5. A relative afferent pupillary defect was present in her left eye. Ocular fundus examination showed optic disc edema in the left eye (Fig. 7), and fluorescein angiography showed hypoperfusion in the upper temporal disc area (Fig. 6). The CRP value was normal (<2.5 mg/dl). She did not have pain during eye movements, and magnetic resonance imaging did not show changes in the optic nerve. From these results, she was diagnosed with NAION in the left eye. Oral aspirin and vitamin B12 were prescribed.

The BCVA in her left eye decreased to 0.15 in December but recovered to 0.2 in January 2003 and remained unchanged for 5 months. In May 2003, TES was performed on the left eye (600 μA, 20 Hz, 30 min), and the BCVA improved to 0.4 in June and to 0.5 in August. However, the BCVA decreased again to 0.3 in September and to 0.1 in December. TES was performed again in March 2004 (700 μA, 20 Hz, 30 min), and the BCVA improved to 0.2 in April and to 0.3 in June (Fig. 7).

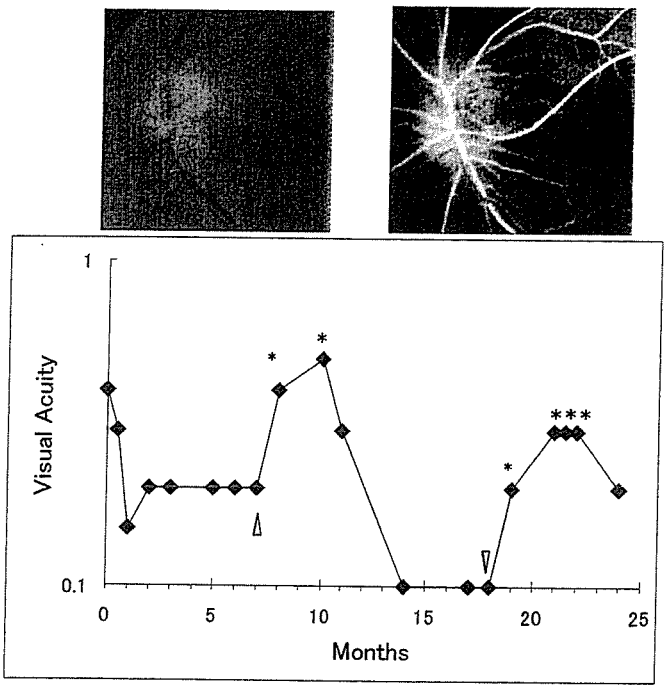

Figure 7. Fundus photograph (*top left*) and early-phase fluorescein angiogram (FA) (*top right*) of the left eye of patient 1 at the first visit. Papilledema of moderate degree can be seen. FA shows hypoperfusion in the upper temporal disc area. The time course of the best corrected visual acuity (BCVA) in the left eye (*bottom*). *Arrowhead* denotes the day of TES treatment. *denotes the improvement of BCVA ≥0.3 logMAR units from the pretreatment value.

Discussion

The visual acuity and CFF improved in two of the three eyes in the NAION group, while the other eye showed no change. In these three eyes, a more sensitive isopter was detected in the visual field after TES (Figs. 2, 4, 5). The IONDT study showed that the mean visual acuity improved up to 3 months after the onset of NAION,[2] and did not improve thereafter during the 24-month follow-up period. Thus, we conclude that TES treatment was effective in some eyes with NAION even if the TES treatment was performed more than 4 months after the onset of NAION.

Our case report shows that in a patient whose visual acuity was stable for more than 5 months, TES can still improve the visual acuity at 3 months after the treatment. Although the visual acuity decreased 6 months later, after a second TES treatment, the vision improved again (Fig. 7). These findings strongly suggest a causal relationship between the treatment and the visual improvement.

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Our case report also shows the limitation of a single TES treatment, because the recovery of vision was obtained for only 3 months, suggesting that repeated treatments might be necessary to maintain the improved vision.

Johnson et al.[6,14] showed that levodopa improved the visual function in 30% of patients with NAION, even if they were treated more than 6 months after the decrease of vision,[14] and in 75% of patients if treated within 2 weeks of the onset of NAION.[6] Therefore, we believe that earlier TES treatment will be more beneficial for the recovery of visual acuity in NAION patients.

This theory is supported by the results in eyes with TON. Three months after TES, the BCVA and CFF improved in all three eyes treated within 4 weeks after onset (patients 4,7,8) but did not improve in the two eyes treated 3 months or more after onset (patients 5, 6; Fig. 2). However, the possibility of spontaneous recovery cannot be ruled out.[15] A randomized controlled study is necessary to confirm the efficacy of TES treatment for TON.

The effective level of electric current for the TES treatment was determined to be the threshold for eliciting phosphenes in both peripheral and central visual fields. This level was selected because it would assure the activation of most retinal ganglion cells and their axons.

Several mechanisms have been proposed to explain the neuroprotective effect of electrical stimulation.[10,16,17] We have demonstrated that insulin-like growth factor 1, a neurotrophic factor, is gradually upregulated in the rat retina up to 2 weeks after TES.[18] This may explain why a single TES treatment is effective in improving vision for up to 3 months. However, additional laboratory studies are necessary to determine more conclusively the mode of action of the electrical stimulation.

To the best of our knowledge, this is the first application of TES for the treatment of optic neuropathy, and we considered it very important to test its safety. The effects of using the Burian-Allen contact lens for the TES treatment are comparable to those following its use for electroretinographic recordings. Only mild corneal punctuate keratopathy was observed after TES treatment in all eyes, and the keratopathy healed by the next day (Fig. 6). The safety of TES on the corneal epithelium may stem from the protective effect of hyaluronic acid and chondroitin sulfate, as well as the balanced charge stimulation using biphasic pulses.

Because the current delivered by the contact lens electrode also stimulated the ciliary body, we carefully checked the size and shape of the pupils, and whether cells or flare was present in the anterior chamber. No significant alterations were observed. The electrical current may also penetrate the cornea, so we confirmed that there were no changes in the corneal endothelium. In particular, no changes were observed in the number of corneal endothelial cells. These observations suggest that TES is a safe method for stimulating the retinal ganglion cells and their axons.

In conclusion, TES led to the improvement of the visual acuity in eyes of some patients with NAION or TON. However, a larger prospective, randomized clinical trial with controls is necessary to confirm conclusively the effectiveness of TES treatment.

Acknowledgments. The authors are grateful to Dr. Yozo Miyake, Dr. Satoshi Suzuki, Dr. Yutaka Fukuda, Dr. Tomomitsu Miyoshi, and Dr. Hiroyuki Kanda for valuable discussions on the development of a system for transcorneal electrical stimulation. This study was partly supported by Health Science Research grants from the Ministry of Health, Labor, and Welfare, Japan, and by a grant from the Ministry of Education, Sports, Culture, Science, and Technology (No. 16591752). T. Morimoto was supported by the Japan Society for the Promotion of Science Research Fellowship for Young Scientists.

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網脈絡膜変性疾患の治療に向けて：人工網膜

Retinal Prosthesis：Toward Curative Therapy for Retinal Degenerative Diseases

不二門　尚*

はじめに

網膜色素変性や加齢黄斑変性などの網脈絡膜変性疾患で，視細胞が変性し失明に至った場合，現在視力回復の手段は存在しない．遺伝子治療や，再生医療などの研究が精力的に行われているが，まだ臨床応用に至っていない．一方，網膜，視神経または大脳皮質への電気刺激によって生じる光覚を利用して，失われた視覚の再建を目指す人工視覚は，これらの疾患に対する有効なアプローチの一つとして期待されており，1日でも早い実用化を目指して，世界各国で精力的に研究開発が進められている．わが国においても2001年度より経済産業省(NEDO)と厚生労働省の連携国家プロジェクトとして人工網膜の研究が始まった．本プロジェクトでは大阪大学，名古屋大学，杏林大学，滋賀医科大学，奈良先端科学技術大学院大学，九州大学，(株)NIDEKが参加し，2011年の実用化を目指して研究開発を進めている．

I　人工視覚の原理

被験者の視覚系伝導路の一部に電気刺激を与えると光感覚を生じる．これを電気閃光(electrical phosphene)とよぶ．Electrical phospheneの現象は古くから知られており，1755年にはLeRoyが眼球への電気刺激によるelectrical phospheneの報告を行っている[1]．その後，1960年代後半よりBrindleyによって大脳皮質を電気刺激した際に生じるelectrical phospheneの研究が行われた[2]．また同時期にPotts & Inoueらのグループや三宅らのグループによって，おもに網膜疾患の検査を目的としてコンタクトレンズ型電極を用いて経角膜で眼球を電気刺激した際のelectrical phospheneに関する数々の研究報告が行われた[3～7]．これら一連の研究から網膜色素変性症で失明に至った患者でも眼球への電気刺激によってelectrical phospheneが生じることが明らかになった．

コンタクトレンズ型電極を用いた電気刺激では網膜全体に電流が拡散するため，electrical phospheneは視野全体に広がる．それに対し，直径数百マイクロメートル程度の単極電極を網膜に接触させ電気刺激を与えた場合，網膜が局所的に刺激されるため，被験者は1個の点状のelectrical phospheneを知覚することが知られている[8]．この刺激電極を多極化しそれぞれの電極で局所刺激を行うと，刺激部位に対応してelectrical phospheneが光の点の集まりとして知覚されるのではないかと考えられている．人工視覚ではテレビカメラなどを用いて体外の画像データを取得し，その情報をもとに刺激部位を多極電極で制御することで，パターン状のelectrical phospheneを生み出し簡単な文字や絵を表現することを目指している(図1)．

II　人工視覚(artificial vision)の種類

さまざまな方式の人工視覚が提案されており，それらは多極電極を埋植する部位によって分類することができる．現在のところ，網膜を刺激するタイプ(人工網膜，retinal prosthesis)，視神経を刺激するタイプ(視神経

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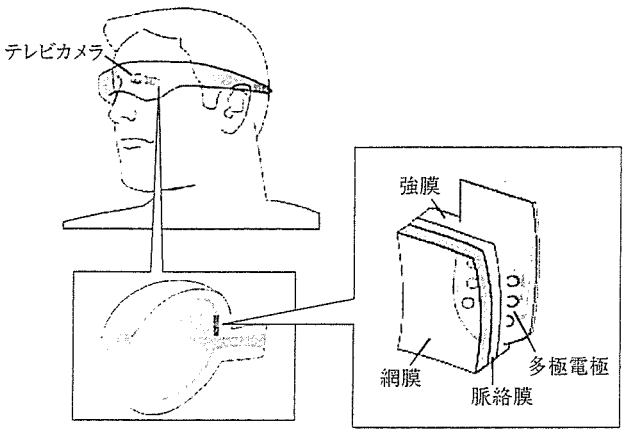

図1　人工視覚の想像図

刺激型人工視覚），そして視覚皮質を刺激するタイプ（皮質刺激型人工視覚）の3種類が提案されている[9]．原理的には視覚伝導路のどこを刺激しても electrical phosphene が生じると考えられるため，上記のほか外側膝状体も刺激場所の候補としてあげられる[9]．

人工視覚はその方式によって適応疾患や手術の安全性が異なる．たとえば，皮質刺激型人工視覚の場合，さまざまな疾患に対して適応が望めるものの，多極電極の埋植時に開頭術を要するため，手術の危険性やその後の感染症のリスクが高い．一方，人工網膜は装置の埋植手術の安全性は高いものの，網膜神経節細胞が変性している場合や視神経の機能が正常に保たれていない場合には，electrical phosphene を生み出すことができず，適応可能な疾患の範囲が限られる．

III　人工網膜（retinal prosthesis）の種類と開発状況

人工網膜では，多極電極を網膜の近傍に設置して電気刺激を行う．前述のとおり刺激する電極の組み合わせを選ぶことによって，患者は文字などのパターンをちょうど電光掲示板のように複数の光点のパターンで知覚することができるのではないかと考えられている．

人工網膜のシステム開発はまだ研究段階にあり，どのグループもいまだ臨床応用には至っていない．それは，実用化に向けて，クリアしなければならない安全面や機能面の課題が残っているためである．たとえば，手術時に電極で網膜を損傷させるリスクを極力抑えなければならない．そして大電流で生じる熱や pH の変動で生じる網膜損傷のリスクを抑えなければならない．また機能面の課題としては，刺激電極の改良や刺激方法の最適化を行うことで，解像度を上げる工夫が必要である．

現在までに考案されてきた人工網膜は多極電極の埋植部位によってさらに網膜上刺激方式，網膜下刺激方式，脈絡膜上-経網膜刺激方式の3つの方式に分類できる（図2）．次節ではそれぞれの方式について詳しく説明する．

1．人工網膜の種類

a．網膜上刺激方式

これは，多極電極を網膜上（多極電極を網膜と硝子体の境界）に設置し網膜を刺激する方式である．多極電極は，網膜タックとよばれる小型の押しピンで網膜上に固定される．この網膜タックの先端は網膜を貫き強膜層まで到達する（図2A）．

1980年代後半に Michelson や Juan & Humayun によって考案された網膜上刺激方式[10,11]は，当初，撮像素子を多極電極と同一基盤内に組み込んだシステムを提案していた．しかし，この場合撮像面（硝子体側）と電極面（網膜側）が反対側を向くため，回路作製に非常に高度な技術を要する．その後多極電極，刺激回路，撮像素子などがそれぞれ分離されたシステムが提案された．

2000年代に入り Humayun が率いる南カリフォルニア大のグループは人工内耳を改造して16極型の多極電

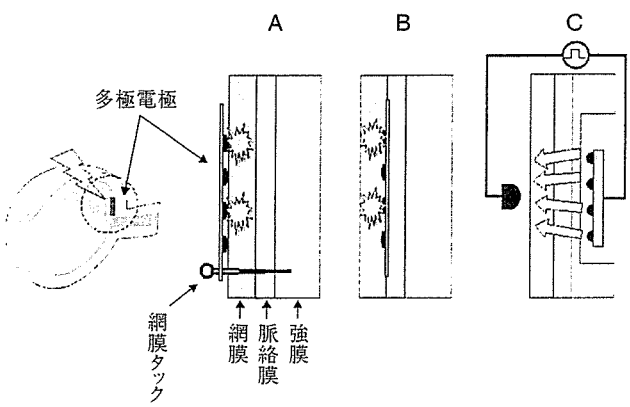

図2　人工網膜の3つの方式

A：網膜上刺激方式，B：網膜下刺激方式，C：脈絡膜上-経網膜刺激方式．

極を装備した網膜上刺激方式の人工網膜を試作した．このシステムを実際に網膜色素変性症患者に埋植し1年以上機能したとの報告を行った[12]．この人工網膜は内部装置と外部装置の2つの装置から構成されている．外部装置は，体外に設置され，外界の画像データを取得するテレビカメラと画像処理を施す回路などから構成される．内部装置は体内に埋植される装置で，刺激装置と多極電極を搭載し，外部装置から受け取った画像データを基に多極電極で網膜の神経細胞を電気刺激し興奮させ画像情報を脳へ伝える．外部装置と内部装置との間はコイルを用いた電磁誘導により画像データと電力の無線伝送が行われる．この内部装置では，多極電極のみ眼内へ埋植しそのほか刺激回路やコイルは眼外（側頭部の皮下）に埋め込む方式が採用されている．側頭部にコイルを設置することによってコイルサイズを大きく設計することができるため大電力供給が可能となる．いかにして電極本数を増やし自然な視覚に近づけるか，また手術手技が複雑なためそれをどのように改良していくかが今後の課題となっている．

一方，Walterらのグループは，内部装置を完全に眼内へ埋植する方式を採用したシステムを開発した[13]．この内部装置ではコイルと刺激装置が前眼部に設置され体外装置から電力と画像情報を取得する．多極電極は黄斑付近の網膜上に網膜タックで固定される．この試作機を実験動物の眼球に埋植したところ，人工網膜による電気刺激で神経興奮を惹起することができたと報告している[13]．移植手術時に眼球への侵襲が大きいため，慢性埋め込みに向けてこれをどのように改良するかが今後の課題である．

b. 網膜下刺激方式

これは，多極電極を網膜下（神経網膜と網膜色素上皮の境界）に埋植し網膜を刺激する方式である（図2B）．網膜上刺激方式に比べて，多極電極の固定は比較的安定し網膜タックを必要としない．また，電極面で眼内入射光を受けることができるため，撮像素子と多極電極を同一基盤上に組み込むことが可能である．したがって，このシステムでは人工網膜で生み出した視覚が眼球運動に対して自然に対応できる．ただ，脈絡膜からの網膜への栄養輸送が電極で遮断されることによる網膜損傷が生じることが危惧される．

Tassickerは1956年に発表した特許のなかで，光感受性をもつ物質を表面に塗布した金属片を網膜下に移植することによって眼内入射光に応じて網膜の神経細胞を刺激する手法を発表した[14]．この特許が網膜下刺激方式の原型となった．その後1991年には半導体シリコン基盤表面に複数のフォトダイオードを作製した人工網膜がChowによって考案された[15]．実現性の高いアイデアであったため，この特許をきっかけに1990年代に半導体シリコンを用いた網膜下刺激方式の人工網膜の研究がChowのグループやZrennerのグループによって精力的に行われた．この装置はASR（artificial silicon retina）またはMPDA（micro-photodiode array）とよばれ，これらは近年急速に発達した集積回路技術を応用することで直径2～3 mmの円形の薄い基板上に撮像素子とそれに対応する刺激電極を数千組搭載することが可能である．

しかし，動物実験による機能評価が進むにつれ，眼内入射光だけでは神経細胞を興奮させるのに必要な電力をまかなうのが困難であることが徐々に明らかになってきた．そこでZrennerのグループは体外装置から赤外線で電力供給を行うことによって不足分の電力を補う新しいMPDAの開発を進めている．このMPDAの場合，赤外線受光部と信号処理回路を増設しなければならないため必然的に内部装置のサイズが大きくなり，装置すべてを眼内に埋植することが困難となる．そこで，経硝子体経由にて人工的に網膜剝離させた部位に装置を移植する従来の術式（ab-interno方式）の代わりに，強膜を貫通して経脈絡膜的に網膜下へアプローチする術式（ab-externo方式）が開発された．この手術を用いて多極電極と赤外線受光部のみ眼内へ，刺激回路の一部が眼外へ飛び出した形での埋植が可能となった．

一方，これまで網膜上刺激方式の研究を進めてきたRizzo & Wyattのグループは最近方針を変更し，網膜下刺激方式による人工網膜の研究に着手した．彼らの提唱する人工網膜は，ChowやZrennerの網膜下刺激方式の人工網膜と異なり，撮像部分が体外に設置される[11]．体外装置で取得した画像データと駆動用電力はコイルを介して内部装置へ伝送される．内部装置中のコイルと刺激

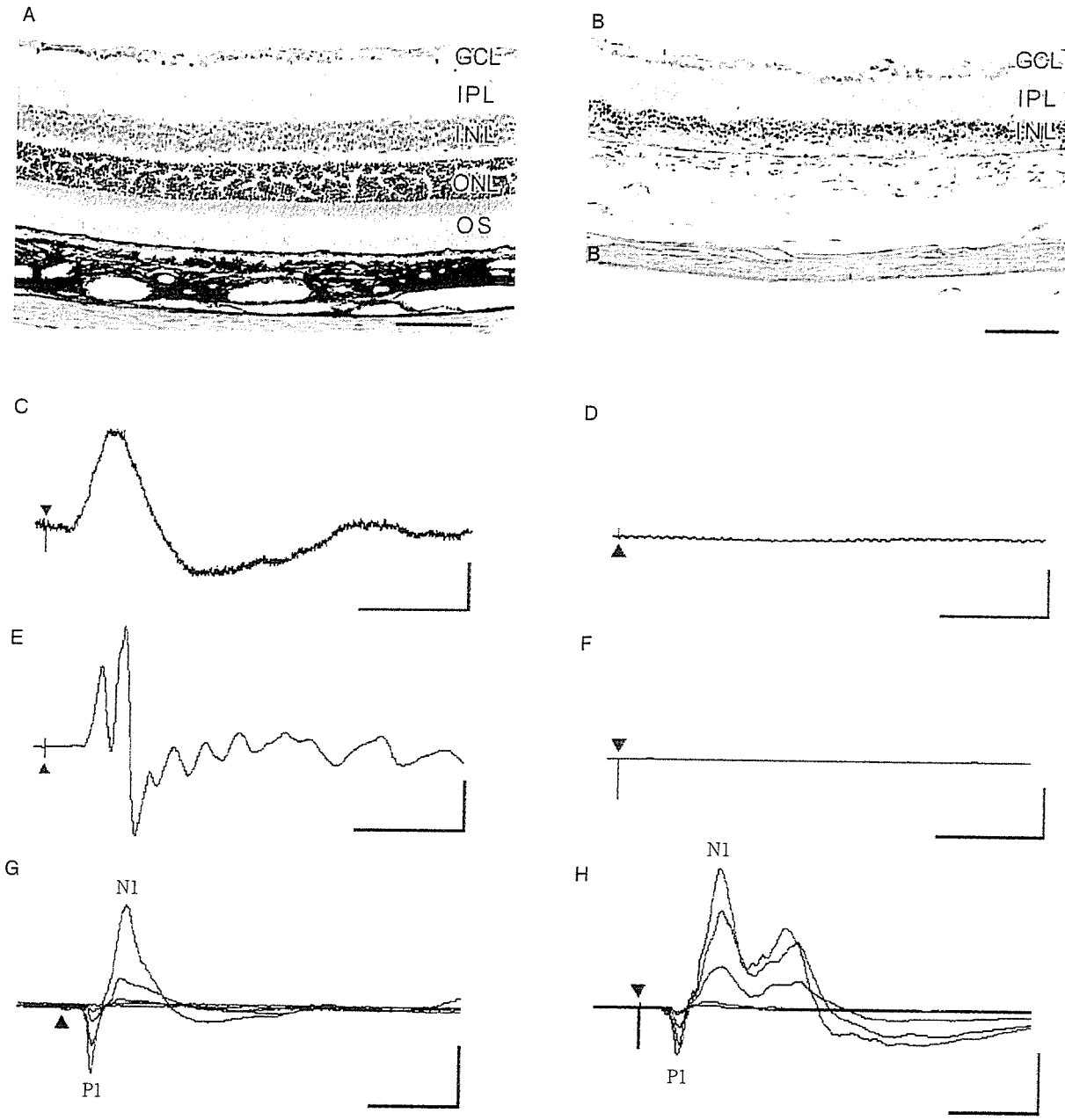

図 3　健常ラットと RCS ラットにおける網膜組織，ERG，光刺激に対する上丘の誘発電位，STS による上丘の誘発電位の比較

ヘマトキシリン-エオジン染色による網膜切片の光顕像から RCS ラットでは外顆粒層，視細胞内節，視細胞外節が消失していることがわかる(**A**：健常，**B**：RCS)．健常ラットではフラッシュ光に対する ERG(**C**)と上丘誘発電位(**E**)の反応が得られているが，RCS ラットでは ERG (**D**) と上丘誘発電位 (**F**) とも反応が得られなかった．STS に対する上丘の誘発電位は健常ラット(**G**)からも RCS ラット(**H**)からも反応が得られた．OS：視細胞外節，ONL：外顆粒層，INL：内顆粒層，IPL：内網状層，GCL：網膜神経節細胞層，スケールバー：100 μm(**A**, **B**)．刺激強度 10，30，60，80，100 μA(**G**, **H**)．矢印は刺激を与えた瞬間を表す．縦軸：20 μV，横軸：100 ms(**C**, **D**)，縦軸：100 μV，横軸：100 ms(**E**, **F**)，縦軸：100 μV，横軸：20 ms(**G**, **H**)．

回路は眼外の強膜上に設置し多極電極部のみab-externo手術で網膜下へ挿入される．これにより，2次コイルの面積を大きく設計することができるため最大1mAの出力が可能である．すでに彼らは試作機を作製し動物実験で安全評価試験を進めている．

2. 脈絡膜上-経網膜刺激方式

網膜上刺激方式と網膜下刺激方式は「多極電極が眼内に埋植され網膜に直接接触している」という特徴を有する．多極電極が接触した状態で網膜に電気刺激を行うことでより高解像度の画像を再現させることを目指している一方で，多極電極の埋植時に網膜へ損傷を与える危険性がある．

この問題を解決できる可能性をもった網膜刺激方式として脈絡膜上-経網膜刺激方式（suprachoroidal-transretinal stimulation：STS方式）が田野らによって考案され[17]，わが国のプロジェクトにて研究開発が進められている．STS方式では，多極電極を眼球「強膜半層切除した部位」または「脈絡膜と強膜の間」に設置し，参照電極を硝子体内に設置する（図2C）．そして，硝子体内に設置した参照電極との間で網膜を貫通するように刺激電流を通電する．多極電極が網膜と接触していない点および多極電極が眼外に設置される点がこの刺激法の大きな特徴である．多極電極が網膜と接触しないため，手術時の網膜への侵襲を低減できると期待できる．さらに，網膜貫通型の電流を用いるため，たとえ多極電極が網膜と離れていても効率的に局所刺激が可能になるのではないかと考えられる．また大きな多極電極を移植することができ広い視野を確保できる．全体のシステムとしてはHumayunのグループやRizzo & Wyattのグループのシステム同様，外部装置と内部装置の2つの装置から構成される．外部装置にはテレビカメラや信号処理回路が搭載され，内部装置には刺激回路や多極電極が搭載される．

STS方式は，まだ基礎研究の段階であるが，これまでのいくつかの研究から徐々に有効性が示されてきている．たとえば，STS方式の機能評価を網膜色素変性症モデル動物（RCSラット）視覚中枢から行った結果，低い刺激強度で限局した誘発電位を惹起できることが確認された[18]（図3）．また，昨年学内倫理委員会の承認を経て2例の網膜色素変性のボランティアに対して急性臨床試験を行い，STS方式により限局したelectrical phosphene が得られ，2点弁別が可能であることを見出した[19]．今後，空間分解能などの機能評価や安全性に関して研究を進めていく予定である．

おわりに

現在開発が進められている人工網膜の電極数は多くても数十極である．そのため，これらが仮にうまく機能したとしても高い解像度は望むことがむずかしい．

ただ，すでに実用化されている人工内耳においては，電極数は22極しかなく，埋め込み直後も患者は音声の認識率が低いものの，手術後長期間のリハビリテーションによって音声の認識率が飛躍的に伸びると報告されている．そのため，人工網膜埋め込み直後は再現できる視力は低くても，訓練やリハビリテーションによって視機能が高まっていくことも考えられる．また，今後人工網膜は，研究が進むことでより解像度を増した人工網膜が開発されていくことと思われる．近い将来人工網膜によって読書可能な程度の視機能を回復させることが可能となるかもしれない．

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PRECLINICAL INVESTIGATION OF INTERNAL LIMITING MEMBRANE STAINING AND PEELING USING INTRAVITREAL BRILLIANT BLUE G

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Purpose: To investigate the effects of intravitreal brilliant blue G (BBG) on the morphology and functions of the retina and its possible use for staining and peeling of the internal limiting membrane (ILM).

Methods: Rat eyes (n = 78) underwent gas compression vitrectomy. BBG solution was then injected into the vitreous cavity. The eyes were enucleated at 2 weeks and 2 months. Light as well as electron microscopy, terminal nick-end labeling staining, and electroretinography (ERG) were used to investigate retinal damage and function. To test the clinical potential of BBG, ILM staining was evaluated in primate eyes after pars plana vitrectomy followed by ILM peeling.

Results: In the rat eyes, no pathologic changes were observed with light microscopy. Electron microscopy revealed that high doses of BBG induced vacuolization in the inner retinal cells, but apoptosis was not detected. There was no reduction in the amplitude of the ERG waves. In the primate eyes, the ILM was clearly visualized after the intravitreous injection of BBG and was peeled off easily from the retina.

Conclusions: These results demonstrate that BBG, which has low potential for toxicity, high staining ability, and ease of handling, is a good candidate dye for ILM peeling.

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The internal limiting membrane (ILM) is the innermost layer of the retina. It forms a boundary between the vitreous and the retina. The ILM acts as a structural support for the Müller cells of the retina. Alterations in the structure of the retina due to cellular proliferation may cause distortion of the ILM, leading to the formation of epiretinal membranes (ERMs) and macular holes. Removal of the ILM can successfully alleviate these vitreoretinal diseases; however, difficulties in visualization of the virtually translucent ILM can present technical challenges in this procedure. It is now widely recognized that without surgical adjuvant it is extremely difficult to remove the membranes due to the poor visibility of the ILM and ERMs. Staining of the ILM is therefore one of the most important developments in surgery for such vitreoretinal diseas-

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es.[1–4] Development of indocyanine green (ICG) staining and trypan blue (TB) staining has greatly facilitated peeling of ILM and ERMs in the treatment of various vitreoretinal diseases, and as a result, these staining procedures are now widely used by many surgeons.[5–7] However, numerous clinical and experimental reports have recently suggested that intravitreal injections of ICG and TB can cause retinal damage.[8–21]

In a previous study, we screened various dyes, focusing on their safety and ability to stain membranes. The results of our screening demonstrated that brilliant blue G (BBG) capsular staining contributed to better visualization during continuous curvilinear capsulorhexis in pig eyes than the other dyes tested.[22] Furthermore, the standard staining dose of the dye resulted in no apparent complications and produced minimal changes on the corneal endothelium in rat eyes.[22] Because BBG has an excellent safety record from its use in corneal endothelium and capsular staining, we selected BBG as a potential dye for ILM peeling.

In the present study, we examined the effects of intravitreal BBG on the retina using morphologic and functional analyses in rat models. We also investigated the ability of BBG to stain the ILM of primate eyes.

Materials and Methods

All procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines for animal care produced by Kyushu University (Fukuoka, Japan).

Surgical Procedure for Intravitreal BBG in Rat Eyes

Brown Norway rats (78 males; age, 8 weeks; Kyudo, Fukuoka, Japan) were anesthetized with an intraperitoneal injection of ketamine hydrochloride at a dose of 75 mg/kg body weight. One eye from each animal (total of 6 per dose group) was vitrectomized using 0.05 mL of pure SF_6 gas as described previously.[8,23–27] After gas injection, 0.05 mL of BBG solution was injected into the vitreous cavity of each vitrectomized eye using a microscope for enhanced magnification during surgery. The BBG solution (Coomassie BBG 250; Sigma–Aldrich, St. Louis, MO) was prepared at concentrations of 0.01 mg/mL, 0.1 mg/mL, 1.0 mg/mL, and 10 mg/mL using dilution in intraocular irrigating solution (OPEGUARD-MA; Senjyu Pharmaceutical, Osaka, Japan) and sterilized through a 0.22-μm syringe filter. The mean osmolarity was determined using an osmotic pressure meter (OSMO STATION; Arkray, Kyoto, Japan), and the pH of each solution was determined for all concentrations prepared (Table 1). The final concentration was determined according to the ICG solution used in vitrectomies for humans (2.5–5.0 mg/mL),[8] to provide the rats with a safe dose of BBG that would also produce good staining. Twenty-four sham-operated eyes (injected with SF_6 followed by 0.05 mL of intraocular irrigating solution) were used as controls.

Table 1. Characterization of BBG Solution

Solution	Osmolarity (mosm/KgH_2O)	pH
Control*	298	7.33
BBG, 10 mg/mL	310	7.41
BBG, 1.0 mg/mL	300	7.42
BBG, 0.1 mg/mL	298	7.41
BBG, 0.01 mg/mL	298	7.41
Saline	285	7.40
BSS plus†	305	7.10

*OPEGUARD-MA (Senjyu Pharmaceutical, Osaka, Japan).
†Santen, Osaka, Japan.
BBG, brilliant blue G; BSS, balanced salt solution.

Light Microscopy

The eyes were enucleated and fixed in 10% paraformaldehyde on day 14 (n = 30; 6 per dose and control group) and at 2 months (n = 30; 6 per dose and control group) after surgery. Whole eyes were cut approximately along the vertical meridian. Paraffin-embedded sections were stained with hematoxylin–eosin, and each section was examined using light microscopy.

Transmission Electron Microscopy

The eyes were enucleated on day 14 and at 2 months after surgery and fixed in 1% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline. The specimens were postfixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon (Epon 812 Resin, CHIYODA JYUNYAKU INC., Tokyo, Japan). Ultrathin sections were cut from blocks and mounted on copper grids. The specimens were observed with a JEM 100CX electron microscope (JEOL, Tokyo, Japan).[28]

TdT-dUTP Terminal Nick-End Labeling (TUNEL)

Apoptotic cell death was detected using TdT-dUTP TUNEL as described previously.[29] A cryostat was used to produce 4-μm sections from samples fixed in 4% paraformaldehyde and embedded in paraffin. TUNEL staining was performed with the ApopTag

Fluorescein Direct In Situ Apoptosis Detection Kit (Intergen, New York) according to the manufacturer's protocol. The sections were costained with propidium iodide (Molecular Probes, Eugene, OR), thus allowing observation of the cell nuclei by a fluorescence microscope (Olympus, Tokyo, Japan). Ten sections from each eye specimen were selected at random and observed using the microscope.

Electroretinography (ERG)

After gas injection, 0.05 mL of BBG solution (1 mg/mL and 10 mg/mL) or intraocular irrigating solution was injected into the vitreous cavity. There were six rats in each dose group and six controls. At time points of 14 days and 2 months, the rats were kept in a dark room for one night, with only dim red illumination, and anesthetized with an intraperitoneal injection of 15 μL/g of body weight of saline solution containing ketamine (1 mg/mL), xylazine (0.4 mg/mL), and urethane (40 mg/mL). ERG was then performed as previously described.[8,27,30,31] The pupils were dilated with 2.5% phenylephrine hydrochloride and 1% tropicamide drops and showed maximal dilatation before ERG recording. The cornea was anesthetized with 1% proparacaine hydrochloride drops, and the rats were then placed on a heating pad throughout the experiment. A wire electrode, coated with 1% methylcellulose, was placed over the cornea for ERG recording. A similar wire electrode placed in the mouth served as a reference electrode, while a needle electrode inserted into the tail was grounded. The responses were differentially amplified (0.8–1,200 Hz), averaged, and stored using a computer. White (xenon) strobe flashes were presented in a Ganzfeld stimulator (VPA-10; Cadwell, Kennewick, WA) against an achromatic adapting field. Dark-adapted (rod-mediated) ERG was performed first to check the response stability at both intensities. Each rat was then adapted to dark background luminance for 20 minutes, a period sufficient to achieve a stable level of response. Thereafter, dark-adapted *a* (rod-mediated) ERG and dark-adapted *b* (bipolar and Müller cell–mediated) ERG were performed at a flash luminance of 1.30 log cd s/m^2. The responses to five successive flashes at an interstimulus interval of 1 minute were then averaged to determine the dark-adapted responses. The rats were then exposed to a white light–adapting field (1.50 log cd/m^2) for at least 25 minutes, and then light-adapted *b* (cone-mediated) ERG was performed at a flash luminance of 1.30 log cd s/m^2 (rod-desensitized condition in rats). The responses to 50 successive flashes made at 2 Hz were averaged. The results of the ERG amplitudes were evaluated using the Student's *t*-test, and $P < 0.05$ was considered statistically significant.

ILM Staining by BBG in Primate Eyes

Because ILM peeling is impossible in rat eyes, we examined the ability of BBG to stain the ILM in primate eyes. Two eyes from two cynomolgus monkeys 3 years of age were used in this study. The animals were restrained in a squeeze cage and injected intramuscularly in the thigh with 20 mg/kg ketamine hydrochloride (Sankyo Yell Pharmaceutical Products, Japan) for general anesthesia. The monkeys were subsequently transported to an operating department. Surgery consisted of standard three-port pars plana vitrectomy with induction of a posterior vitreous detachment by suction with a vitrectomy cutter using triamcinolone acetonide injection for vitreous visualization.[32–35]

Ten milligrams of BBG was dissolved in 20 mL of intraocular irrigating solution and sterilized with a syringe filter. The final concentration of BBG was 0.5 mg/mL. The prepared BBG solution (0.5 mL) was then injected gently into the vitreous cavity and washed out immediately with balanced salt solution (BSS plus; Santen, Osaka, Japan). Removal of the ILM was performed using ILM forceps. The instruments were then removed, and the sclerotomy ports were closed using 7-0 polygalactin sutures. Postoperative examinations included slit-lamp microscopy and ophthalmoscopy on days 1, 3, and 14. Fluorescein angiography was performed on day 14.

Results

Characterization of the BBG Solution

BBG is also known as acid blue 90 and Coomassie BBG. Aside from our previous publication, to our knowledge, there are no reported studies investigating the toxicity of BBG for ophthalmic use.[22] Table 1 shows the osmolarity and pH of the BBG solution at different concentrations. The osmolarity and pH of BBG were found to be similar to those of intraocular irrigating solutions.

Effect of Intravitreal BBG on the Retina

Light Microscopy

After intravitreal injection of BBG, no toxic effects of BBG, such as corneal edema, severe retinal edema, or endophthalmitis, were observed by surgical microscopy over a period of 2 months. The eyes were enucleated on day 14 and at 2 months after surgery. The normal structure of the retina was preserved in the

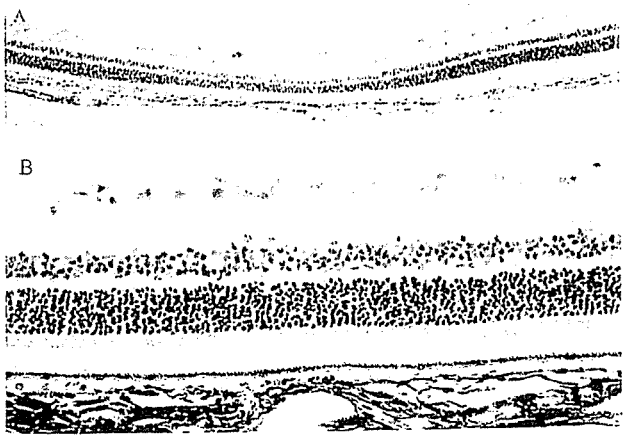

Fig. 1. Light microscopic photography of rat eyes injected with intravitreal brilliant blue G (10 mg/mL; 0.05 mL per eye) visualized at 14 days. No significant change in the retinal construction was observed (original magnification: **A**, ×200; **B**, ×400).

eyes injected with the highest doses of BBG (10 mg/mL) both on day 14 and at 2 months. In addition, no infiltration of inflammatory cells was observed (Fig. 1; day 14). The normal structure of retina was also retained in the groups injected with lower doses of BBG, and no sign of cellular degeneration was observed in the sections on day 14 or at 2 months.

Transmission Electron Microscopy

Some specimens injected with the highest dose of BBG (10 mg/mL) showed vacuolization in the ganglion cells and Müller cell processes of nerve fibers both on day 14 (Fig. 2A; day 14) and at 2 months. Although the same changes were also found in the group injected with 1 mg/mL BBG, the grade of vacuolization was less than in the 10 mg/mL group (Fig. 2B). Vacuolization was not observed in the groups receiving lower doses or in the controls. Among all groups, no remarkable changes were observed in the retina, including the inner nuclear, outer nuclear, and retinal pigment epithelial cell layers.

Apoptotic Cell Death Detected by TUNEL

Because there have been several recent reports regarding damage of the retinal cells by ICG and TB dependent on apoptosis,[16–21] we investigated apoptotic cell death by TUNEL. In the group administered the highest doses of BBG (10 mg/mL), 1 case of apoptotic cell death was observed from among 10 sections. However, the apoptotic cell ratio was not significantly different from that observed in control sections (Fig. 3; day 14). In groups injected with lower doses of BBG, no TUNEL staining was observed in the retina on day 14. Furthermore, retinal cells in all BBG dose groups had not undergone apoptotic cell death after 2 months.

ERG

To evaluate the retinal function after BBG injection, we also performed ERG in the high-dose groups (1.0 mg/mL and 10 mg/mL). The absence of cataracts was confirmed in all rats before measurements were taken. Figure 4A represents the dark-adapted and light-adapted ERG waveforms of the responses of the control and high-dose groups on day 14. The ampli-

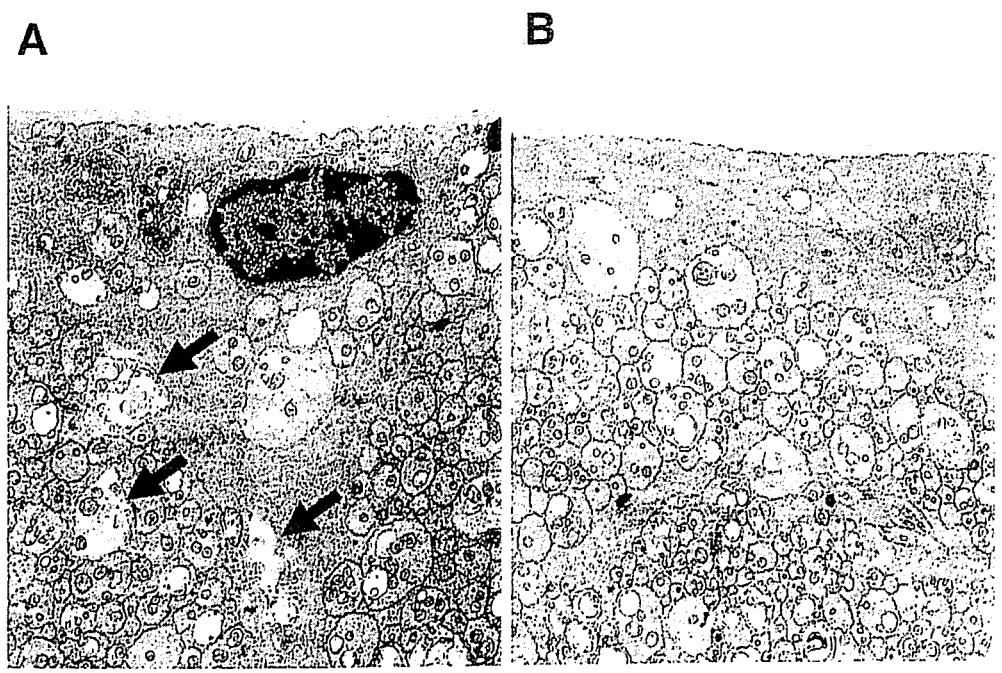

Fig. 2. Transmission electron microscopic photography of the rat eyes injected with intravitreal brilliant blue G (10 mg/mL and 1 mg/mL; 0.05 mL per eye) visualized at 14 days. In the highest dose group (10 mg/mL), arrows show vacuolization in the ganglion cells and Müller cell processes of some specimens at day 14 (A). Although the same changes were also found in the 1 mg/mL group (B), the grade of vacuolization was less than in the 10 mg/mL group (original magnification, ×2,000).

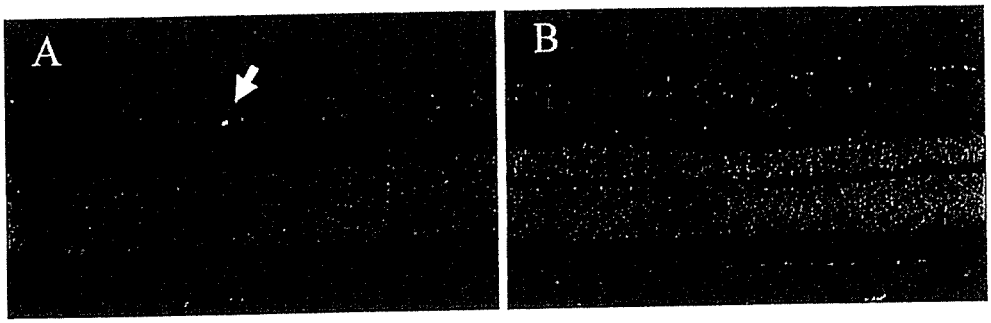

Fig. 3. Apoptotic cell death detected by terminal nick-end labeling. In the highest dose (10 mg/mL) group, 1 case of apoptotic cell death was observed in 10 sections (**A**; day 14); however, the apoptotic cell ratio was not significantly higher than that of the control specimens (**B**; day 14) (original magnification, ×400).

tudes of dark-adapted responses obtained at the beginning of the experiments showed a low variability between groups. Although a slight reduction in the mean maximal amplitude of the dark-adapted *a* waves (Fig. 4B; day 14) on day 14 was observed in a dose-dependent manner in the high-dose groups, there was no significant difference in the maximal wave amplitude compared with the controls (10 mg/mL, $P = 0.054$; and 1.0 mg/mL, $P = 0.063$; *t* test). In addition, dark-adapted *b* waves (10 mg/mL, $P = 0.553$; and 1.0

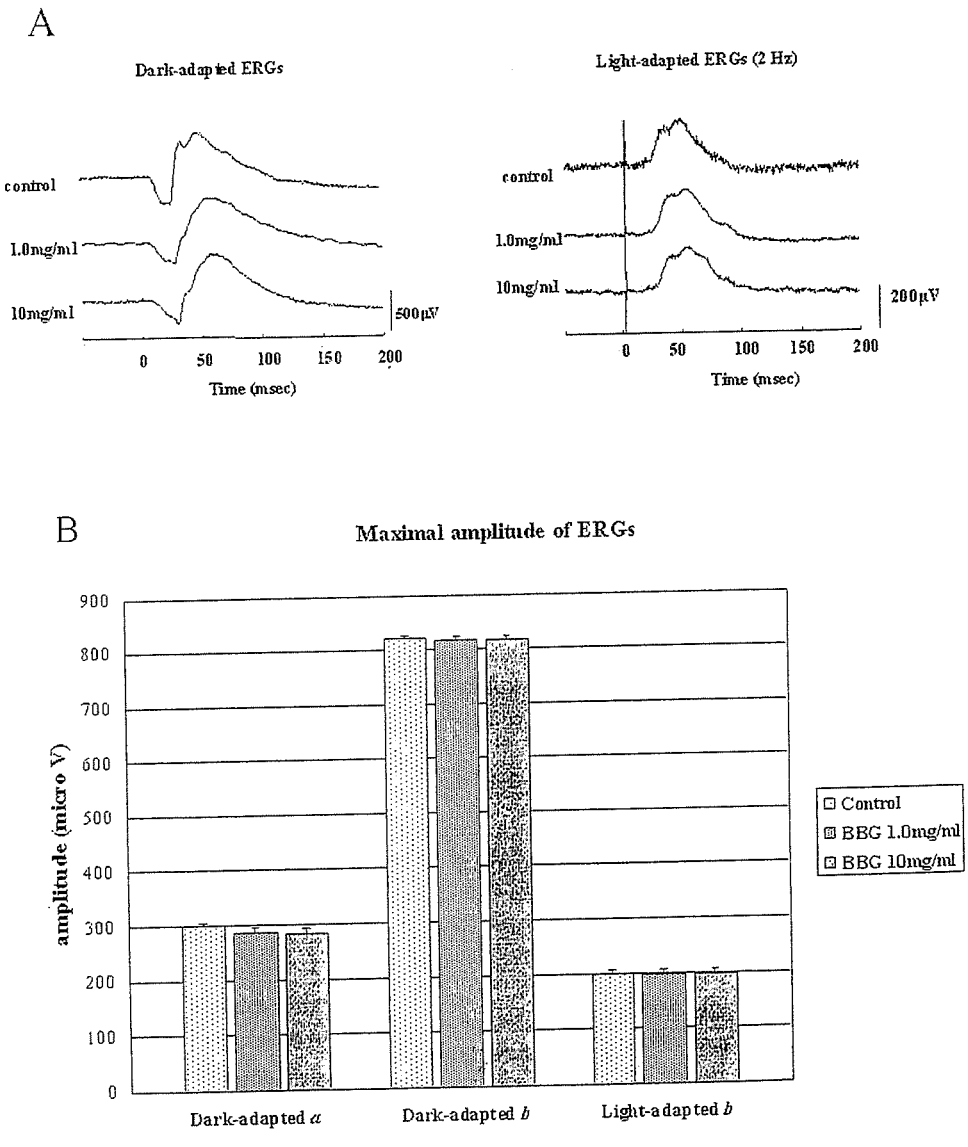

Fig. 4. Waveforms and maximal amplitude of electroretinograms (ERGs) in rat eyes. **A**, ERG traces showing the dark-adapted responses (**left**) and the light-adapted responses (**right**) of the control and intravitreal high-dose brilliant blue G (BBG) injection groups at 14 days after injection. All waves were clearly recorded. Although a slight reduction in the mean maximal amplitude of the dark-adapted *a* waves was observed in a dose-dependent manner on day 14, there was no significant difference when compared with the control group (10 mg/mL, $P = 0.054$; and 1.0 mg/mL, $P = 0.063$; *t* test). Dark-adapted *b* waves and light-adapted *b* wave ERGs demonstrated no remarkable reduction. There was no statistically significant difference between the amplitudes (**B**; day 14). Data are expressed as mean ± SEM of the amplitude as compared with the control group.

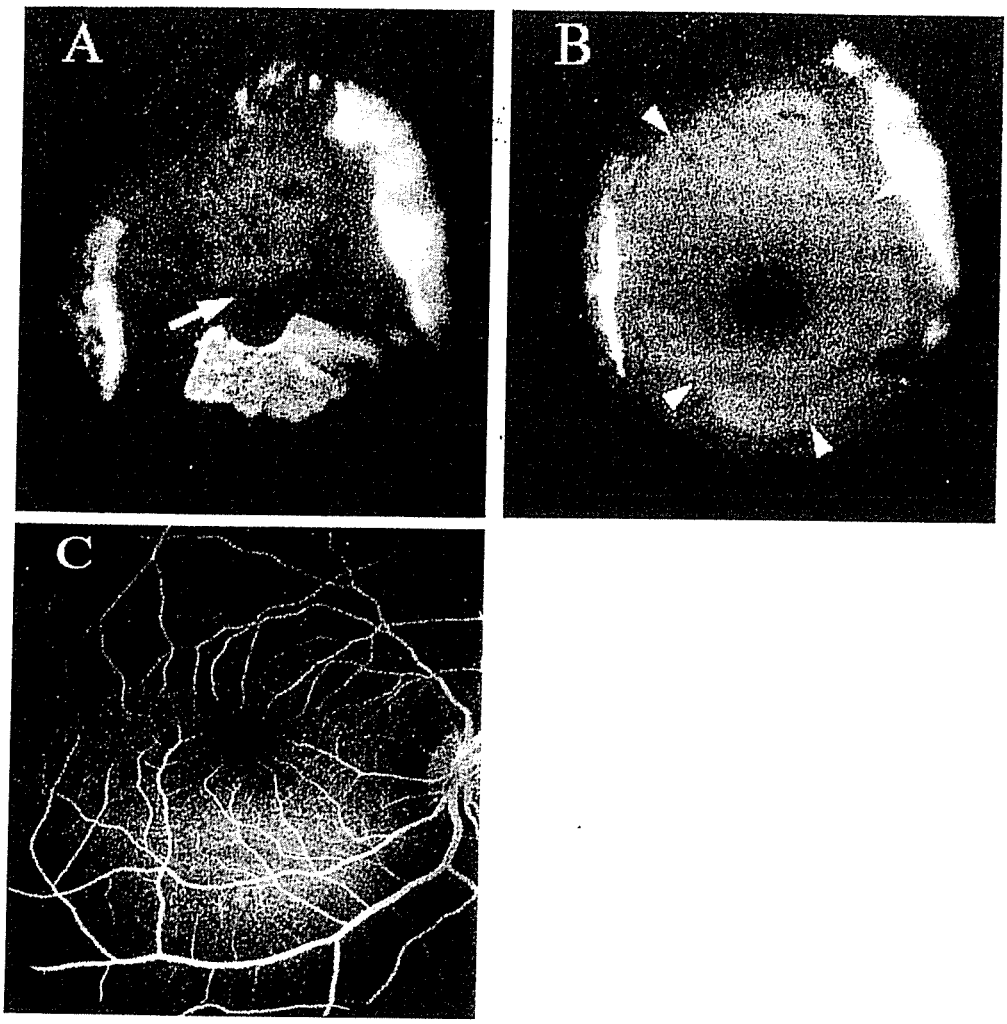

Fig. 5. Internal limiting membrane (ILM) staining by brilliant blue G (BBG) in primate eyes. The ILM was visualized by intravitreal injection of BBG. The arrow shows the ILM being removed from the retina with ILM forceps (A). The triamcinolone granules were trapped in the gel structure of the residual vitreous. After ILM removal, the difference in the retinal surface color between the area from which the ILM had been removed (arrowheads) and the surrounding area was clearly visible (B). No adverse effects of BBG on the retina were observed by fluorescein angiography (C; day 14).

mg/mL, $P = 0.508$; t test) and light-adapted b wave ERG (10 mg/mL, $P = 0.451$; and 1.0 mg/mL, $P = 0.550$; t test) demonstrated no remarkable reduction, with no statistically significant difference between the amplitudes (Fig. 4B; day 14). After 2 months, ERG recordings in the same dose groups (1.0 mg/mL and 10 mg/mL) were measured, and reduction of the amplitude of the dark-adapted a waves was found to recover in a manner similar to that in the control group.

BBG-Assisted ILM Peeling and Postoperative Examinations in Primate Eyes

The prepared BBG solution (0.5 mL) was injected gently into the vitreous cavity and washed out immediately with balanced salt solution. After irrigation of the vitreous cavity, the ILM was stained a light blue color. The edge and flap of the ILM were clearly visible during ILM peeling (Fig. 5A). The circular area underlying the ILM was clearly visible after ILM peeling (Fig. 5B).

Postoperatively, toxic effects of BBG, such as a corneal edema, severe retinal edema, and endophthalmitis, were not observed during slit-lamp microscopy and ophthalmoscopy at day 14. Fluorescein angiography also revealed that there was no apparent retinal damage by BBG on day 14 (Fig. 5C). Further ophthalmoscopic examinations showed no further changes in the retina during the 6-month follow-up period.

Discussion

The use of dyes such as ICG and TB has become a popular method to facilitate removal of the ILM and ERMs for treatment of various vitreoretinal diseases.[1–4] This technique has enabled surgeons to perform ILM and ERM peeling procedures with improved safety and ease.[5–7] However, adverse effects of these dyes on the retina have been widely reported in recent years.[8–21] Our previous studies have also shown the adverse effects of intravitreous ICG on the retina,[8] demonstrating that retinal damage can be caused by

phototoxicity[9] and the osmolarity[10] of ICG. TB, the other dye frequently used to assist in membrane peeling, is inferior to ICG in its ability to stain the ILM, and the surgical technique is also complicated by the need for fluid–gas exchange.[3,7] Furthermore, TB has also been recently reported to have a toxic effect on retinal cells.[14,20,21]

To select a suitable candidate for staining the ILM, we screened various dyes that were superior to ICG and TB in terms of their safety and membrane staining potential. Recently, we reported that BBG capsular staining contributed to better visualization during continuous curvilinear capsulorhexis in pig eyes.[22] BBG is a blue dye (color index 42655) with the formula $C_{47}H_{48}N_3O_7S_2Na$ (molecular weight, 854.0) that is also known as acid blue 90 and Coomassie BBG. BBG has been used for protein staining in biologic fields, because it binds nonspecifically to virtually all proteins. It is also used as a protein electrophoresis dye. The standard staining dose of the dye produced no apparent complications, with minimal changes in the corneal endothelium of rat eyes.[22] Furthermore, the staining ability of BBG solution is similar to that of ICG, thereby enabling a relatively simple surgical procedure to be performed.[22] The staining mechanism of BBG at the ILM still remains unknown. However, to the best of our knowledge, there have been no reports examining the clinical use of BBG in humans. The pharmacological function of the BBG still remains unconfirmed. However, although there are no reports on the medical use of this dye, there is a long history of biologic use in which no apparent toxicity has been reported. We therefore performed this study to investigate the possible use of BBG for safer membrane peeling in human eyes, because BBG is for experimental use only at present.

In our previous study, high doses (2.5 mg/mL and 25 mg/mL) of intravitreous ICG were found to cause morphologic damage in the rat retina when observed by light microscopy. In groups injected with low doses (0.025 and 0.25 mg/L), there was no apparent histologic damage, but the amplitude of the dark-adapted *b* waves decreased in a dose-dependent manner, producing a significant difference when compared with the controls. Similar findings were also observed in the rat eyes at 2 months after injection.[8] In the present study examining the safety of intravitreal BBG, the normal structure of the retina was preserved with all doses tested, both in groups enucleated on day 14 and at 2 months when examined using light microscopy. However, transmission electron microscopic observations revealed that the high-dose groups (10 mg/mL and 1 mg/mL) showed vacuolization in the ganglion cells and Müller cell processes in the nerve fiber layer. Because there have been several reports suggesting that damage of the retinal cells by ICG and TB is dependent on apoptotic cell death,[16–21] we investigated apoptotic cell death by TUNEL. Even in the group injected with the highest dose of BBG (10 mg/mL), however, no apparent apoptotic cell death was detected in the retina. In addition, we performed ERG to investigate retinal function. A slight reduction in the maximal amplitude of the dark-adapted *a* waves was observed in a dose-dependent manner on day 14 in the high-dose groups (1.0 mg/mL and 10 mg/mL); however, the difference between the high-dose and control groups was not significant. Because these changes had recovered to a level similar to that of the control group 2 months later, the influence of physical problems such as the high osmolarity of the high-dose BBG solution or technical problems of the ERG or operative stress were considered to cause the temporal dysfunction. From these results, we concluded that BBG has a better biocompatibility than ICG.

Because ILM peeling is impossible in rat eyes, we examined the ability of BBG to stain the ILM in primate eyes. After injecting 0.5 mg/mL BBG solution into primate eyes, the ILM was instantly stained light blue and was clearly visible. We were then able to easily remove the ILM with ILM forceps. Fluorescein angiography on day 14 also demonstrated that there was no apparent damage to the retina of primate eyes.

BBG also has a number of advantages over both ICG and TB in terms of handling. ICG is packaged as lyophilized powder and will not dissolve properly in intraocular irrigating solution alone. It therefore must be diluted in 0.5 mL of aqueous solvent before adding 4.5 mL of intraocular irrigating solution. BBG granules, by contrast, can be easily dissolved in intraocular irrigating solution alone and subsequently sterilized with a 0.22-μm syringe filter. The osmolarity and pH of the BBG solution are also very stable. Because BBG is not a fluorescent dye, the presence of light toxicity such as that found in ICG is highly unlikely. Furthermore, the staining process requires no additional techniques such as fluid–gas exchange that is necessary for TB application.

Although the surgical results of ILM or ERM peeling have been improved with the aid of ICG or TB staining,[1–7] surgeons should be aware of the possible adverse effects of these dyes when treating vitreoretinal diseases. The BBG concentration required for ILM staining in primates (0.5 mg/mL) is one tenth of that required for ICG (5 mg/mL). Further investigation is necessary before any clinical recommendation can be given. From our results, we conclude that BBG, which has low potential for toxicity, high staining ability,

and ease of handling, is a good candidate dye for ILM peeling.

Key words: brilliant blue G, vitrectomy, internal limiting membrane peeling, preclinical investigation, retinal function, retinal damage.

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BRILLIANT BLUE G SELECTIVELY STAINS THE INTERNAL LIMITING MEMBRANE/BRILLIANT BLUE G–ASSISTED MEMBRANE PEELING

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Purpose: To report the use of the dye brilliant blue G (BBG) for staining of the internal limiting membrane (ILM) during macular hole (MH) and epiretinal membrane (ERM) surgery.

Methods: This study was designed as an interventional, noncomparative, prospective, clinical case series. Twenty eyes from 20 consecutive patients with MH or ERM underwent BBG-assisted ILM and ERM removal. In MH cases, a posterior vitreous detachment was created, followed by the injection of 0.25 mg/mL BBG solution into the vitreous cavity and immediate washout of the BBG. This technique improved visualization of the ILM, enabling peeling and surgery to be performed successfully. However, in ERM cases, staining of the ERM could not be confirmed at this concentration. Finally, the ILM including the ERM was removed in all cases. Preoperative and postoperative ophthalmic examinations were performed.

Results: Postoperatively, 17 patients (85%) had visual acuity improved by at least 2 Snellen lines. No adverse effects were observed postoperatively during the observation period (mean follow-up ± SD, 7.3 ± 1.0 months).

Conclusions: BBG selectively stains the ILM. This technique can facilitate the management of MH and ERM surgery without any adverse effects, as was shown in this short-term study.

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Indocyanine green (ICG) staining and trypan blue (TB) staining have greatly facilitated internal limiting membrane (ILM) peeling in various vitreoretinal diseases.[1–6] However, numerous reports have emerged regarding retinal damage caused by ICG and TB in both experimental models and clinical use.[7–16] A dye with both satisfactory staining ability at low concentrations and minimal toxicity is required for effective membrane staining. We have screened various dyes focusing on their safety and ability to stain membranes during vitrectomy. From the results of our preclinical studies, we reported that brilliant blue G (BBG) membrane staining contributed to better visualization in continuous curvilinear capsulorhexis and ILM peeling in animal eyes.[17,18] The satisfactory staining dose of the dye provided no apparent complications, with minimal changes on the corneal endothelium and retinal cells of rat eyes.[17,18]

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