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網膜ニューロンの緑内障性障害　ーそれに対する保護と再生—に関する研究

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研究要旨：

昨今、基本的な生命現象のみならず疾患における病態の解明においても遺伝子改変動物存在が多く貢献している。我々は緑内障による網膜神経細胞死のメカニズムを探求するにあたり、アポトーシス機構の解明、生体下での評価、それぞれに有用であると思われる遺伝子改変マウスを用いて研究を行なった。以下にプロジェクトを示した。

１、　緑内障モデルマウスにおける、NMDA 受容体 ε 1 サブユニットの役割の解明

２、　蛍光蛋白発現マウスを用いた、生体下網膜神経節細胞死評価法の確立

プロジェクト１：緑内障モデルマウスにおける、NMDA受容体 ε 1サブユニットの役割の解明

A. 研究目的

NMDA受容体 ε 1サブユニットの網膜における機能の解析と網膜神経節細胞死への関与の解明を目的とする。 ε 1サブユニットノックアウトマウスを用い臨床的緑内障に近いモデルマウスを作製し、 ε 1サブユニットの神経保護作用が認められれば、新しく緑内障治療に結びつくことができるのではないかと考えている。

B. 研究方法

マウス眼圧測定法の確立。従来の眼圧測定機器は人間を対象としているため、角膜径が約4mmのマウスに流用することは不可能である。そこで、内径0.04mmのガラス管を用いて水銀圧力計を作製した。

慢性眼圧上昇モデルの作製と評価。房水流出経路の一つである上強膜静脈をナイロン糸にて結紮することで慢性眼圧上昇モデルを作製した。眼圧上昇処置後、1ヶ月、3ヶ月後にマウス眼球を摘出し、網膜切片および視神経割断切片を作製した。網膜各層の細胞数や視神経髄鞘数を測定し、その変化を統計学的に評価した。

この慢性眼圧上昇モデルにおける網膜神経細胞死に、NMDA受容体が関与しているのかを明らかにするために，NMDA受容体を構成しているサブユニットの一つであるGluRε1 (NR2A) サブユニットが欠損したマウスに先述の上強膜静脈血流遮断法を適用し，この分子の役割を組織学的に検討した。

C. 研究結果

マウスの眼圧を測定するため、水銀マノメーターを作製した．マノメーターの較正をするために，摘出した眼球を用いて負荷した静水圧を正確に測定できることを確認した．このマノメーターで測定したC57BL / 6マウスの眼圧は12.9±0.3mmHgであった．

慢性的な高眼圧を引き起こすために，耳上下側2方向の上強膜静脈の血流を遮断した．その結果，遮断3日後に眼圧は23.7±1.5mmHgと上昇した．血流遮断後3日、1週、2週、4週の時点で正常より有意な上昇を示した．遮断後8週，12週，16週，20週では血流遮断前と有意な差を認めなくなった．この結果，眼圧を少なくとも4週にわたって20mmHg以上に保持することができた．

この慢性的高眼圧による病理学的な変化を検討した．遮断4週後の網膜において，網膜神経節細胞の脱落や核の変性と思われる濃縮像と，内網状層の菲薄化を認めた．この病的変化を定量的に測定するため，網膜を中心部と周辺部に分け各領域の細胞核数を測定した．RGC数は，遮断4週後において無処置眼と比して中心部88.3.%，周辺部71.8%と共に減少が認められた．高

眼圧による病理学的な変化がほとんど認められないとされているONC数を用いたRGC / ONCの比較では，中心部での減少率86.0%，遠位部74.6%となりRGC数と同様の結果が得られた．また，INC数に大きな変化はなかった．遮断12週後は，４週後と比してRGC数に有意な減少を認めなかったものの，残存した細胞核に膨潤や萎縮など変性したものが多く認められた．INC数の減少は認めなかったが，内網状層の厚みは明らかに減少しており，網膜全体の変性が進んでいることが観察された．RGC / ONC では中心部82.8%，周辺部で72.0%の減少率を示した．さらに，球後視神経の横断面では，強拡大において有髄神経の減少が認められた．

NMDA受容体が慢性的な高眼圧によって惹起される網膜神経細胞死に関与するかを調べるため，NMDA受容体GluRε1（NR2A）サブユニットが遺伝的に欠損したマウスに，先述の上強膜静脈血流遮断法を適用した．遮断4週後の眼圧は20.1±1.1mmHgであり野生型マウスの値と差はなかったが，組織学的な変性はほとんどを認められなかった．一方，遮断12週後になると眼圧は14.9±1.2mmHgと野生型マウスと同様であったが，遮断4週後では認められなかった網膜神経節細胞の変性脱落や内網状層の厚みの減少が認められた．この変化を細胞数で見ると，GluRε1ノックアウトマウスでは，遮断4週後の眼圧上昇群と対照群の間で差が認められない．しかし，遮断12週後では，網膜神経節細胞核の膨潤や萎縮などの変性像が多く認められ，内網状層の厚みの減少もおこっており野生型の病変と同様の結果となった．RGC / ONCは，中心部85.0%，周辺部75.9%と減少しており，野生型マウスと差が認められなかった．したがってNMDA受容体GluRε1サブユニットの欠損は，4週間までの細胞死を防ぐことはできるが，それ以後の病変には関与しなかったと考えられた．

D.　考察

緑内障病態によって生じる網膜神経細胞死の原因の一つに、興奮性アミノ酸であるグルタミン酸の興奮毒性の関与が考えられている。これに対し、これまでグルタミン酸の硝子体内投与や、グルタミン酸に対する阻害剤の投与などによる間接的な研究がなされてきている。しかし、グルタミン酸に対する受容体についての直接的な研究の報告がなく、その詳細な解析は神経細

胞死のメカニズムの解明に不可欠であると考えられる。本研究は、グルタミン酸受容体であるNMDA受容体のサブユニットをノックアウトしたマウスを用いて慢性眼圧上昇モデルを作製し、その網膜の組織学的な解析から受容体が網膜神経細胞死に関与していることを明らかにした。現在まで用いてきた眼圧測定法はマウス前房内に針を挿入して測定するために、同一眼での測定は一回のみである。そこで、同一個体の眼圧を連続して測定するために、マウス用のTonoLabトノメーターを用いて眼圧を測定するべくその精度を予備実験にて測定中である。

E．　結論

緑内障における視神経障害の発症・進展における神経細胞死のメカニズムの一端がグルタミン酸であることは既に明らかではあるが、その分子機構を解明することは、将来的な抗緑内障薬の開発に不可欠なものである。本研究によってグルタミン酸受容体の一つであるNMDA受容体が、神経細胞死に主要な役割を果たしていることが直接的に示され、さらにその中でもε1サブユニットが重要な働きを持つことが証明された。選択的ε1サブユニット阻害剤の開発により、緑内障における網膜神経節細胞死に対する保護効果が期待される。

F.健康危険情報

特記すべきことなし

G.研究発表

未

H.知的財産権の出願・登録状況

なし

プロジェクト２　　蛍光蛋白発現マウスを用いた、生体下網膜神経節細胞死評価法の確立

Ａ．研究目的

緑内障は視野欠損を主症候とする慢性進行性疾患であり、組織学的には網膜神経節細胞死に伴う視神経脱落が認められる。緑内障の原因解明には網膜神経節細胞死の生ずるアポトーシスメカニズムの解明が必要である。この点において、動物実験による網膜神経節細胞死の評価が必要となるが、従来の評価方法では同一固体での経時的変化が追えず、定量性が低いと考えられ、より良いモデルマウス作製が必要と考えた。このような背景から、本研究では網膜神経節細胞膜表面抗原であるThy-1に蛍光物質を遺伝子導入し、網膜神経節細胞死を評価するための新たなモデルマウスの作製を行い、そのモデルマウスを用い、網膜神経節細胞死の新たな評価系を確立する事を目的とした。

Ｂ．研究方法

①　プラスミドベクターの作製

ｔｈｙ１．２を含む既存のプラスミドベクターからｔｈｙ１．２のcording regionを制限酵素で切り出し、ｔｈｙ１．２のプロモーター領域の下流にPCRで増幅したEGFP(蛍光発色蛋白)cording regionを導入し、新たなベクターを作製する。そのベクターをマウス卵細胞にmicroinjectionした。

②　Tgマウスゲノム解析

離乳子の尻尾よりゲノム抽出を行い、PCR及びsouthern blotting法にて、導入遺伝子発現の確認を行った。Thy1-EGFP遺伝子導入が確認されたマウスを育成し、蛍光顕微鏡にて網膜神経節細胞の蛍光発色を組織学的手法で確認した。網膜神経節細胞のみが発色した系統を確立し、以後の実験に使用した。

③　障害モデルによる検証

網膜神経節細胞障害モデルのひとつである虚血再灌流モデルを作製し、その眼底蛍光をScanning

Laser Opthalmoscope (SLO)を用いて生体下で経時的に撮影し、その眼底蛍光輝度の変化と網膜神経節細胞死の関係を検討した。

動物実験に際してはAssociation for Research of Vision and Ophthalmology (ARVO)決議を順守し充分な倫理的配慮を加えた。

C. 研究結果

網膜神経節細胞特異的マーカーであるｔｈｙ１．２遺伝子を含むプラスミドベクターを作製し、培養細胞にて本ベクターの蛍光発色を確認した。

このベクターをマウス卵細胞にmicroinjectionし、トランスジェニックマウスを作製した。42匹の産仔の尻尾よりゲノム抽出を行いPCRにて、導入遺伝子発現の確認を行った。7匹のファンダーマウスを得ることができ、そのうち3系統で目的の網膜神経節細胞の蛍光発色が組織学的に確認できた。2系統でほぼ全ての網膜神経節細胞の蛍光発色を、1系統で一部の蛍光発色を認めた。

本トランスジェニックマウスに４５分間の一過性虚血を負荷した。虚血解除後４日後から著明に眼底蛍光が減少し、７日後ではさらに減少した。１４日後は７日後と比較して著変は認められなかった。眼底蛍光を定量的に評価するため、虚血負荷前をコントロールとしてSLO画像の蛍光輝度を計測した。蛍光輝度は虚血解除後４日後で約６５％、７日後では約４０％と統計学的に有意な減少を示し、それ以後１４日後まで変化はなかった。

また、虚血再灌流モデルにおける網膜神経節細胞の組織学的変化を定量化するため、網膜各層の細胞数を計測し、網膜神経節細胞/外顆粒細胞X100 (GCL/ONL比) を算出し、網膜神経節細胞への障害の指標とした。その結果、GCL/ONL比は、虚血負荷前が4.85±0.4であったのに対し、虚血解除後4日で2.73±0.68と統計学的に有意な減少を認め ($p<0.05$) 、7日後、14日後では1.05±0.23、1.10±0.29にまで減少した。

この結果をSLO画像の蛍光輝度の減少と比較検討すると、7日以後の減少率に軽度の差が認められるものの、両者の変化には同様の傾向が認められた。

D. 考察

今回の実験において、マウスthy1.2遺伝子を用いることで、網膜神経節細胞が特異的に標識されるトランスジェニックマウスを作製することができた。トランスジェニックマウス系統ごとに様々なGFP発現パターンが確認されたが、ほぼ全ての網膜神経節細胞のみが蛍光発色するトランスジェニックマウス系統を確立し、実験に使用した。

虚血再灌流モデルは網膜神経節細胞死を誘導するモデルとしてよく用いられる。このモデルは眼内圧を上昇させ、網脈絡膜循環を完全に遮断し網膜全層に虚血を負荷することで網膜からのグルタミン酸の遊離が急上昇し、このグルタミン酸がNMDA受容体を介して網膜内層に分布する神経細胞に遅発性の障害を与えると考えられている。このモデルにおいて、Thy1.2-GFPトランスジェニックマウスの眼底蛍光輝度の減少は、虚血解除後4日で約65%、7日以後で約40%であったのに対し、組織学的な網膜神経節細胞の低下は4日後で約55%、7日以後で約20%であった。両者は同様の減少傾向を示したが、眼底蛍光輝度の減少が組織学的変化と比較して若干少なくなった。この両者の差は、蛍光発色していた網膜神経節細胞が変性し細胞死を起こした後も、そのGFP蛋白の残骸をマイクログリアが貪食するまで蛍光が残存するために生じていると考えられる。今回報告する網膜神経節細胞死の新たな評価法の特徴は、非侵襲的に且つ生体下で繰り返し同一部位の網膜神経節細胞を観察できる点である。従来の評価法は、組織学的手法にしても生化学的手法にしても抜眼が必須であるため、多くのサンプルを必要とした上に、同一個体での経時的変化を測定することは不可能であった。本研究での方法では慢性眼圧上昇モデルや神経保護作用を持つ薬剤の評価など、長期間のフォローが必要な実験にも対応できる点が優れていると考えられる。

また、従来の評価法では網膜神経節細胞の同定のため、逆行性標識のため上丘への蛍光色素注入といった侵襲的操作が必要であり、間接的な影響も無視できなかった。加えて、生体下で網膜神経節細胞死が評価できるため、同じ組織を他の組織学的もしくは生化学的実験に用いることも可能となる。

近年、本法同様に蛍光顕微鏡を用いた生体下での網膜神経節細胞の観察も報告されているが、これらはラットを用いた手技である。ラットと異なり、マウスではトランスジェニックマウスを含め、数多くの遺伝的背景が明確な系統の利用が可能である。今後、各種ノックアウトマウスと本トランスジェニックマウスとの交配により、特定の遺伝子の網膜神経節細胞死への関与の解析が大いに期待できる。

以上のことから、本トランスジェニックマウスを用いた網膜神経節細胞死の新たな評価法は今後の緑内障をはじめとする網膜神経節細胞死を誘導する眼疾患の研究において有用であると思われる。

E. 結論

網膜神経節細胞の特異的マーカーであるthy1遺伝子のプロモーター下流にGFPを発現させ、網膜神経節細胞が特異的に標識されるトランスジェニックマウスを作製した。

本マウスの眼底蛍光はSLOで生体下の経時的観察が可能であり、虚血再灌流モデルにおける眼底の蛍光輝度の減少は、組織切片における網膜神経節細胞数の減少と同様の傾向を示した。

この結果より、本トランスジェニックマウスを用いた眼底蛍光撮影は網膜神経節細胞死の長期評価の経済的かつ有用な方法となりうる。

F.健康危険情報

特記すべきことなし

G. 研究発表

平成15年5月　ARVO meeting Florida USA.

Live imaging of retinal ganglion cells in transgenic mice expressing GFP as an index of RGC damage under anesthetized mice

H.知的財産権の出願・登録状況

なし

別紙４

研究成果の刊行に関する一覧表

書籍
該当なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yanagi Y, Inoue Y, Kawase Y, Uchida S, Tamaki Y, Araie M, Okochi H	Properties of growth and molecular profiles of rat progenitor cells from ciliary epithelium.	Exp Eye Res.	82	471-478	2006
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Properties of growth and molecular profiles of rat progenitor cells from ciliary epithelium

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Abstract

Recent studies have demonstrated that multipotent retinal stem or progenitor cells can be isolated from the ciliary epithelium (CE) of the eye using a neurosphere culture. In this study, we investigated the properties of growth and differentiation, and molecular profiles of rat adult ciliary epithelium (CE)-derived retinal progenitors and forebrain (FB) derived neurospheres. Under clonogenic culture conditions, we found that the CE-derived neurospheres contained fewer undifferentiated cells compared with the FB-derived neurospheres, and that CE-derived neurospheres initially expressed the set of Notch pathway molecules genes including Notch 1 and Delta 1, HES-1 and HES-5, but partially lose their expression after passaging. Furthermore, we found that the CE-derived neurospheres did not express several markers for in vivo embryonic retinal progenitors. Additionally, when the eye was divided into four subregions along its dorsoventral and nasotemporal axes and progenitor cells were obtained from the subregions, the progenitor cells did not express the subregion specific transcription factors, suggesting that subregional specificity is not maintained in vitro. Together, our results demonstrate that CE-derived progenitor cells may have intrinsic limitations in the production of cell types.

Keywords: retinal cell differentiation; Retinal cell lineages; Ciliary epithelium; Retinal cell culture

1. Introduction

Recent studies have demonstrated that multipotent retinal stem or progenitor cells can be isolated from the ciliary epithelium (CE) using a neurosphere culture (Ahmad et al., 2000; Tropepe et al., 2000). A study using mice (Tropepe et al., 2000) demonstrated that one in 500 CE cells give rise to a neurosphere consisting of ~12 000 cells with retinal neural properties and that an individual neurosphere contains cells which can generate six to eight daughter neurosphere colonies. This may imply that a smaller number of the daughter neurosphere colonies is generated from individual CE-derived neurospheres compared with the neurospheres from other regions including embryonic forebrain (FB) (Reynolds et al., 1992; Reynolds and Weiss, 1996) and adult subventricular zone (Morshead et al., 1994), from which approximately 20% of the cells within a neurosphere can generate daughter colonies. However, the growth properties of the rat CE-derived retinal progenitor cells have not been extensively described (Ahmad et al., 2000; Tropepe et al., 2000).

Much less is known about the molecules expressed to define regional specificity of CE-derived retinal progenitor cells. Previous studies have shown that CE-derived neurosphere colonies in mice and rats express one of the retinal specific transcription factors, Chx10 (Ahmad et al., 2000; Tropepe et al., 2000). It is well-known that several other transcription factors are essential for embryonic or postnatal retinal progenitors to generate complete sets of mature retinal cell types (Cepko et al., 1996). For example, transcription factors including homeodomain transcription factors such as Pax2, Pax6, Six3 and Rx (Dressler et al., 1990; Furukawa et al., 1997; Gruss and Walther, 1992; Mathers et al., 1997; Nornes et al., 1990; Oliver et al., 1995)

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define specification of the retinal primordial. The embryonic or postnatal retinal progenitor cells also express region-specific transcriptional factors such as Chx10, RORβ and Foxn4 (Burmeister et al., 1996; Chow et al., 1998; Gouge et al., 2001). Notably, the retina itself is also patterned along dorsoventral and nasotemporal axes. In the dorso-ventral axis of the retina, graded expressions of homeobox gene Tbx5 determines dorsal identity, whereas another class of homeobox gene, Vax2, defines the ventral identity (Barbieri et al., 2002; Schulte et al., 1999). EphAs are expressed in a low to high naso-temporal gradient in retina, whereas ephrins are expressed in a high to low naso-temporal gradient (Pittman and Chien, 2002). Previous studies have demonstrated that isolated neural stem cells from spinal cord (Yamamoto et al., 2001) or different regions of brain (Hitoshi et al., 2002) express different regional markers after culturing. It is also important to examine which regional specific transciption factors are also expressed in the CE-derived retinal progenitors to estimate their differentiation potential.

In the current study, we obtained progenitor cells from four subdivision of the rat CE and the embryonic forebrain (FB) (Reynolds et al., 1992; Reynolds and Weiss, 1996), and compared their growth properties and expression pattern of region specific transcription factors. Concomitantly, we examined the expression of stem cell factors defining the 'stemness'(Ramalho-Santos et al., 2002) such as Notch signaling pathway molecules (Bao and Cepko, 1997; Tomita et al., 1996).

2. Materials and methods

2.1. Animals

Wister rats (6–8 weeks old) were obtained from Saitama Experimental Animal Supply Co. Ltd (Saitama, Japan). All experiments were conducted in accordance with the Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Isolation of progenitor cells from ciliary epithelium

Ciliary epithelium (CE)-derived progenitor cell was isolated as described elsewhere (Ahmad et al., 2000; Tropepe et al., 2000). Briefly, the ciliary epithelium was dissected and digested with 0.25% trypsin for 10 min. To divide the eye into four subregions, a small incision made by scissors to mark the dorsal side of the eye before enucleation, and the eye was carefully removed and divided into four subdomains along its dorso-ventral and naso-temporal axes. Then, the ciliary epithelial cells were isolated from each region. The tissue was then mechanically dissociated in DMEM and F-12 medium (Invitrogen, Rockville, MD), washed once with soy bean trypsin inhibitor, and the cell suspension was poured through a 40 μM cell strainer (Beckton Dickinson Labwere, Franklin Lakes, NJ). Primary culture was performed as previously described (Reynolds et al., 1992) with some modifications. We used medium containing methylcellulose to prevent cell re-aggregation similar to our previous studies (Inoue et al., 2005; Kawase et al., 2004), which was established by previous investigators to isolate clonogenic neural stem cells (Gritti et al., 1999; Gritti et al., 1996). In brief, cell culture was performed using methylcellulose gel matrix (Wako, Osaka, Japan) dissolved in a DMEM and F-12 medium. The cells were seeded at a density of 10 viable cells/μl in a DMEM and F-12 medium supplemented with the B-27 culture supplement (Invitrogen), 20 ng/ml bovine basic fibroblast growth factor (bFGF) (Peprotech EC LTD, London, U.K), 20 ng/ml mouse epidermal growth factor (EGF) (Antigenix America Inc., NY, U.S.A.), 2 g/ml heparin (Sigma, St. Louis, MO)

2.3. Isolation of stem cells from the embryonic forebrain

The striatal primordia was dissected from the embryonic day 14 (E14) embryonic forebrain as described previously (Reynolds et al., 1992). The tissue from each embryo was transferred to a DMEM and F-12 medium and mechanically triturated into single cell. The cells were cultured under the same conditions, i.e. they were seeded in methylcellulose gel matrix dissolved in a DMEM and F-12 medium supplemented with the B-27 culture supplement, 20 ng/ml bFGF, 20 ng/ml EGF and 2 g/ml heparin at a density of 10 viable cells/μl.

2.4. Cell passaging

To passage the neurospheres, sphere colonies obtained after 7 days in culture were collected, dissociated into single cells by trypsinization, plated into 24 well dishes at a cell density of 10 cells/μl in 24 well dish plates and cultured for further 7 days under the same condition. Cells were passaged every 7 days thereafter.

To measure the diameter of the neurosphere colonies, the cultures were observed with an inverted microscope (Olympus BX-51, Tokyo, Japan) and the images were imported into a Macintosh computer with an aid of CCD camera (Olympus DP50) and were analyzed by NIH image program developed at National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

2.5. Differentiation of sphere-derived cells

For differentiation, spheres or singly dissociated cells from the neurospheres were allowed to attach to poly-D-lysine/laminin coated 24 well dishes for 7 days in the presence of 1% fetal bovine serum (FBS) and bFGF at a final concentration of 10 ng/ml.

2.6. Immunocytochemistry

Neurospheres or cells were washed with PBS, and fixed in absolute methanol for 30 min at 4 °C and blocked in PBS containing 1% skim milk for 10 min. The samples were then incubated with the primary antibodies at room temperature for two hours, rinsed twice with the blocking buffer, and incubated with the secondary antibody for two hours. Dilutions and sources of antibodies were as follows. The primary antibodies were: mouse monoclonal antibody against neurofilament-M (NF-M; Sigma 1:200), rabbit polyclonal antibody against GFAP (Dako, Glostrup, Denmark, 1:500) and mouse monoclonal antibody against O4. The secondary antibodies were Alexa 488 conjugated donkey IgG against mouse IgG and Alexa 488 conjugated donkey IgG against rabbit IgG. All the secondary antibodies were obtained from Molecular Probes at Eugene in OR and used at a 1:200 dilution. Negative control slides were made by omitting the primary antibody from the reaction. The samples were observed under a confocal microscope LSM510 (Carl Zeiss Thornwood, NY) or an epifluorescent microscope (IX70, Olympus).

2.7. RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using a kit (SV total RNA extraction kit, Promega) following the manufacturer's instructions. The RNA was treated with DNase I to eliminate possible DNA contamination. Two microgram of mRNA was then converted to cDNA in a 20 μl reaction mixture using Superscript II reverse transcriptase (Gibco BRL, Rockville, MD USA) as recommended by the manufacturer. One micro litre of each RT reaction was then added to a standard 50 μl PCR mixture. After 5 min of preincubation at 95 °C, amplification was performed for 30 cycles consisting of 1 min of denaturing at 95 °C, 1 min of annealing at appropriate temperature, and 1 min extension at 72 °C. The sequences of cDNA primers and annealing temperature are available at request from authors. As dorsoventral markers, Vax2 and Tbx5 were employed. However, our preliminary experiments failed to demonstrate any Eph and ephrins to be expressed in a graded fashion along its nasotemporal axis in rats, and nasotemporal retinal cell markers were unavailable. PCR products were separated on 2% agarose gel. The sequences of these fragments were completely identical to transcripts. Preliminary experiments established that under this condition of PCR, the amounts of each transcript were semiquantitative.

3. Results

3.1. Potential of differentiation and proliferation of the rat ciliary epithelium derived-progenitor cells

First, we compared the potential of differentiation of progenitor cells from the adult ciliary epithelium and embryonic forebrain. Embryonic forebrain contains highly proliferative stem cells that have been extensively studied and well characterized (Reynolds et al., 1992; Reynolds and Weiss, 1996). Tissues from the adult ciliary epithelium and embryonic forebrain were subjected to primary cultures, and proliferative progenitors were enriched as neurospheres in the presence of FGF2 and EGF as described (Reynolds et al., 1992; Reynolds and Weiss, 1996; Tropepe et al., 2000). These neurosphere-forming cells could be maintained by repeated passages for 6 weeks, and nearly all the neurosphere-forming cells from both groups expressed Nestin, an intermediate filament expressed in, but not specific for, undifferentiated neural precursors, similar to the previous studies (data not shown) (Reynolds et al., 1992; Reynolds and Weiss, 1996; Tropepe et al., 2000). Differentiated progenies from the CE-derived neurospheres expressed a neuronal marker, NF-M, and an astrocytic marker, GFAP (Fig. 1A and B). Expression of a photoreceptor-cell specific homeobox gene, CRX, was also detected by RT-PCR (Fig. 1C). In contrast, the progeny from the embryonic forebrain derived neurospheres expressed NF-M, GFAP and O4 as demonstrated by immunocytochemistry, but did not express detectable levels of CRX by RT-PCR (Fig. 1C–F), consistent with the previous studies (Tropepe et al., 2000). Progenitors in the adult ciliary epithelium did not generate neurons expressing Thy-1 and HPC-1, specific molecular markers for retinal ganglion cells and amacrine cells, respectively, and O4-positive oligodendrocyte. The morphology of the neuronal cells from the ciliary epithelium and forebrain was different. Most NF-M positive cells from the ciliary epithelium are bipolar; in contrast, NF-M positive cells from the embryonic forebrain have multiple cell processes (compare Fig. 1A and D). Thus, the potential of differentiation was different between progenitors isolated from ciliary epithelium and embryonic forebrain.

Next, we compared the proliferative potential of progenitor cells from the adult ciliary epithelium and embryonic forebrain. The number of secondary and tertiary neurosphere colonies from the adult ciliary epithelium was decreased compared with those from embryonic forebrain (Fig. 2A) and the diameter of the primary, secondary, tertiary neurosphere was smaller compared with those derived from embryonic forebrain (Fig. 2B), suggesting that the ciliary epithelium derived-neurospheres proliferate poorly.

Because Nestin expression is known to decrease gradually upon differentiation (Reynolds et al., 1992), we speculated that cells in a more differentiated state lose its expression more rapidly compared to the cells in an undifferentiated state. Hence, we sought to determine whether the percent of Nestin negative cells are different between progenitors from ciliary epithelium and embryonic forebrain. Although almost all the cells within the neurospheres expressed Nestin, neurosphere-forming cells from the ciliary epithelium showed tendency to lose Nestin

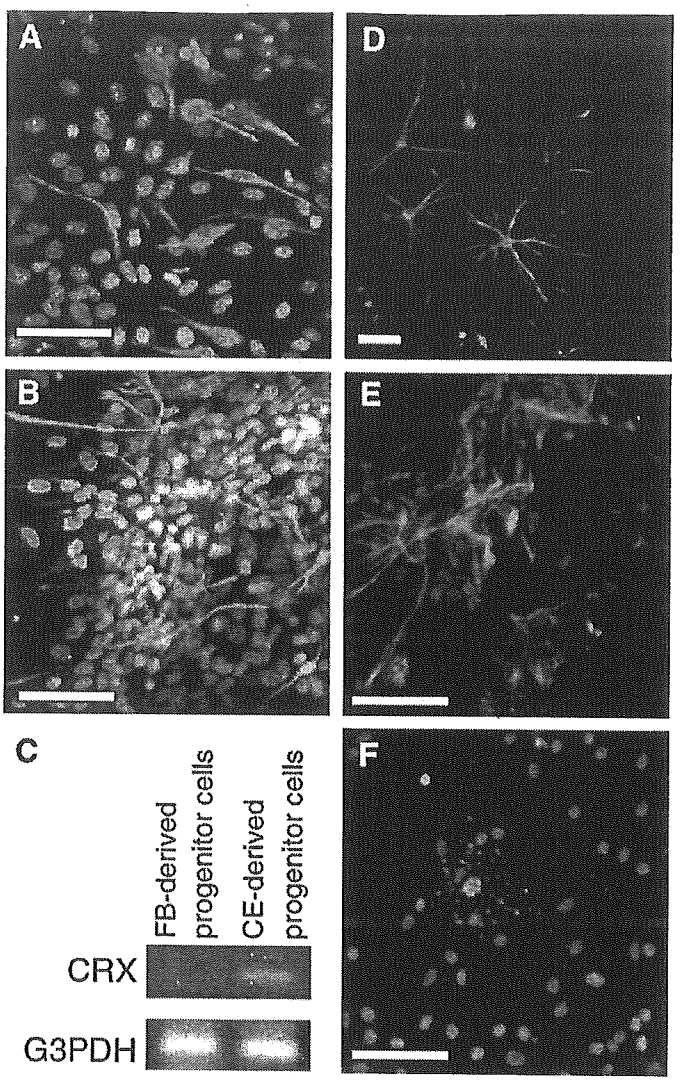

Fig. 1. Potential of differentiation of the progenitors derived from ciliary epithelium and embryonic forebrain. (A, B, D, E and F) Immunocytochemistry of the progenies derived from CE-derived retinal progenitor cells (A and B) and FB-derived progenitor cells (D–F). Both progenitors generate NF-M-positive putative neurons (A and D), GFAP-positive glia (B and E). FB-derived progenitor cells also generate O4-positive oligodendrocyte (F). The nucleus is counterstained with Hoechst 33258 and is depicted in blue. (C) RT-PCR analysis demonstrates that adult CE-derived retinal progenitor cells express CRX, whereas FB-derived progenitor cells do not. Note that the potency of differentiation of the progenies from the ciliary epithelium and embryonic forebrain are different in terms of the marker gene expressions and their morphology (see text for details.)

expression compared with the embryonic forebrain derived neurosphere forming cells when evaluated by immunocytochemistry 24 h after plating in the differentiating condition (Table 1). Next, we examined Notch signaling pathway molecules and other transcription factors that are considered as core factors defining the 'stemness' (Ramalho-Santos et al., 2002) by means of semi-quantitative RT-PCR using the secondary and passage 4 neurospheres from the ciliary epithelium. The result demonstrated that the expressions of several core factors defining stemness such as CyclinD1, Pten, ERCC-5 were not perturbed, while the expressions of Notch signaling pathway molecules, including Delta1, Notch1 and HES-5, were decreased in the passage 4 neurospheres compared with the passage 2 neurospheres (Fig. 3). Although the expression of a downstream effector of Notch signaling pathway, HES-1, was not decreased in the passage 4 neurospheres, these results demonstrated that the CE-derived neurospheres initially express the set of genes implicated in the maintenance of stem cells at least partly, but lose their expression after passaging. Taken together, CE-derived neurospheres contained fewer undifferentiated cells and more differentiated cells compared with the embryonic forebrain-derived neurospheres, and the undifferentiated cells within the neurospheres from the ciliary epithelium were reduced after passaging.

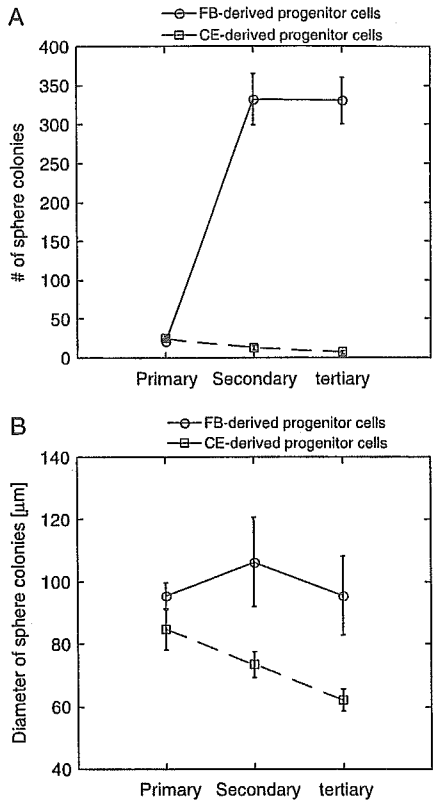

Fig. 2. Number and diameter of neurosphere colonies derived from embryonic forebrain and ciliarly epithelium. Sphere colonies obtained after 7 days in culture were collected, dissociated into single cells by trypsinization, plated 24 well dishes at a cell density 10 cells/μl in 24 well dish plates and cultured for further 7 days under the same condition. Cells were passaged every 7 days thereafter. (A) The number of neurosphere colonies per well was counted to give the value for one experiment. The data are mean from 6 experiments; error bars indicate S.E.M. (B) To measure the diameter of the neurosphere colonies, the cultures were observed with an inverted microscope and the images were analyzed by NIH image program. The diameter of at least 50 sphere colonies was measured. The data are mean from 6 experiments; error bars indicate S.E.M. Note that the neurospheres from the embryonic forebrain generate more neurosphres and are more proliferative compared with those derived from ciliary tpithelium. FB; forebrain, CE; ciliary epithelium.

Table 1
Precentage of Nestin positive cells 24 h after plating onto poly-L-lys/lamin coated dishes in the presence of 1% FBS

	Primary	Secondary	Tertiary
N	12	12	1
Neurospheres from the ciliary epithelium	93.7±3.5	64.6±3.5	67.8
N	3	3	3
Neurospheres from the embryonic forebrain	100±0.0	100±0.0	100±0.0

Data are presented as mean±SEM. The cells from the neurosphre were dissociated and plated onto poly-L-lys/laminine coated dishes. They were evaluated by immunocytochemistry 24 h after plating in the differentiating condition.

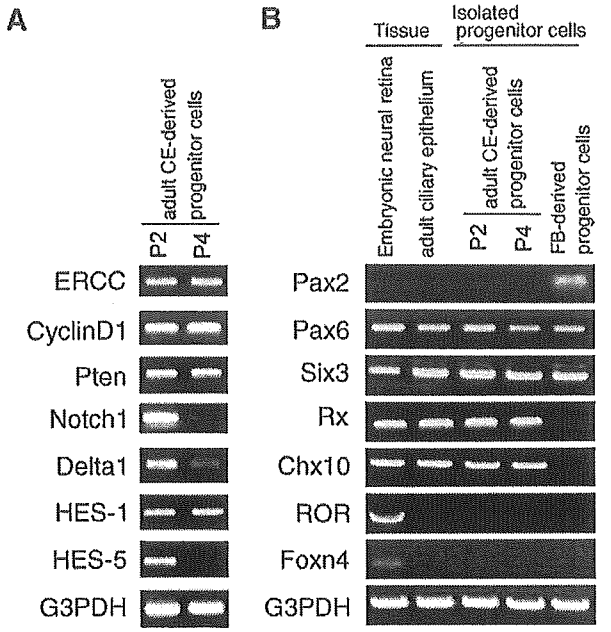

Fig. 3. Regulation of undifferentiated cell markers and transcription factors of the progenitors derived from ciliary epithelium during culture. (A) Expression levels of core stem cell factors including CyclinD1, Pten, ERCC-5, and Notch pathway singnaling molecules, Delta1, Notch1, HES-1 and HES-5, were examined by means of semi-quantitative RT-PCR using the secondary and passage 4 CE-derived retinal progenitor cells. Note that the expression levels of Delta1, Notch1 and HES-5 are decreased in the passage 4 progenitors. (B) Expressions of homeodomein factors including Pax2, Pax6, Six3, Rx and Chx10, and region-specific factors, RORβ and Foxn4 were examined using the mRNA from the ciliary epithelium and embryonic neural retina, and CE- and FB-derived progenitor cells. Three independent experiments were performed, which gave identical results. CE; ciliary epithelium, FB; forebrain, P2; passgage 2, P4; passage 4.

4. Expression of region specific transcription factors in progenitors from rat ciliary epithelium and embryonic forebrain

The potential of differentiation and proliferation of the progenitors from the ciliary epithelium differs from that of the progenitors from the embryonic forebrain (Fig. 1), raising the possibility that each progenitor expresses different sets of genes. Thus, we analyzed region-specific transcriptional factors expressed in the progenitors from the ciliary epithelium and embryonic forebrain. Concomitantly, we examined whether these factors are expressed in the embryonic day 17.5 neural retina and the adult ciliary epithelium. First, expressions of homeodomain factors including Pax2, Pax6, Six3, Rx and Chx10 were examined (Fig. 3B). The embryonic retina expressed Pax6, Six3, Rx and Chx10 as expected. The adult ciliary epithelium also expressed Pax6, Six3, Rx and Chx10. Neither embryonic retinal cells nor adult ciliary epithelium expressed Pax2. Progenitors from the ciliary epithelium expressed Pax6, Six3, Rx and Chx10 even after passaging, but not Pax2, whereas progenitors from the embryonic forebrain

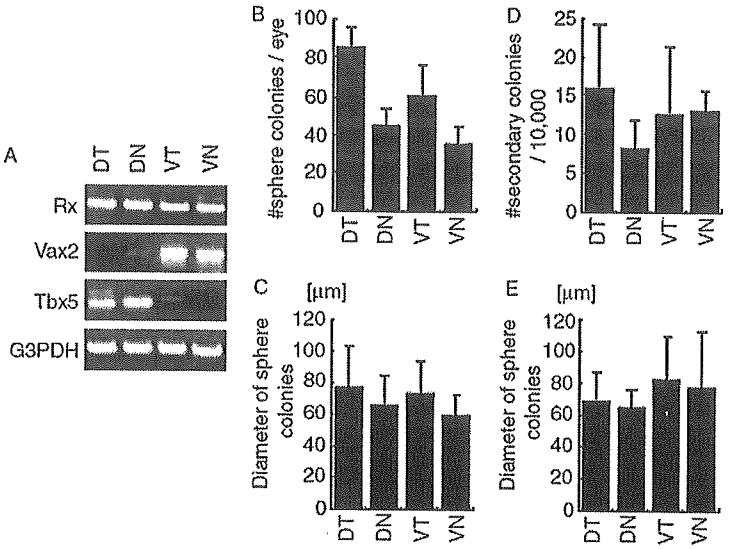

Fig. 4. Growth and molecular properties of sphere colonies from the ciliary epithelium subdivided into four subregions. (A) RT-PCR showing the expression pattern of Vax2 and Tbx5 before culture, suggesting that the eye was precisely divided along its dorsoventral and nasotemporal axes. (B and D) The number of primary (B) and secondary (D) neurosphere colonies per well was counted to give the value for one experiment. The data are mean from 6 experiments; error bars indicate S.E.M. (C and E) To measure the diameter of the primary (C) and secondary (E) neurosphere colonies, the cultures were observed with an inverted microscope and the images were analyzed by NIH image program. The diameter of at least 50 sphere colonies was measured. The data are mean from 6 experiments; error bars indicate S.E.M. D; dorsal, N; nasal, T; temporal, V; ventral

expressed Pax2, Pax6 and Six3, but not Rx and Chx10. Next, expressions of retinal specific transcription factors including RORβ and Foxn4 were examined (Fig. 3B). The embryonic retina expressed RORβ and Foxn4 as expected. However, the adult ciliary epithelium expressed neither of them. Neither progenitors from the ciliary epithelium nor embryonic forebrain expressed RORβ and Foxn4.

Next, we subdivided the CE into four subdivisions along its dorsoventral and nasotemporal axes and examined the sphere formation from these subdomains. To confirm the eye was precisely divided along its axes, the expression of dorsoventral markers markers, i.e. Vax2 and Tbx5, were examined before culturing (Fig. 4A). Interestingly, there was a trend for the ciliary epithelium from the temporal side to generate a greater number of colonies with larger diameter (Fig. 4B). The number and diameter of the secondary colonies from each subregion was similar (Fig. 4C). RT-PCR demonstrated that neither the primary and secondary colonies nor their progeny expressed detectable levels of Vax2 and Tbx5.

5. Discussion

Previous studies demonstrated that neural progenitor cells are present in the adult ciliary epithelium (Ahmad et al., 2000; Tropepe et al., 2000). In the current study, we isolated CE-derived retinal progenitor cells from adult rat ciliary epithelium (adult CE-derived retinal progenitor cells) using reaggregation-free methylcellulose gel matrix. Under our condition, it was not determined whether these progenitor cells from the ciliary epithelium possessed the properties of stem cells in vitro. Instead, our results support that the neurospheres from the ciliary epithelium contained fewer undifferentiated cells compared with the neurospheres from the embryonic forebrain, for several reasons. First, the number of neurosphere-initiating cells in the nuerospheres from the ciliary epithelium was few relative to those from the embryonic forebrain. Second, although the expression of Nestin was detected almost all the cells within the neurospheres, the number of Nestin positive cells was decreased compared to the embryonic forebrain-derived neurospheres when evaluated 24 h after differentiation. In addition, our results demonstrated that the relative expression levels of several components of the Notch pathway signaling molecules were decreased after the passaging of the neurospheres from the ciliary epithelium, suggesting that the undifferentiated cells within the neurospheres were reduced after passaging. To maintain retinal progenitor cells in an undifferentiated state in vivo, Notch signaling pathway molecules, such as Delta 1, Notch1, HES-1 and Hes-5 are essential. Lack of either gene in mice causes retinal progenitor cells to differentiate immaturely. (Bao and Cepko, 1997; Ohtsuka et al., 1999; Tomita et al., 1996) Taken together, our results demonstrated that the neurospheres from the ciliary epithelium contained fewer undifferentiated cells compared to the embryonic forebrain-derived neurospheres in rats.

We explored molecular properties of adult CE-derived retinal progenitor cells. Embryonic or postnatal retinal progenitor cells express regulatory molecules such as Pax6, Rx, Chx10, Foxn4 and RORβ in vivo (Chow et al., 1998;

Furukawa et al., 1997; Gouge et al., 2001; Liu et al., 1994; Marquardt et al., 2001; Mathers et al., 1997). In addition, expression of Pax2 and Six3 is detected in the developing neural retina (Dressler et al., 1990; Oliver et al., 1995). In the adult ciliary epithelium, we found that Pax6, Rx, Six3 and Chx10 are expressed. We have demonstrated that expression of these transcription factors can be recapitulated in vitro, i.e. CE-derived retinal progenitor cells express Pax6, Six3, Rx and Chx10. Even after passaging, expressions of these factors are retained, suggesting that their expression is not confined within the Notch1-positive undifferentiated progenitor cells in vitro. In the ventricular zone where forebrain neural progenitor cells are localized, expressions of Pax2, Pax6, Six3, Rx, Chx10 and RORβ are detected from the early stage of neurulation to immediately after optic vesicle evangination (Chow et al., 1998; Dressler et al., 1990; Furukawa et al., 1997; Gouge et al., 2001; Liu et al., 1994; Marquardt et al., 2001; Mathers et al., 1997; Oliver et al., 1995). However, expressions of Rx, Chx10, RORβ and Foxn4 fade in the late embryonic period (Chow et al., 1998; Furukawa et al., 1997; Gouge et al., 2001; Liu et al., 1994). In agreement with this, we found that the Pax6 and Six3 can be detected in the neurospheres from the E14 embryonic forebrain, but expressions of Rx, Chx10 RORβ and Foxn4 were not detected. Thus, adult CE-derived retinal progenitor cells in vitro express, at least to some extent, similar a set of transctription factors from the proliferatve retinal progenitors in vivo (Chow et al., 1998; Furukawa et al., 1997; Gouge et al., 2001; Liu et al., 1994; Marquardt et al., 2001; Mathers et al., 1997), which are different from those expressed in forebrain-derived neural progenitor cells. This is in good accordance with the previous studies demonstrating that the isolated neural stem cells from spinal cord (Yamamoto et al., 2001) or different regions of brain (Hitoshi et al., 2002) express different regional markers after culturing. Although further studies are necessary, it is tempting to speculate that such region-specific transcription factors might regulate the potential of differentiation of the progenitor cells.

We also found different properties between adult CE-derived retinal progenitor cells and embryonic or postnatal retinal progenitor cells in vivo in the following aspects. First, most embryonic retinal progenitor cells in vivo are considered multipotent and produce most of the cells in the neural retina (Cepko et al., 1996). However, adult CE-derived retinal progenitor cells did not generate neurons expressing markers specific for retinal ganglion cells and amacrine cells in vitro. Second, embryonic proliferative retinal progenitor cell cultures usually contain morphologically undifferentiated cells and principally two differentiated cells, i.e. multipolar neurons and photorecepotors having one short neurite (Kelley et al., 1995; Stenkamp and Adler, 1993). On the contrary, most of the neurons from the adult ciliary epithelium are bipolar. This is good accordance with previous studies (Ahmad et al., 2000; Tropepe et al., 2000); the differentiated progenies from the CE-derived retinal progenitor cells from mice and rats are stained with the some retinal selective markers, however, their morphologies seem different from those observed in embryonic retinal cultures (Kelley et al., 1995; Stenkamp and Adler, 1993). In addition, progenitor cells derived from the ciliary epithelium do not express transcription factors, such as RORβ and Foxn4 that in vivo proliferatve retinal progenitor cells express. Moreover, subregion analysis demonstrated that retinal progenitor cells did not express detectable levels of subregion specific transcription factors such as Vax2 and Tbx5. Thus, our results suggest that CE-derived retinal progenitors may have intrinsic limitations in the production of cell types.

Recent studies have shown that growth factor can induce neurogeneis in vivo in the CE cells. (Fischer and Reh, 2000) Furthermore, many of the molecular markers used to define differentiated retinal cells in vitro, such as markers from the phototransduction pathway or retinoid pathways, are already expressed in vivo and in vitro in adult CE cells (Bertazolli-Filho et al., 2001; Ghosh et al., 2004; Salvador-Silva et al., 2005), raising the possibility that adult CE, in vivo, may not contain retinal progenitor or stem cells, and that the cell and molecular characteristics, in vitro, described in this work as well as in previous studies, may simply reflect the embryological neural origin of the CE and its embryological relationship with the retina.

In summary, our study confirmed the previous studies that retinal progenitor cells can be isolated from the ciliary epithelium using reaggregation-free methylcellulose medium. As demonstrated in this study, CE-derived retinal progenitor cells contain fewer undifferentiated cells have limited capacity of self-renewal and lack the ability to produce specific types of retinal neurons in vitro. Despite these limitations, CE-derived retinal progenitor cells may be an attractive source of transplantation therapy for retinal diseases. Indeed, a recent study demonstrated that the CE-derived retinal progenitor cells can be incorporated into the injured retina and differentiate to mature retinal neurons (Chacko et al., 2003). To facilitate growth and/or differentiation of CE-derived retinal progenitor cells and to utilize CE-derived retinal progenitor cells as a cell-source of retinal repair, further studies regarding factors regulating their differentiation are necessary.

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Short Communication

Neuroprotective effect of calcium channel blocker against retinal ganglion cell damage under hypoxia

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ABSTRACT

The purpose of this study was to determine whether iganidipine, nimodipine and lomerizine, potentially useful calcium channel blockers for ophthalmic treatment, have direct retinal neuroprotective effects against hypoxic damage in experimental in vitro model. We used purified retinal ganglion cells (RGCs) from newborn rats. RGCs were incubated in controlled-atmosphere incubator in which oxygen levels were reduced to 5% normal partial pressure and cell viability was assessed. We also examined the effect of calcium channel blockers on the calcium ion concentration in RGC under hypoxic stress by calcium imaging. Iganidipine, nimodipine and lomerizine (0.01–1 μM) increased the RGC viability. Increase in intra-RGC calcium ion concentration by hypoxic damage was reduced by these calcium channel blockers. In conclusion, iganidipine, nimodipine and lomerizine were effective against hypoxic RGC damage in vitro. This neuroprotective effect was thought to be mediated by blocking calcium ion influx into RGC. These findings suggest that iganidipine, nimodipine and lomerizine have a direct neuroprotective effect against RGC damage related to hypoxia.

Retinal ganglion cell (RGC) is damaged in various ocular diseases, including glaucoma. Although not always confirmed, many investigators reported beneficial effects of systemic calcium channel blocker on the prognosis at least in a part of glaucoma patients (Bose et al., 1995; Gaspar et al., 1994; Kitazawa et al., 1989; Netland et al., 1993). It has been suggested that the beneficial effect was mainly due to increase in ocular blood flow (Tomita et al., 1999; Yamamoto et al., 1998) or direct effect to RGC survival (Boehm et al., 2003).

RGC culture system is a useful experimental model to assess the direct effect of calcium channel blockers to RGC (Otori et al., 2003). Using retinal cell or pure RGC culture model, neuroprotective effect of calcium blockers against neuronal damage induced by glutamate has been discussed (Otori et al., 2003; Toriu et al., 2000). However, several recent studies cast doubt on the primary role of glutamate in glaucoma (Carter-Dawson et al., 2002; Honkanen et al., 2003; Levkovitch-Verbin et al., 2002). Tissue hypoxia has been postulated to occur in glaucomatous eye in the basis of blood flow and histopathologic studies (Chung et al., 1999; Cioffi and Wang, 1999; Flammer et al., 2002; Flammer, 1994; Osborne et al., 1999; Tezel and Wax, 2004). In addition, it is reported that hypoxia lead to selective RGC death (Luo et al., 2001) in vitro.

Thus, we hypothesized that the damage of RGC by hypoxia would be at least partly caused by intracellar

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calcium increase through calcium channel. To verify this hypothesis, we have developed a hypoxia damage model using purified RGC culture system (Goto et al., 2002; Inatani et al., 2001; Kashiwagi et al., 2001; Otori et al., 2003; Otori et al., 1998; Pielen et al., 2004; Tezel and Yang, 2004) and investigated direct neuroprotective effect of calcium channel blockers on hypoxia-induced cell death.

Iganidipine hydrochloride, a new dihydropyridine-derivative calcium channel blocker, is relatively water soluble and is the only dihydropyridine-derivative calcium channel blocker presently available that is easily prepared as an ophthalmic solution (Ishii et al., 2003). Instillation of iganidipine increased the blood flow in the ipsilateral optic nerve head (ONH) in rabbit (Ishii et al., 2004; Waki et al., 2000) and monkey (Ishii et al., 2004), and inhibited the endothelin-1-induced contraction of retinal vessels (Ishii et al., 2003).

Nimodipine is a dihydropyridine calcium channel blocker and clinically used to treat cerebral vasospasm and migraine headaches. Several researchers recently reported that nimodipine oral administration had beneficial effect in visual field testing, color vision (Piltz et al., 1998) and contrast sensitivity (Boehm et al., 2003).

Lomerizine, a dual L/T-type calcium channel blocker (Hara et al., 1999), has been clinically used for treatment of migraine (Hara et al., 1995). It has shown selective enhancement of retinal and ONH blood flow in humans and rabbits (Tamaki et al., 2003) and neuroprotective effect in rabbits (Danielisova

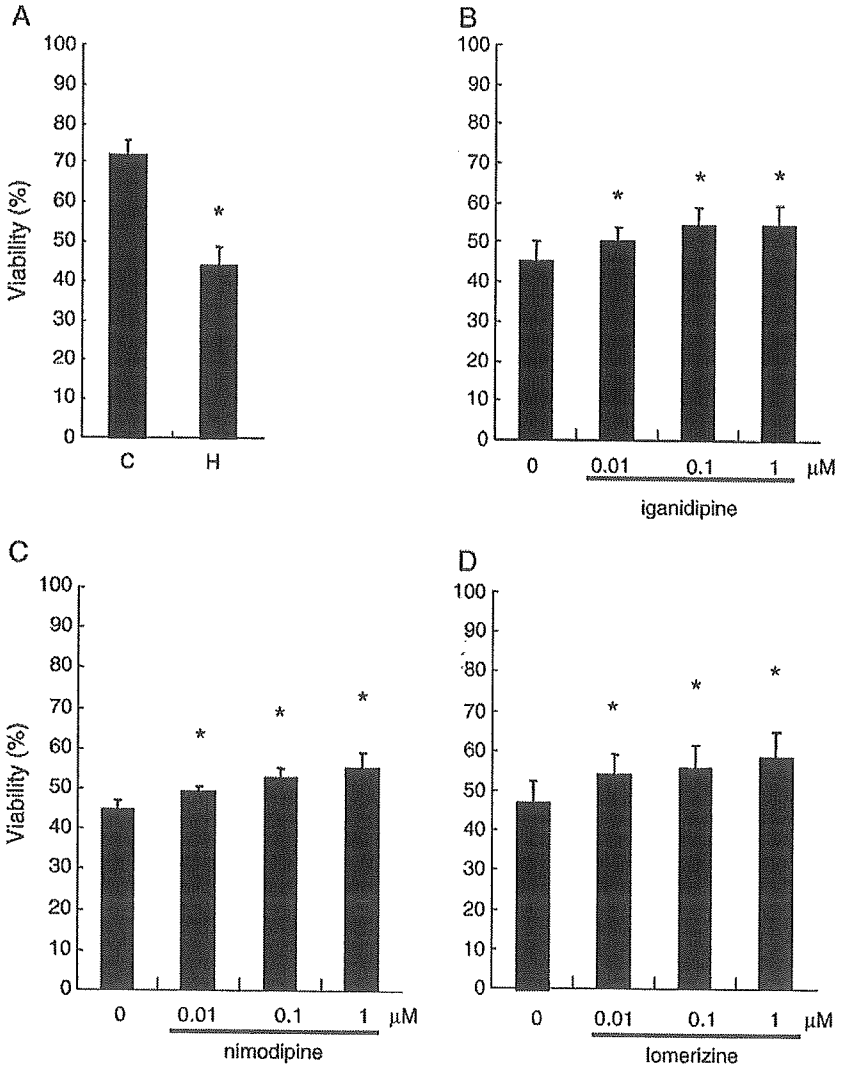

Fig. 1 – Effect of iganidipine, nimodipine and lomerizine to hypoxia-induced RGC loss. (A) C: Survival rate (viability) of RGC under normoxia, H: survival rate under hypoxic condition for 12 h. $n = 10$, Mann–Whitney U test. *$P < 0.01$ versus C. (B) Survival rate of RGC with iganidipine. RGCs were cultured under hypoxic condition for 12 h in the medium including iganidipine. $n = 8$. (C) Survival rate of RGC with nimodipine. RGCs were cultured under hypoxic condition for 12 h in the medium including nimodipine. $n = 8$. (D) Survival rate of RGC with lomerizine. RGCs were cultured under hypoxic condition for 12 h in the medium including lomerizine. $n = 8$. ANOVA followed by Dunnett's multiple comparison test for panels B, C and D. *$P < 0.01$ versus 0 μM. Viability is indicated as mean ± SD.