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（分担）研究報告書

# デバイスの移植と網膜機能評価に関する研究

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研究要旨

本研究の目的は、網膜疾患治療デバイスとして、強膜上に置くだけの低侵襲な方法で移植できる経強膜ドラッグデリバリーシステム（DDS）を作製し、さらに多剤併用療法に対応するために複数の薬物を徐放できるマルチ DDS を開発することである。本分担研究は、ラット眼強膜上へのデバイス移植と網膜電図（ERG）による網膜機能評価を目的とした。Edaravone（EDV）および Unoprostine（UNO）のデバイスを移植し、1 週間後に網膜光障害（8000Lux、18h）を実施し、暗順応 4 日間後に ERG 検査を行った。その結果、コントロールの Phosphate-buffered saline（PBS）デバイスでは、光障害によって a 波、b 波ともに 7 割以上低下したが、EDV および UNO デバイスでは有意に ERG 振幅値の低下が抑制されていた。また、EDV/UNO のマルチ徐放デバイスでは、シングル徐放デバイスと比較して、ERG 振幅値の低下が抑制されていた。網膜組織の評価ではデバイス移植群で網膜厚みが維持されていた。また、デバイス移植群の網膜では細胞死に関わる p38 のリン酸化が抑制されていた。

## A．研究目的

本課題の目的は、失明疾患の上位を占める網膜疾患の治療デバイスとして、強膜上に置くだけの低侵襲な方法で移植できる経強膜ドラッグデリバリーシステム（DDS）を作製し、多剤併用療法に対応するために、複数の薬物を任意の速度で徐放できるマルチDDSデバイスを開発することである。本研究は分担研究として、ラット眼強膜上へのデバイスの移植および網膜機能評価を目的とした。

視覚はヒトの情報の8割を占めるため、視覚障害はQuality of life（生活の質）を著しく低下させる。2006年の厚生労働省難治性疾患克服事業の統計結果では、失明疾患の上位はすべて網膜疾患（1位 緑内障、2位 糖尿病網膜症、3位 網膜色素変性症、4位 黄斑変性症）である。加齢性疾患が多い網膜疾患においては、超高齢化社会を迎え今後さらに増加する可能性がある。網膜は主に視細胞、双極細胞、水平細胞、アマクリン細胞、神経節細胞からなる神経組織である。一般に神経細胞は再生が難しく、一度障害されると治療が難しい場合が多い。

眼から入った光は光受容細胞である視細胞で神経信号へ変換され、神経節細胞から

視神経を経て脳中枢で情報が伝えられる。この神経信号は活動電位という生体電気パルスとして伝達される。眼球には角膜側をプラス、網膜側をマイナスとする静止電位が存在するが、光を受容すると活動電位が生じて電位変化が生じる。この変動を記録したものが網膜電図（Electroretinogram：ERG）である。一般にコンタクトレンズ型の電極を角膜に装着して、電極から強い光を網膜に当て、心電図のように電位波形を記録する。ERGは白内障など眼底検査が行えない場合に有効な網膜機能評価方法である。

本分担研究では、ERGを用いてEDV、UNO、およびEDV/UNOマルチ徐放デバイスの網膜保護効果をERG評価することを目的とした。ERGは動物実験用に開発されたMayo. Co.のPuRECを使用した。

B．研究方法

（1）デバイスの作成

デバイスはリザーバー、薬物ペレット、徐放膜から構成される。CAD-CAMでリザーバーと薬物ペレットのデザインを作製し、小型NC微細加工機Micro MC-2（株式会社PMT）でアクリル板に鋳型を作製した。このアクリル板をフルオロシアンでコートし鋳型Aとした。この鋳型Aにポリジメチルシロキサン（PDMS）をキャストし60℃で30分加熱して硬化させた。このPDMS鋳型をフルオロシアンでコートし鋳型Bとした。鋳型BにPDMSをキャストし60℃で30分加熱して硬化させた。このPDMS鋳型をリザーバーを作製するための最終鋳型Cとした。このPDMS鋳型Cに、TEGDM 1mlに2-Hydroxy-2-methyl-propiophenone 10μlを混合したプレポリマーをキャストし、UV架橋（10mW/cm$^2$、3min [浜松ホトニクス、LC8]）して硬化させた。鋳型CからTEGDMリザーバーを剥がして完成した。作成したリザーバーのサイズは、縦1.5mm×横1.5mm×高さ0.6mm、薬剤充填部容量は1.2μlである。

薬物ペレットは薬物をPEGDMとTEGDMの混合プレポリマーに混合し、あらかじめ作っておいたペレット用PDMS鋳型にキャスト（1.2μL）してUV硬化（10mW/cm$^2$、1.5min）して作成した。ペレットサイズはリザーバー内にぴったり合うように外径が縦1.5mm×横1.5mm×高さ0.6mmで作製した。

薬物はEdaravone（EDV、WAKO）、Unoprostone（UNO、アールテック・ウエノ(株)から譲渡）を使用した。ペレットに各薬物が250mg/mlになるように調整した。プレポリマー中のPEGDMとTEGDMの比率は0:100から100:0の間で調整した。

シングル徐放デバイスとして、EDVおよびUNOのペレット1個をリザーバーに詰めて徐放膜で蓋をした。徐放膜は、PEGDMとTEGDMを混合したプレポリマーで作製した。上記の薬物ペレットを充填したリザーバー上にプレポリマーを1uL滴下し、ガラス板でカバーした後、UV硬化して作成した。プレポリマー中のPEGDMとTEGDMの比率は0:100から100:0の間で調整した。

マルチ徐放デバイスは、EDVおよびUNOの半割ペレットを1個ずつ充填し、徐放膜で蓋をした。各ペレットのPEGDMとTEGDMの比率をさまざまになるように充填した。徐放膜はP100（PEGDM：TEGDM＝100:0）で固定した。

（2）動物

動物実験操作は、ARVOの眼科研究の動物

使用に関する声明のガイドラインに従い、東北大学大学院医学系研究科の動物管理委員会の承認を得た。200から250gの雄のSDラットを使用した。すべての過程においてケタミン塩酸塩（90mg/kg）とキシラジン塩酸塩（10mg/kg）の腹腔内注射で麻酔をした。瞳孔は2.5%phenylephrinと1%ttropicamideで拡大した。Oxybuprocaine hydrochloride（0.4%）を局所麻酔として使用した。

（３）デバイスの移植

麻酔後、実体顕微鏡で観察しながら、ラットの上方結膜を切開しテノン嚢を鈍的に剥離し強膜を露出させた。デバイスを挿入し強膜上に接着するように固定した。結膜を縫合し、タリビッド眼軟膏を点入し終了とした。

（４）網膜光障害モデル

デバイスを移植したラットに光障害を行った。ラットを2.5%phenylephrinと1%ttropicamideで散瞳してから、空調を有するLED光障害装置（特注モデル）内で、デバイスを移植したラットを飼育した（22℃、8000Lux）。予備実験で光障害時間は、24時間が適当と判断した。この条件では、完全に視力を失うわけではなく動物の行動に異常は見られなかった。光照射後、LEDを消灯し、装置内で4日間暗順応した。暗順応後、暗室内でラットをケタミン塩酸塩（90mg/kg）とキシラジン塩酸塩（10mg/kg）の腹腔内注射で麻酔し、2.5%phenylephrinと1%ttropicamideで散瞳してから網膜電図（ERG；Purec、Mayo㈱）を評価した。

（５）網膜電図（ERG）

コンタクトレンズ電極（2mmベースカーブ、Mayo）を角膜に当て、Identical reference 電極を口に、Ground電極をしっぽに置いた。Single flash light（1000cds/$m^2$、3msec）を刺激にERG波形を計測した（Dark-adapted maximal rod/cone combined response）。a波（ベースラインからa波の振幅）およびb波（a波とb波の最大振幅）の振幅を計測した。コントロールとして、Phosphate-buffered saline（PBS）を含有するデバイスを移植したラット、および未移植のラットを使用した。

シングル徐放デバイスでは、カバー条件の異なるデバイスを移植し、移植1週目に光障害を行って、カバー条件と網膜保護効果の関係を調べた。シングル徐放デバイスでは、カバー条件は統一し、EDVとUNOのペレット条件を変えたデバイスを移植し評価した。

（６）組織学的、分子生物学的評価

ERG評価後7日目にラットを安楽死し、眼球を摘出した。パラフィン包埋によって標本を作製し、HE染色およびTUNEL染色を行った。また、網膜を分画し、ホモジネートのウェスタンブロットを行った。細胞死関連シグナルのp38とMAPKのリン酸化、および内部標準のβ-tubulinを評価した。Bio-rad製ゲルでSDS-PAGEを行い、Bio-rad製トランスブロットでPVDF膜に転写した。スキムミルクでブロッキング後、各一次抗体（Cell signaling）で反応後、二次抗体（Cell signaling）で反応し、ECL plus（GE healthcare）で検出した。バンド強度をImageJソフトウェアで解析した。

（7）統計学的解析

測定結果はエクセル統計2012を用いて、One-way ANOVA with Tukey testによる有意差検定を行った。95%の信頼度（$p<0.05$）のときに統計学的に有意差があると判断した。

（倫理面への配慮）

動物実験操作は、ARVOの眼科研究の動物使用に関する声明のガイドラインに従い、東北大学大学院医学系研究科の動物管理委員会の承認を得た。

C．研究結果

（1）デバイスの移植

デバイスの移植性に問題はなかった。移植後のデバイスはマイルドなFibrosisで覆われていたが、周辺組織への著名な炎症や眼内への副作用は認められなかった。デバイス除去後の強膜はネクローシス等の異常は全く見られなかった。

（2）網膜光障害モデル

デバイスをラット強膜上に移植し、1週間後に光障害を行った後、暗順応4日後にERG検査を行った。コントロールのPBS-DDSでは、光障害によってa波、b波ともに7割以上低下した。一方、EDV-DDSでは、EDV放出の多いカバーのデバイスで有意に波形値の低下が抑制されていた。また、EDV放出の少ないカバーのデバイスでは波形値低下の抑制は見られなかった。UNO-DDSにおいても同様に、UNO放出の多いカバーのデバイスで有意に波形値の低下が抑制されていた。以上より、EDVおよびUNOのシングル徐放デバイスは網膜光障害に対して保護効果があり、その効果は薬剤の放出条件と関係があることが示唆された。

次にEDV/UNOマルチ徐放デバイスをラット強膜上に移植し1週間後に光障害を行った後、暗順応4日後にERG検査を行った。PBS-DDSおよび未処理群では光障害によってa波、b波ともに7割以上低下していた。一方、EDV/UNOデバイス群では、波形値の低下が有意に抑制されていた。この抑制効果はシングル徐放デバイス群より高かった。以上より、マルチ徐放デバイスはシングル徐放デバイスと比較して相乗効果的に網膜保護効果を示すことが示唆された。

（3）組織学的、分子生物学的評価

ERG測定後7日目の網膜組織評価を行った。HE染色標本の網膜外顆粒層（ONL）厚みを顕微鏡観察で評価した。その結果、デバイス移植部位周辺部位において、コントロールデバイスと比較して有意にONL厚みが維持されていた。また、EDV/UNOマルチ徐放デバイス群ではシングル徐放デバイス群よりも厚みが維持されており、網膜全体にわたって厚みが維持傾向であった。

網膜保護の考察として、網膜の細胞死関連シグナルの発現を評価した。その結果、コントロールデバイス移植群ではp38のリン酸化が上昇し、MAPKのリン酸化は変化がなかった。一方、薬物デバイス移植群では、p38のリン酸化が減少しており、MAPKのリン酸化が上昇していた。また、EDV/UNOマルチ徐放デバイスでは、シングルデバイスと比較して、p38のリン酸化の減少がより低く、MAPKのリン酸化の上昇がより高い傾向にあった。

D．考察

本研究はデバイスの移植とERGによる網膜機能評価を行った。デバイス移植に伴う炎症や眼内への副作用はなく、網膜光障害モデルではデバイスの網膜機能保護効果が確認された。また、マルチ徐放デバイスでは、相乗効果的に網膜保護効果を有する可能性が示唆された。

網膜光障害モデルは、過剰光に曝すことによって網膜にダメージを与えるモデルである。光によるダメージの蓄積は、加齢黄斑変性症などの網膜障害に関与することが指摘されている。そのためマウスやラットで網膜光障害モデルが利用されている。過去にEDVおよびGGAの全身投与が光障害に対して保護効果があることが報告されている（EDV：Invset Ophthalmol Vis Res, 52, 7289-7297, 2011、Eur J Pharm, 642, 77-85, 2010、GGA：J Neurosci, 25, 2396-2404, 2005）。いずれも約1週間毎日2回以上の全身投与を続けたときに網膜保護効果を報告している。一方、我々のデバイスは一度の投与で薬物を持続投与し網膜保護効果を示した。さらにマルチ徐放化によって、シングル投与よりも相乗的に網膜保護できる可能性が示された。これらの結果は、本デバイスを使用することで複数薬剤の点眼や静脈投与の反復投与の必要がなくなり、かつ網膜に局所的に投与できるため全身性の副作用を抑えることができることを示唆している。

E．結論

EDVおよびUNO徐放デバイスは、網膜光障害から網膜を保護する可能性を示唆した。また、その効果はマルチ徐放化することで、より効果が高いことが示唆された。本デバイスは持続性のあるマルチ薬剤眼内投与デバイスとして有用であることが示唆された。

F．健康危険情報

該当なし

G．研究発表

1. 論文発表

① Hideyuki Onami, Nobuhiro Nagai, Shigeki Machida, Norihiro Kumasaka, Ryosuke Wakusawa, Yumi Ishikawa, Hikaru Sonoda, Yasufumi Sato, **Toshiaki Abe**. “Reduction of laser-induced choroidal neovascularization by intravitreal vasohibin-1 in monkey eyes” RETINA The Journal of Retinal and Vitreous Diseases, 32(6), 1204-1213 (2012).

② Yumi Ishikawa, Nobuhiro Nagai, Hideyuki Onami, Norihiro Kumasaka, Ryosuke Wakusawa, Hikaru Sonoda, Yasufumi Sato, **Toshiaki Abe**. “Vasohibin-1 and retinal pigment epithelium” Adv Exp Med Biol, 723, 305-310 (2012).

2. 学会発表

（国際学会発表）

① **Toshiaki Abe**, Yumi Ishikawa, Hideyuki Onami, Yuki Katsukura, Nobuhiro Nagai “Intra-scleral transplantation of collagen sheet with cultured brain-derived neurotrophic factor expressing cells partially rescued the retina from the damage of acute high intraocular pressure” RD2012 XV International Symposium on Retinal Degeneration, Bad Gogging, Bavaria, Germany (July 16-21, 2012)

② Nobuhiro Nagai, Hideyuki Onami, Hiro

kazu Kaji, Takuya Yamada, Yuki Katsukura, Machiko Sato, Yumi Ishikawa, Toru Nakazawa, Matsuhiko Nishizawa, and **Toshiaki Abe** "Protective Effects of Transscleral Drug Delivery Device Against Light-induced Retinal Damage in Rats" 2012 ARVO annual meeting, Fort Lauderdale, Florida (May 6-10, 2012)

③ Hideyuki Onami, Nobuhiro Nagai, Ryosuke Wakusawa , Hirokazu Kaji, Takuya Yamada, Yumi Ishikawa, Matsuhiko Nishizawa, Yasufumi Sato, Toru Nakazawa,and **Toshiaki Abe** "Suppression of Rat Choroidal Neovascularization by Transscleral Vasohibin-1 Delivery Device" 2012 ARVO annual meeting, Fort Lauderdale, Florida (May 6-10, 2012)

（国内学会発表）

① 永井展裕、大浪英之、梶弘和、山田琢也、勝倉由樹、小柳恵理、西澤松彦、阿部俊明：「経強膜マルチドラッグ徐放デバイスの作製と網膜保護効果の検討」日本バイオマテリアル学会シンポジウム2012、仙台国際センター（2012年11月26-27日）

② 伊藤俊太郎、永井展裕、長峰邦明、西澤松彦、阿部俊明、梶弘和：「マイクロ流路デバイスを用いる眼底組織培養モデルの開発」日本バイオマテリアル学会シンポジウム2012、仙台国際センター（2012年11月26-27日）

③ 永井展裕、大浪英之、梶弘和、山田琢也、勝倉由樹、小柳恵理、西澤松彦、阿部俊明：「薬物徐放デバイスの作製と網膜光障害モデルに対する網膜保護効果の検討」第32回日本眼薬理学会学術集会、ピアザ淡海（2012年9月15日～16日）

④ 永井展裕、大浪英之、梶弘和、山田琢也、勝倉由樹、小柳恵理、西澤松彦、阿部俊明：「網膜光障害モデルに対する経強膜DDSの網膜保護効果」第28回日本DDS学会学術集会、札幌コンベンションセンター（2012年7月4日～5日）

⑤ 大浪英之、永井展裕、梶弘和、山田琢也、勝倉由樹、西澤松彦、中澤徹、阿部俊明：「プロテインドラッグ眼内徐放デバイスによる加齢黄斑変性治療の試み」第28回日本DDS学会学術集会、札幌コンベンションセンター（2012年7月4日～5日）

⑥ 大浪英之、永井展裕、梶弘和、西澤松彦、涌沢亮介、佐藤靖史、中澤徹、阿部俊明：「分子徐放デバイス作製と網膜保護」第63回東北臨床超微形態懇話会、東北大学医学部（2012年6月28日）

⑦ 永井展裕、大浪英之、梶弘和、山田琢也、勝倉由樹、佐藤真智子、中澤徹、西澤松彦、阿部俊明：「網膜光障害モデルに対する経強膜ドラッグデリバリーデバイスの網膜保護効果」第116回日本眼科学会総会、東京国際フォーラム（2012年4月5日～8日）

⑧ 大浪英之、永井展裕、梶弘和、西澤松彦、涌沢亮介、佐藤靖史、中澤徹、阿部俊明：「経強膜vasohibin徐放デバイスによるラット脈絡膜新生血管抑制」第116回日本眼科学会総会、東京国際フォーラム（2012年4月5日～8日）

H．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

別紙５

研究成果の刊行に関する一覧表

書籍

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# REDUCTION OF LASER-INDUCED CHOROIDAL NEOVASCULARIZATION BY INTRAVITREAL VASOHIBIN-1 IN MONKEY EYES

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**Purpose:** To determine whether intravitreal vasohibin-1 will reduce the grade of the choroidal neovascularization in monkey eyes.

**Methods:** Choroidal neovascularizations were induced in 12 monkey eyes by laser photocoagulation. Three monkeys were evaluated for the safety of the vasohibin-1 injections, 6 monkeys for the effects of a single injection, and 3 monkeys for repeated injections of vasohibin-1. Ophthalmoscopy, fluorescein angiography, focal electroretinograms, and optical coherence tomography were used for the evaluations. The level of vascular endothelial growth factor in the aqueous was determined by enzyme-linked immunosorbent assay. Immunohistochemistry was performed.

**Results:** An intravitreal injection of 10 μg of vasohibin-1 induced mild intraocular inflammation. Eyes with an intravitreal injection of 0.1 μg and 1.0 μg of vasohibin-1 had significant less fluorescein leakage from the choroidal neovascularizations and larger amplitude focal electroretinograms than that of vehicle-injected eyes. Similar results were obtained by repeated injections of 0.1 μg of vasohibin-1. Immunohistochemistry showed that vasohibin-1 was expressed mainly in the endothelial cells within the choroidal neovascularizations. The vascular endothelial growth factor level was not significantly altered by intravitreal vasohibin-1.

**Conclusion:** The reduction of the laser-induced choroidal neovascularizations and preservation of macular function in monkey by intravitreal vasohibin-1 suggest that it should be considered for suppressing choroidal neovascularizations in humans.

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Age-related macular degeneration (AMD) is one of the most common sight-threatening disease in developed countries.[1] A choroidal neovascularization (CNV) is a typical finding in eyes with the wet-type AMD, and the CNV can lead to subretinal hemorrhages, exudative lesions, serous retinal detachment, and disciform scars.[2] Many different types of treatments have been used to treat AMD, for example, laser photocoagulation,[3] surgery,[4,5] transpupillary thermotherapy,[6] photodynamic therapy,[7] and intravitreal injection of anti–vascular endothelial growth factor (VEGF).[8,9] Each of these treatments has advantages and disadvantages, and the best treatment of AMD has still not been determined.

Different pro- and antiangiogenic factors play important roles in the development and progression of CNVs.[10] Among the proangiogenic factors, VEGF has been shown to play a major role.[11] Thus, anti-VEGF therapy is being used to successfully treat CNVs in patients with AMD.[8,9] However, this method requires repeated injections that can lead to irritation, infection, and other adverse side effects.[12] In addition, not all patients respond to the therapy.[13] Thus, other types of therapy need to be developed to treat AMD eyes with a CNV.

Vasohibin-1 is a VEGF-inducible molecule expressed by human cultured endothelial cells (ECs) and has antiangiogenic properties.[14] Its expression is selectively induced in ECs not only by VEGF but also by several other proangiogenic factors such as basic fibroblast growth factor.[15] Vasohibin-1 inhibits the formation of EC networks in vitro, corneal neovascularization,[14] and retinal neovascularization in a mouse model of oxygen-induced ischemic retinopathy.[16] Vasohibin-1 is found in the vitreous and in

proliferative membranes of patients with diabetic retinopathy. The level of vasohibin-1 is significantly correlated with the VEGF level in the vitreous of patients with proliferative diabetic retinopathy.[17] Vasohibin-1 is also expressed in the CNV membranes of patients with AMD.[18] Eyes with lower vasohibin-1/VEGF expression ratios tend to have larger CNV lesions, whereas those with higher vasohibin-1/VEGF ratios have subretinal fibrosislike lesions.[18]

We have found that the laser-induced CNVs were less active in mice injected intravitreally with vasohibin-1 than those injected with the vehicle.[19] Thus, the purpose of this study was to determine the effect of intravitreal vasohibin-1 on the laser-induced CNVs in monkey eyes. We shall show that the intravitreal vasohibin-1 was safe and reduced the degree of the CNVs in monkey eyes.

## Methods

### *Animals*

The procedures used in the animal experiments followed the guidelines of the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and they were approved by the Animal Care Committee of Tohoku University Graduate School of Medicine. Twelve Japanese macaque monkeys (*Macaca fuscata*) between ages 4 and 6 years and weighing between 4.2 kg and 10.1 kg were used (Table 1). For all procedures, the monkeys were anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), and the pupils were dilated with topical 2.5% phenylephrine and 1% tropicamide. Oxybuprocaine hydrochloride (0.4%) was also used for local anesthesia. Three monkeys were used to evaluate the safety of intravitreal vasohibin-1, 6 monkeys for dose dependency of a single injection of vasohibin-1, and 3 monkeys for repeated injections of vasohibin-1.

Table 1. Monkey Eyes Used in This Study

| | Vasohibin (mg) | Number of Eyes | Inflammation |
|---|---|---|---|
| Nontreated | 0 | 1 | 0/1 |
| | 0.01 | 1 | 0/1 |
| | 0.1 | 1 | 0/1 |
| | 1 | 1 | 0/1 |
| | 10 | 1 | 1/1 |
| | 100 | 1 | 1/1 |
| Laser application | 0 | 3 | 0/3 |
| | 0.01 | 3 | 0/3 |
| | 0.1 | 3 | 0/3 |
| | 1 | 3 | 1/3 |
| Laser application | 0 | 3 | 0/3 |
| | 0.1 | 3 | 0/3 |
| Total | | 24 | 3/24 |

Inflammation shows clinical inflammation signs that were observed during the experiments.

### *Experimental Choroidal Neovascularization*

An argon green laser was used to rupture of the choroidal membrane using a slit-lamp delivery system (Ultima 2000SE; Lumenis, Yokneam, Israel) with a contact lens.[20] The laser settings were as follows: 50-$\mu$m diameter, 0.1-second duration, and 650-mW to 750-mW intensity. Five laser burns were made around the macula within 15° of the fovea. The foveola was not treated. Each burn was confirmed to have induced subretinal bubbles indicating a rupture of Bruch membrane.

### *Expression and Purification of Human Vasohibin-1 Polypeptide*

Human vasohibin-1 was purified from *Escherichia coli* as described.[21] Human vasohibin-1 was isolated as a thioredoxin fusion protein. The fusion protein was dialyzed and digested with blood coagulation Factor Xa (Novagen, Darmstadf, Germany). The released vasohibin-1 was collected, eluted, and dialyzed against 20 mM glycine–HCl buffer (pH 3.5). Then, the vasohibin-1 was resolubilized with 50 mM Tris–HCl buffer containing 50 mM NaCl, 5 mM tris(2-carboxyethyl)phosphine, 0.5 mM ethylenediaminetetraacetic acid, 5% glycerol, and 4.4% *N*-lauroylsarcosine (pH 8.0) and was dialyzed against 20 mM sodium phosphate buffer at pH 8.0. This buffer was also used as the vehicle.

The protein concentration was determined by the Bradford method with a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as a standard protein.

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This study was performed at the Tohoku University. Monkeys were supplied by National BioResource Project for breeding and supply.

The authors declare no conflict of interest.

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*Intravitreal Injection of Recombinant Vasohibin-1 Polypeptide*

Vasohibin-1 was injected intravitreally in 3 groups of monkeys (Table 1). The first group of 6 eyes did not have a laser burn and received a single injection of vehicle, or 0.01, 0.1, 1, 10, or 100 $\mu$g of vasohibin-1/50 $\mu$L of vehicle. The second group of 12 eyes of 6 monkeys (3 eyes for each concentration) received a single injection of vehicle or 0.01, 0.1, and 1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle 4 days after the laser burn. The third group of 3 eyes had 3 injections of 0.1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle in the right eyes and 50 $\mu$L of vehicle in 3 fellow eyes on 0, 4, and 7 days after the laser burn. We examined the natural course of laser-induced CNVs in mice, and the CNVs were most active around Day 14 after the laser burn, and then gradually regressed, especially 28 days after laser burn. When we injected vasohibin-1 into the vitreous of mice after laser burns, we found that the injection of vasohibin-1 on Day 4 after the laser burn was most effective, followed by Days 7 and 1. Other days were less effective. In addition, immunohistochemical studies for vasohibin-1 in the mouse CNV membranes showed that the later the laser burn, the more vasohibin-1 staining was observed.[19] So we decided to do the repeated vasohibin-1 injections on 0, 4, and 7 days after the laser burn (relatively early days after laser burn).

For the intravitreal injections, the monkeys were anesthetized and pupils were dilated. The intravitreal injections were made with a 30-gauge needle attached to a 1-mL syringe. The needle was inserted through the sclera into the vitreous cavity ~1.5 mm posterior to the limbus while observing the eye with an operating microscope. The fundus was examined after the injection to confirm that the retina and lens were not damaged.

*Ophthalmic Examinations*

In addition to the routine ophthalmologic examinations, fluorescein angiography (FA) with an imaging system (GENESIS-Df; Kowa, Tokyo, Japan), optical coherence tomography (OCT, RS3000; NIDEK, Tokyo, Japan), and focal and full-field electroretinography (ERG) were performed on the selected days. Fluorescein angiography was performed 1, 2, and 4 weeks after the laser photocoagulation. Two retinal specialists (R.W. and T.A.) graded the angiograms in a masked way using a grading system[22]: Grade 1, no hyperfluorescence; Grade 2, hyperfluorescence without leakage; Grade 3, hyperfluorescence in the early or middle phase and leakage in the late phase; and Grade 4, bright hyperfluorescence in the transit and leakage in late phase beyond the treated areas.

The central macular thickness was determined from the macular thickness maps (3.45 mm in diameter) of the scans by OCT 4 weeks after the laser photocoagulation. The volume of the lesion was also calculated using the same program.

The pupils were maximally dilated for the ERG recordings 4 weeks after intravitreal vasohibin-1 injections. The ERGs were amplified and digitally band-pass filtered from 0.5 Hz to 500 Hz for the full-field ERGs and from 5 Hz to 500 Hz for the focal ERGs (PuREC; Mayo, Aichi, Japan). The animals were dark adapted for at least 30 minutes before the full-field ERG recordings. The light for the stimulus was obtained from light-emitting diodes (EW-102; Mayo Co., Nagoya, Japan) embedded in a contact lens electrode. The intensity and duration of the stimuli were controlled by an electronic stimulator (WLS-20; Mayo Co.). Chlorided silver plate electrodes were placed on the forehead and right ear lobe as reference and ground electrodes, respectively. The intensity of the stimulus was 1,000 cd/m$^2$ and the duration was 3 milliseconds.

Focal ERGs were recorded 4 weeks after the laser photocoagulation with a focal ERG system (PuREC; Mayo; ER-80; Kowa) that was integrated into an infrared fundus camera. This system was developed and described in detail by Miyake et al.[23,24] The stimulus spot was 15° in diameter and was placed on the macula by viewing the ocular fundus on a monitor. The intensity of the stimulus was 1,000 cd/m$^2$, and the background light was 1.5 cd/m$^2$. The stimulus duration was 100 milliseconds. A Burian–Allen bipolar contact lens electrode (Hansen Ophthalmic Laboratories, Iowa City, IA) was inserted into the anesthesized conjunctival sac to record the focal ERGs. A chlorided silver electrode was placed on the left ear lobe as the ground electrode. Two hundred to 300 responses were averaged at a stimulation rate of 5 Hz.

The a-waves were measured from the baseline to the trough of the first negative response, and the b-wave from the first trough to the peak of the following positive wave. The amplitudes of a-waves and b-waves from the three untreated monkeys were used as control. The number of monkeys used in this experiment was not added to the total number of monkeys.

*Immunohistochemistry*

Immunostaining for vasohibin-1was done on eyes with laser-induced CNVs 28 days after the laser application. From the results of CNV experiments on

mice,[19] the laser-induced CNV lesions were self-resolved >28 days after the laser burn. Thus, we decided to enucleate the eyes 28 days after the laser burn, although there may be differences between mice and monkeys. The eyes were enucleated and fixed in 4% paraformaldehyde overnight, and the anterior segment and lens were removed. The posterior segment was embedded in paraffin, and 3-$\mu$m serial sections were cut, and adjacent sections were stained with hematoxylin and eosin.

The immunohistochemical staining for vasohibin-1 was performed with the peroxidase method and for cytokeratin by the alkaline phosphatase method. Mouse monoclonal antibodies against vasohibin-1 (1:400) and mouse monoclonal anti-pan cytokeratin (1:200; Sigma-Aldrich, St. Louis, MO) were applied to the sections overnight at 4°C. Then the sections were incubated in biotin-conjugated anti-mouse immunoglobulin (Histfine SAB-PO(M) kit; Nichirei, Tokyo, Japan). The slides for vasohibin were incubated with peroxidase-conjugated streptavidin (Histfine SAB-PO(M) kit; Nichirei), and the slides for cytokeratin were incubated with alkaline phosphatase–conjugated streptavidin (Histfine; Nichirei). HistoGreen (HISTOPRIME HistoGreen substrate kit for peroxidase; Ab Cys SA) was used for the chromogen of vasohibin, and VECTOR RED (alkaline phosphatase substrate kit 1; Vector, Burlingame, CA) was used for the chromogen of cytokeratin. The slides were counterstained with hematoxylin. For control, preimmune mouse immunoglobulin G was used instead of the primary antibody.

### *Enzyme-Linked Immunosorbent Assay for Vascular Endothelial Growth Factor*

Aqueous was collected by a 30-gauge needle from the anterior chamber of each monkey 4 weeks after the laser photocoagulation. The level of the VEGF peptide was quantified by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R & D Systems, Mckinley, MN; Quantikine Human VEGF immunoassay) using 50 $\mu$L of aqueous. The intensity of the color of the reaction products was measured with a MAXline microplate reader (Molecular Devices Corporation, Palo Alto, CA). The measurements were made in duplicate, and the mean was used. The concentration of VEGF was expressed as the amount of protein in picograms per milliliter (pg/mL).

### *Statistical Analyses*

Analysis of variance with Scheffe test for post hoc analysis was used to examine the differences in the leakage and intensity of the CNVs in the fluorescein angiograms, amplitudes of the ERGs, mean central thickness, and volume of the CNV. The differences were also compared using the Student two-sample *t*-tests.

## Results

### *Safety Evaluations and Outcomes*

Before any of the procedures, the retina and choroid were normal in all the monkeys. Then 6 nontreated eyes were injected intravitreally with vehicle or 0.01, 0.1, 1, 10, or 100 $\mu$g of vasohibin-1/50 $\mu$L. After 0.01, 0.1, and 1 $\mu$g of vasohibin-1, the appearance of the retina and choroid did not differ from that of the vehicle-injected eyes. When 10 $\mu$g or 100 $\mu$g/50 $\mu$L of vasohibin-1 polypeptide was injected, a mild inflammation (Grade 1)[25] was detected in the vitreous on the day after the injection. The inflammation was less with 10 $\mu$g than with 100 $\mu$g of vasohibin, and the inflammation was resolved in 2 days after 10 $\mu$g and in 1 week after 100 $\mu$g (Table 1). When we injected 1 $\mu$g/50 $\mu$L of vasohibin-1 once in the laser-treated eyes, 1 of the 3 eyes developed inflammation in the aqueous. An inflammation was not observed when 0.1 $\mu$g of vasohibin-1 was injected even after 3 injections. When we injected 50 $\mu$L of vehicle with almost the same amount of endotoxin (400 U/mL) as that of 100 $\mu$g of vasohibin-1, no inflammation was detected. These results indicated that mild inflammation can develop with $\geq$10 $\mu$g of vasohibin-1 injection into the vitreous in nontreated monkey eyes.

The amplitudes of the a- and b-waves of the full-field ERGs of eyes injected with 0.01 $\mu$g to 100 $\mu$g of vasohibin-1 did not differ significantly from the vehicle-injected eyes. The a-wave amplitudes ranged from 87.3 $\mu$V to 180.3 $\mu$V (average, 119.3 ± 36.6 $\mu$V) before and from 100.7 $\mu$V to 195.8 $\mu$V (average, 131.3 ± 53.7 $\mu$V; $P = 0.444$) after the vasohibin-1 injection. The b-wave amplitudes ranged from 219.6 $\mu$V to 340.6 $\mu$V (average 250.6 ± 54.7 $\mu$V) before and from 240.8 $\mu$V to 345.2 $\mu$V (average 274.4 ± 82.0 $\mu$V, $P = 0.801$) after the vasohibin-1 injection.

### *Effect of Different Concentrations of Vasohibin-1*

After the laser photocoagulation, we injected vehicle or 0.01, 0.1, or 1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle in 3 eyes of each dosage for a total of 12 eyes (Table 1). From the results of safety evaluations, we selected the maximum amount of vasohibin-1 as 1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle. Representative results of FA at 1, 2, and 4 weeks after the laser application for each dose of vasohibin-1 are shown in Figure 1. Color fundus photographs and focal ERGs recorded at 4 weeks are also shown.

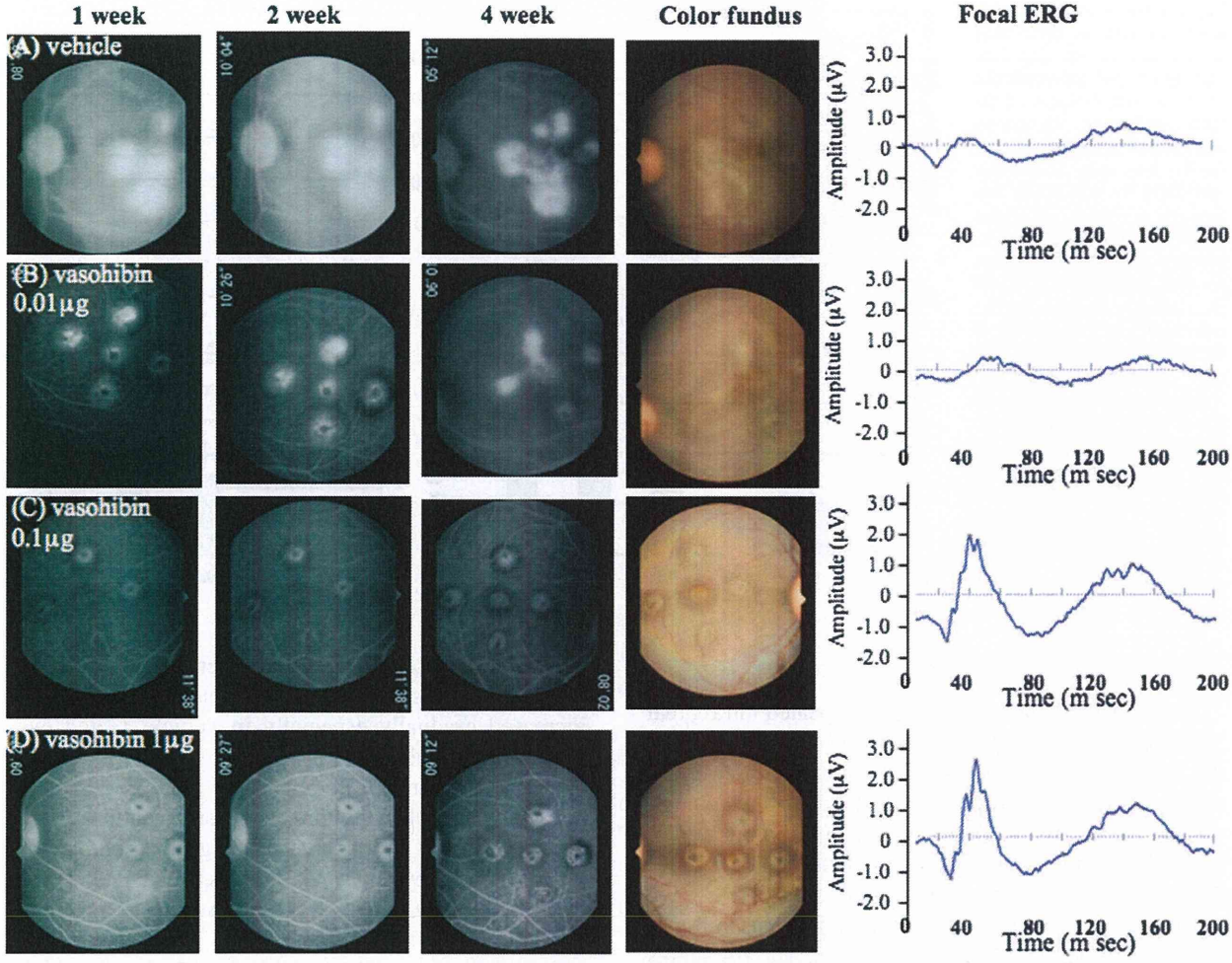


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**Fig. 1.** Representative FAs, fundus photographs, and focal ERGs from 6 monkey eyes are shown. Vehicle or 0.01, 0.1, 1 μg of vasohibin-1/50 μL of vehicle was injected intravitreally, and representative results at 1, 2, and 4 weeks after laser treatment are shown (see quantitative values in Figure 2, A–D). The FA images are those at around 10 minutes after the fluorescein injection. Color fundus photographs were taken 4 weeks after the laser application. Focal ERGs recorded 4 weeks after the laser photocoagulation are shown in the right column for each eye.

The CNV activity was scored using the FA grading system[22] for all five laser spots in each eye. The FA score for each spot was summed and compared with each other (Figure 2A). Our findings showed that there was significantly less leakage after 0.1 μg of vasohibin-1 than that for vehicle ($P = 0.016$) and for 0.01 μg ($P = 0.035$) of vasohibin-1 at 1 week. Significantly less leakage after 0.1 μg of vasohibin-1 than that of vehicle was also observed at 4 weeks ($P = 0.0307$). Because 1 μg of vasohibin-1 showed mild inflammation in 1 eye, we did not analyze the CNV in these eyes. The percentage of eyes with FA scores of 4 is also listed in Figure 2B. Our results showed that 45% of vehicle-treated eyes had Grade 4 leakage, and it was 45% in 0.01 μg of vasohibin-1–treated eyes, 7% with 0.1 μg of vasohibin-1–treated eyes, and none in the 1-μg vasohibin-1–treated eyes (only 2 eyes) at 1 week. Similarly, the percentage of eyes with Grade 4 leakage was 27%, 13%, 7%, and 0% at 2 weeks and 20%, 20%, 0% and 0% at 4 weeks after the vasohibin-1 injection (Figure 2B).

The amplitudes of the a-waves of the focal ERGs after 0.01 μg of vasohibin-1 were significantly smaller than those of the controls ($P = 0.041$) (Figure 2C). The amplitudes of the b-waves of the focal ERG b amplitudes in the vehicle-injected eyes ($P = 0.0085$) and in the 0.01-μg vasohibin-1–injected eye ($P = 0.0184$) were significantly smaller than those of the controls (Figure 2D). The results of inflammation, FA leakage, and ERG amplitudes led us to select 0.1 μg of vasohibin-1 as the optimal concentration for intravitreal injection to reduce the laser-induced CNV in our monkeys.

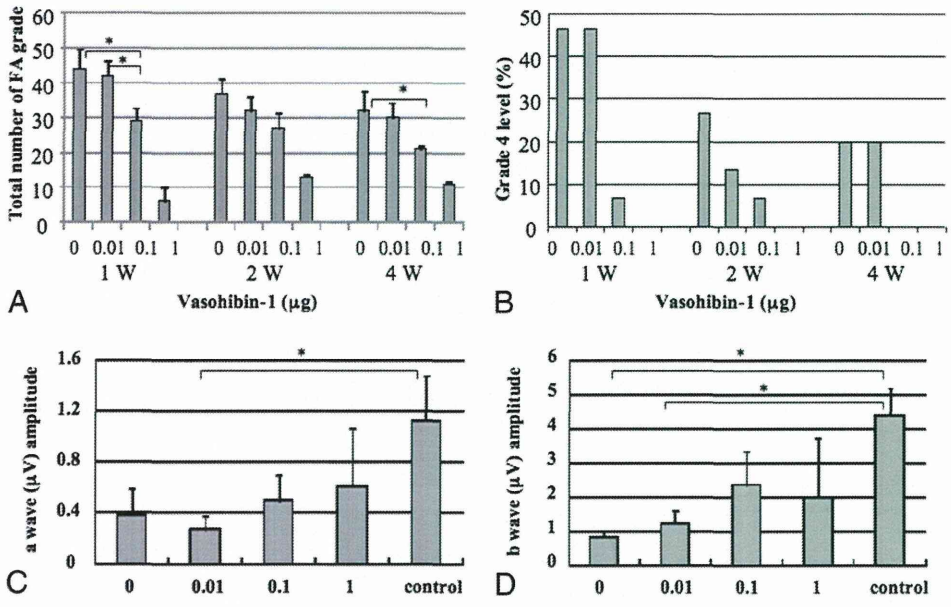


**Fig. 2.** Fluorescein angiographic scores for each of the 5 laser spots in each eye are plotted for each group, and the amplitudes of the a- and b-waves of the focal ERGs. **A.** Fluorescein angiographic scores for each of the five laser spots in each eye are plotted for each group. Statistically significant differences are shown as asterisks. **B.** Distribution of Grade 4 FA scores for each group is shown. **C** and **D.** Average amplitude of the a-waves (**C**) and b-waves (**D**) of the focal ERG recorded 4 weeks after intravitreal vasohibin-1. Vehicle (0) or 0.01, 0.10, or 1.00 $\mu$g of vasohibin-1 was injected in control eyes or eyes after the laser burns. Untreated controls show the effects before laser treatment. The averages ± standard deviations of the amplitudes of the a- and b-waves are plotted on the ordinate.

*Effects of Repeated Injections of Vasohibin-1*

Next, we examined the effects of repeated intravitreal injections of 0.1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle in the right eyes on 0, 4, and 7 days after the laser application while the fellow eyes received an injection of the vehicle on the same days. We studied three eyes in each group. Representative fundus photographs, FAs, and OCT images after vehicle alone are shown in Figure 3 (A and B) and after 0.1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle in Figure 3 (C and D). The FA scores were significantly lower in the vasohibin-1–injected eyes than in the vehicle-injected eyes at 4 weeks ($P$ = 0.009; Figures 3 and 4A). At 1 week and 2 weeks after the vasohibin-1 injections, the FA scores were not significantly different ($P$ = 0.07). The percentage of eyes scored as Grade 4 was 13.3% at 1 week, 26.7% at 2 weeks, and 26.7% at 4 weeks in the vehicle-treated eyes, whereas no Grade 4 eyes were observed in the 0.1 $\mu$g of vasohibin-1/50 $\mu$L of vehicle-treated eyes at any time (Figure 4B).

Although statistical significance was not observed in the a-wave amplitude of the focal ERGs, statistically significant larger b-wave amplitudes were observed in the vasohibin-1–treated eyes than that of vehicle ($P$ = 0.039) (Figure 4, C and D).

Optical coherence tomography examinations showed that the retinal pigment epithelium and Bruch membrane were disrupted in the laser-treated eyes at 1 week and 2 weeks after the laser application (Figure 3, B and D) as was found in histologic preparations.[22] At 4 weeks, an retinal pigment epithelium–like membrane appeared over the CNV lesion (Figure 3, B and D). This line was shown to be cytokeratin positive. The OCT images showed that the size of the CNV increased gradually especially in vehicle-treated eyes as was seen in the FA images.

Optical coherence tomography also showed that the macular thickness (Figure 4E) and volume (Figure 4F) of the CNV lesions after 0.1 $\mu$g of vasohibin-1/50 $\mu$L of treated eyes was ~30% less than the vehicle-treated eyes in the central 1 mm. When we examined the volume of the central 6 mm, no difference was observed between the vasohibin-1–treated and vehicle-treated eyes.

*Histology and Immunostaining of Choroidal Neovascularization*

Histopathologic analyses showed that the retina and choroid surrounding the CNV had normal architecture in both the vehicle and vasohibin-1–treated eyes as reported.[26] The vehicle-treated eyes after the laser application showed a disruption of the Bruch membrane and retinal pigment epithelium complex, and the eyes had different degrees of fibrous tissues and vessels (Figure 5, C and E). Eyes treated with vasohibin-1 tended to have smaller CNV than that of vehicle-treated eyes.

Cytokeratin labeling demonstrated that retinal pigment epithelial cells from the edges of the wound had proliferated and covered the laser wound to different degrees. Although a disruption of the cytokeratin labeling was present in the vehicle-treated eyes (Figure 5, D and F), we could not find any significant difference from that of the vasohibin-1–injected eyes. Different

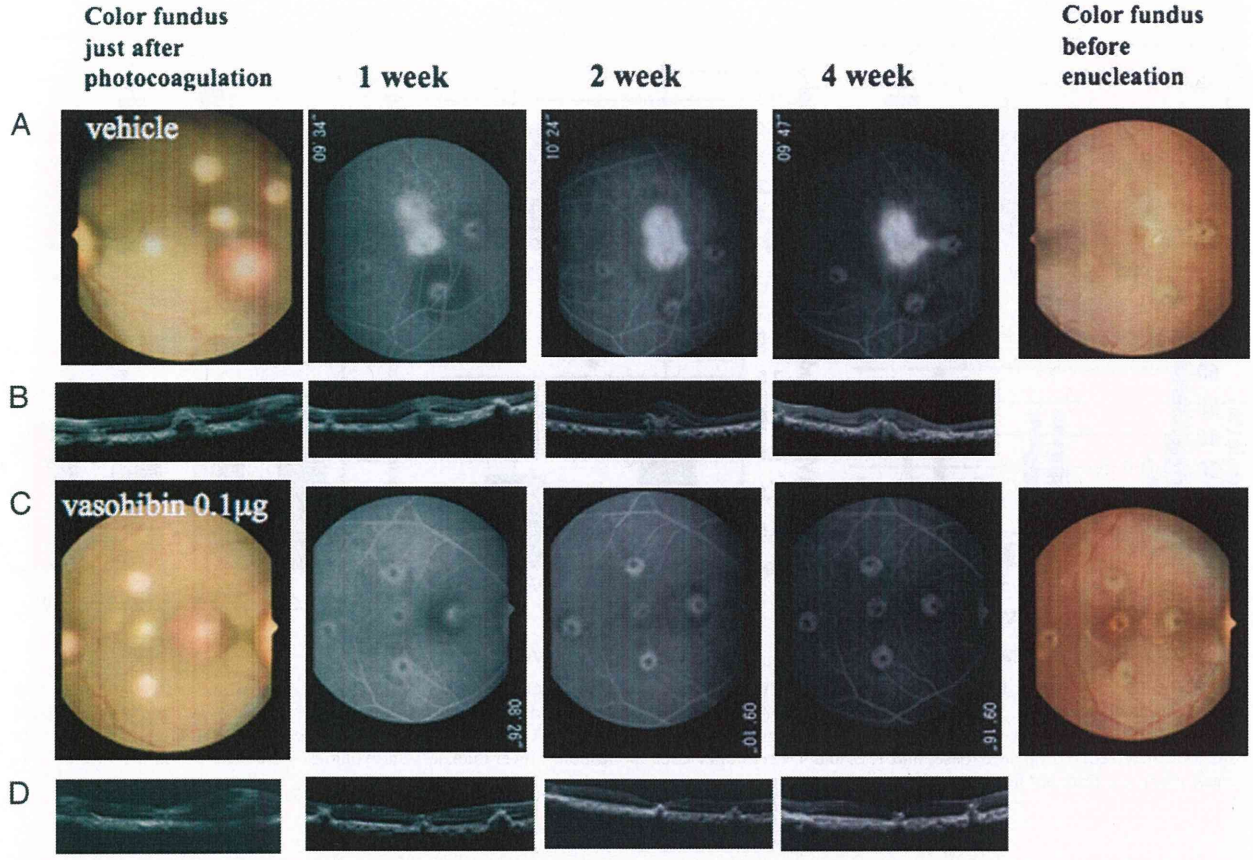


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**Fig. 3.** Fluorescein angiograms, ocular coherence tomographic images, color fundus photographs, and focal ERGs are shown. Vasohibin-1 (0.1 μg/50 μL) was injected into the vitreous of the right eyes 3 times on 0, 4, and 7 days after laser application, and the same amount of vehicle was injected into left eyes on the same days. Photographs show the fundus just after the laser application and the day of enucleation. Fluorescein angiographs recorded 1, 2, and 4 weeks after laser application. Photographs of the right (**A**) and left (**C**) eyes are shown. The results of OCT on the indicated days are shown in the same vertical columns for the indicated day (**B**) and (**D**).

numbers of macrophage-like cells were also observed in the neural retina.[21]

In immunostained eyes, vasohibin-1 positivity was found mainly in the CNV especially on the ECs in the CNV (Figure 5B). The regions surrounding the CNV showed little vasohibin-1–positive staining. Some monkeys showed no vasohibin-1 expression by immunohistochemistry even in the CNV at 28 day after laser application. Positive staining for vasohibin-1 appeared to be greater in the more active CNVs (Figure 5A), and it was more obvious in nontreated monkey eyes, although we could not determine whether the staining was significantly greater because only 3 monkey eyes were studied.

### *Vascular Endothelial Growth Factor in Aqueous During Experiments*

The level of VEGF was determined by enzyme-linked immunosorbent assay. The average VEGF level in the aqueous in the vasohibin-1–injected eyes was 15.3 pg/mL, and it was 20.6 pg/mL in the vehicle-treated eyes at 4 days after laser application. The average VEGF level in the vasohibin-1– and vehicle-treated eyes were 7.0 pg/mL and 8.9 pg/mL, respectively, at 4 weeks after laser application (Figure 6). For both times, the differences were not significant.

## Discussion

Our results demonstrated that when 10 μg or 100 μg of vasohibin-1 was injected intravitreally into nontreated normal monkey eyes, a mild anterior chamber inflammation developed. No signs of inflammation or any adverse effects were found when <1 μg of vasohibin-1 was injected into nonlaser treated eyes, although we used only 1 eye for each dose. However when 1 μg of vasohibin-1 was injected into laser-treated eyes, a mild inflammation developed in 1 of the 3 eyes. Inflammation has also been reported in monkey

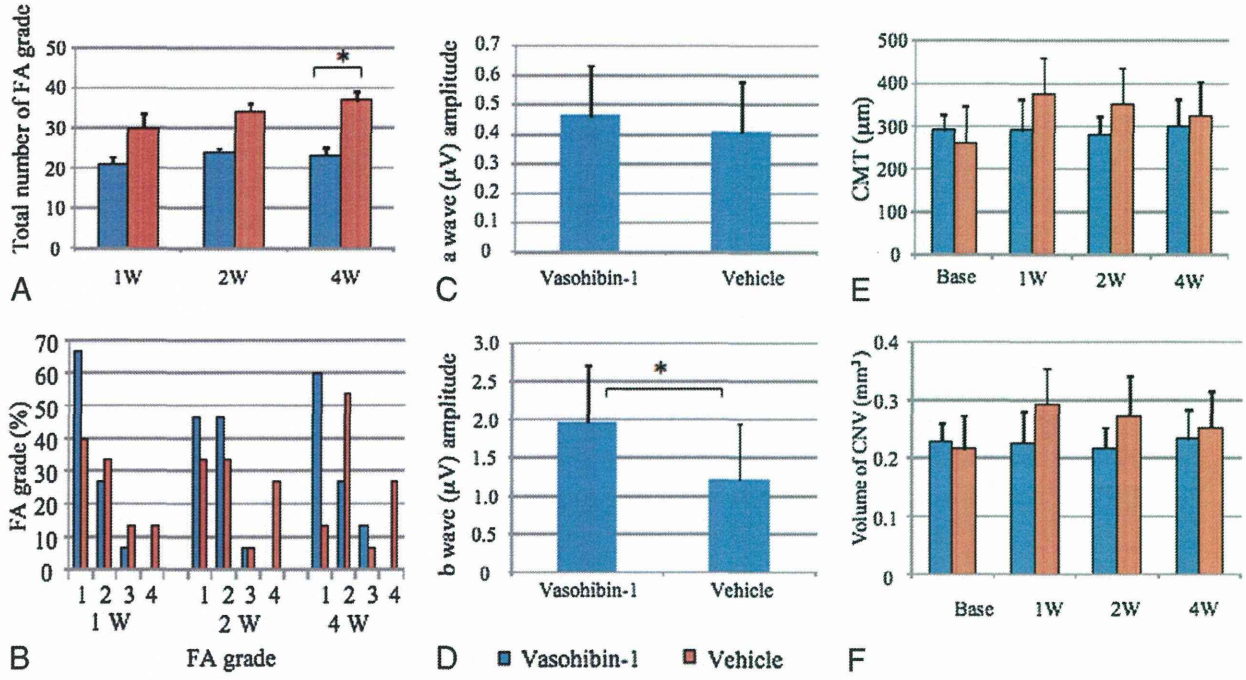


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**Fig. 4.** Results of FA, focal ERG, and OCT are shown. **A.** Significantly less FA leakage was observed after 0.1 μg/50 μL of vasohibin-1 than after vehicle treatment at 4 weeks. **B.** Distribution of Grade 4 FA eyes for each group. **C** and **D.** Average amplitudes of the a-waves (**C**) and b-waves (**D**) of the focal ERGs. **E** and **F.** Average central macular thickness (CMT) and the central 3.4 mm and volume of area of either vasohibin-1–treated (blue) or vehicle-treated (red) eyes before (base) and 1, 2, and 4 weeks after laser application. Lower thickness and volumes were observed in the vasohibin-1–treated monkeys. Data are the standard deviations.

eyes after intravitreal injections of fragments of mouse and human chimera antibodies against VEGF.[22,27]

Fluorescein angiography examination after vasohibin-1 injection in laser-treated eyes showed significantly lower FA scores in eyes that received 0.1 μg and 1 μg of vasohibin-1 than the vehicle-injected eyes, although the number of eyes may have affected the statistics. Fluorescein leakage from the laser spots close to the macula was greater than that of the other laser spots. These results are compatible with the results of Shen et al,[28] who also found that the laser spot was larger and the leakage was greater for lesions closer to the macula. We also found that fluorescein leakage was different among monkeys, even though we applied the same amount of vasohibin-1.[22] This variability may be because the body weight ranged from 4.1 kg to 10.1 kg and age from 4 years to 6 years among the monkeys.

After we injected 0.1 μg of vasohibin-1 3 times in the right eyes and vehicle into the left eyes of 3 monkeys, we found significantly less fluorescein leakage in the vasohibin-1–treated right eyes than in the vehicle-treated eyes. The results of focal ERGs and OCT were well correlated with the results of FA findings, although the quantitative values were not significantly different.

Taken together, these results showed that intravitreal vasohibin-1 is able to reduce the activity of the laser-induced CNV in monkeys. With 3 injections of 0.1 μg of vasohibin-1, the results were not so different from that of only 1 injection at 4 days after the laser application. This may indicate that there may be an optimum time for the vasohibin-1 to affect the course of the laser-induced CNV. Alternatively, the results may be related to the half-life of vasohibin-1.

We found that vasohibin-1 was expressed on ECs especially those in the CNV lesions. Careful examinations showed that vasohibin-1 expression was limited to the CNV lesion and may not show extensive expression in other regions under normal physiologic conditions. Although we have not followed the expression of vasohibin-1 during the course of CNV development in monkeys, vasohibin-1 expression may be enhanced in the new vessels as was reported.[29] The vasohibin-1 expression appeared stronger in nontreated monkey eyes, although this could not be quantified. Vasohibin-1 has been reported to be present on the ECs only in the stroma of tumors and not in the noncancerous regions of the tissue in surgically resected tissues of the same patient.[29] These findings suggest that vasohibin-1 may be expressed mainly in the new vessels as it was in our laser-induced CNVs.

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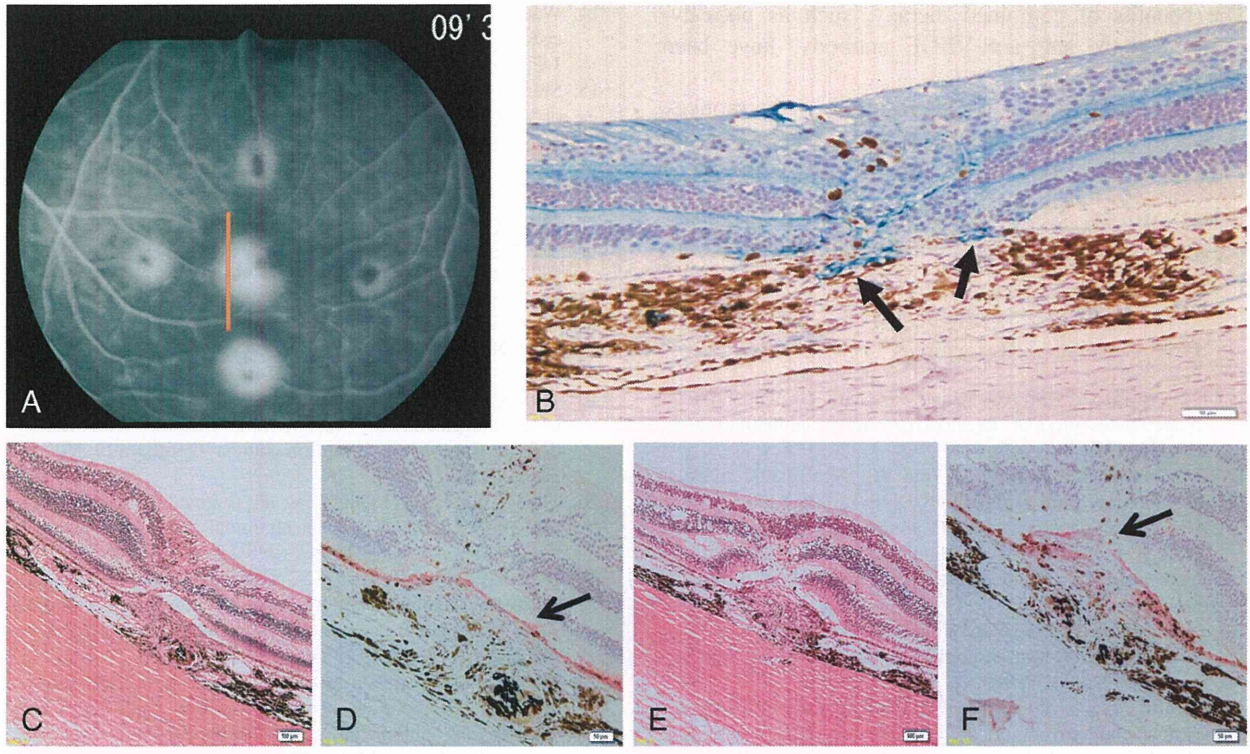


**Fig. 5.** Fluorescein angiograms 4 weeks after laser application or vehicle injection are shown. **A.** Immunohistochemistry for vasohibin-1 (**B**), the same eye as shown in (**A**) at the red line, is shown. Arrows indicate vasohibin-1 labeling. Vasohibin-1 expression is concentrated on the vessels around the CNV (arrows), but markedly less than in the CNV. Vaoshibin-1 expression was observed at active CNV (red line in **A**). The subretinal space is an artifact of histologic processing. Cytokeratin labeling is also shown with vasohibin-treated eye (**D**) and vehicle-treated eye (**F**). Arrows show labeling of cytokeratin. Bar = 50 $\mu$m. **C** and **E.** Hematoxylin and eosin staining of vasohibin-1–treated and vehicle-treated eyes, respectively, are shown. Bar = 100 $\mu$m. Cytokeratin labeling shows that retinal pigment epithelium covers CNV in the vasohibin-1–treated eyes (**D**), and a disruption of cytokeratin labeling is observed in vehicle-treated eye (**F**).

Hosaka et al[29] reported that exogenous vasohibin-1 blocked angiogenesis and maturation of not only the cancerous tissue but also the surrounding vessels and, thus, enhanced the antitumor effects of vasohibin-1. Intravitreal injection of vasohibin-1 may also suppress angiogenesis in CNVs by the same mechanism.

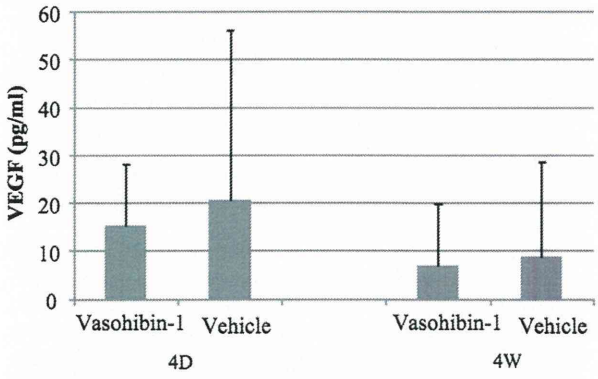


**Fig. 6.** Concentration of VEGF in aqueous in laser-treated monkey eyes 4 days and 4 weeks after laser application is shown. Vertical axis shows VEGF concentration in picograms per milliliter, and horizontal axis is the day of examination. Vascular endothelial growth factor in vasohibin-1–treated eyes (blue boxes) and vehicle-treated eyes (red boxes) show no significant difference at any times.

The amount of VEGF in the aqueous in the vasohibin-1–treated eyes did not differ from that in vehicle-treated eyes. Thus, Zhou et al[30] reported that external vasohibin-1 had no effect on the level of VEGF when they used adenovirus encoding human vasohibin-1 on mouse corneal neovascularization induced by alkali burn. They also reported that vasohibin-1 may downregulate the VEGF receptor 2 (VEGFR2). Shen et al[16] also reported a downregulation of VEGFR2 by vasohibin-1 during mouse ischemic retinopathy. Our previous studies have also shown a downregulation of VEGFR2 by external vasohibin-1 in laser-induced mouse CNVs.[19] Thus, vasohibin-1 may reduce the activity of a CNV by partially downregulating VEGFR2 in the eyes. If this is correct, vasohibin-1 may not affect the favorable aspects of VEGF such as its neuroprotective effect,[31] especially if VEGF works through VEGFR1 rather than VEGFR2. Vasohibin-1 also can be used with anti-VEGF antibody for CNV therapy.

The benefits of combined therapy, such as photodynamic therapy and anti-VEGF antibody, have been discussed.[27]

In conclusion, intravitreal vasohibin-1 in monkey eyes is safe and can reduce the activity of laser-induced CNVs and thus preserve the function of the macula.

**Key words:** choroidal neovascularization, laser-induced, monkey, vascular endothelial growth factor, vasohibin-1.

## References

1. Klein R, Peto T, Bird AC, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004; 137:486–495.
2. Bressler NM, Bressler SB, Fine SL. Age-related macular degeneration. Surv Ophthalmol 1998;32:375–413.
3. Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials Macular Photocoagulation Study Group. Arch Ophthalmol 1986;104: 694–701.
4. Thomas MA, Grand MG, Williams DF, et al. Surgical management of subfoveal choroidal neovascularization. Ophthalmology 1992;99:952–968.
5. Eckardt C, Eckardt U, Conrad HG. Macular rotation with and without counter-rotation of the globe in patients with age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 1999;237:313–325.
6. Reichel E, Berrocal AM, Ip M, et al. Transpupillary thermotherapy of occult subfoveal choroidal neovascularization in patients with age-related macular degeneration. Ophthalmology 1999; 106:1908–1914.
7. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one year results of 2 randomized clinical trials-TAP report Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP). Study Group. Arch Ophthalmol 1999;117:1329–1345.
8. Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. Prog Retin Eye Res 2008;27:372–390.
9. Miller JW, Adamis AP, Shima DT, et al. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 1994;145:574–584.
10. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.
11. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
12. Pilli S, Kotsolis A, Spaide RF, et al. Endophthalmitis associated with intravitreal anti-vascular endothelial growth factor therapy injections in an office setting. Am J Ophthalmol 2008;145:879–882.
13. Lux A, Llacer H, Heussen FMA, Joussen AM. Non-responders to bevacizumab (Avastin) therapy of choroidal neovascular lesions. Am J Ophthalmol 2007;91:1318–1322.
14. Watanabe K, Hasegawa Y, Yamashita H, et al. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. J Clin Invest 2004;114:898–907.
15. Shimizu K, Watanabe K, Yamashita H, et al. Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. Biochem Biophys Res Commun 2005;327:700–706.
16. Shen J, Yang X, Xiao WH, et al. Vasohibin is up-regulated by VEGF in the retina and suppresses VEGF receptor 2 and retinal neovascularization. FASEB J 2006;20:723–725.
17. Sato H, Abe T, Wakusawa R, et al. Vitreous levels of vasohibin-1 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Diabetologia 2009;52:359–361.
18. Wakusawa R, Abe T, Sato H, et al. Expression of vasohibin, an antiangiogenic factor, in human choroidal neovascular membranes. Am J Ophthalmol 2008;146:235–243.
19. Wakusawa R, Abe T, Sato H, et al. Suppression of choroidal neovascularization by vasohibin-1, vascular endothelium-derived angiogenic inhibitor. Invest Ophthalmol Vis Sci 2011;52:3272–3280.
20. Tobe T, Ortega S, Luna JD, et al. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am J Pathol 1998;153:1641–1646.
21. Heishi T, Hosaka T, Suzuki Y, et al. Endogenous angiogenesis inhibitor vasohibin1 exhibits broad-spectrum antilymphangiogenic activity and suppresses lymph node metastasis. Am J Pathol 2010;176:1950–1958.
22. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.
23. Miyake Y, Yanagida K, Yagasaki K, et al. Subjective scotometry and recording of local electroretinogram and visual evoked response. System with television monitor of the fundus. Jpn J Ophthalmol 1981;25:439–448.
24. Kondo M, Ueno S, Piao CH, et al. Comparison of focal macular cone ERGs in complete-type congenital stationary night blindness and APB-treated monkeys. Vision Res 2008;48: 273–280.
25. Hogan MJ, Kimura SJ, Thygeson P. Signs and symptoms of uveitis. I. Anterior uveitis. Am J Ophthalmol 1959;47:155–170.
26. Zhang M, Zhang J, Yan M, et al. Recombinant anti-vascular endothelial growth factor fusion protein efficiently suppresses choroidal neovascularization in monkeys. Mol Vision 2008;14:37–49.
27. Husain D, Kim I, Gauthier D, et al. Safety and efficacy of intravitreal injection of ranibizumab in combination with verteporfin PDT on experimental choroidal neovascularization in the monkey. Arch Ophthalmol 2005;123:509–516.
28. Shen WY, Lee SY, Yeo I, et al. Predilection of the macular region to high incidence of choroidal neovascularization after intense laser photocoagulation in the monkey. Arch Ophthalmol 2004;122:353–360.
29. Hosaka T, Kimura H, Heishi T, et al. Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. Am J Pathol 2009;175:430–439.
30. Zhou SY, Xie ZL, Xiao O, et al. Inhibition of mouse alkali burn induced-corneal neovascularization by recombinant adenovirus human vasohibin-1. Mol Vision 2010;16:1389–1398.
31. Alon T, Hemo I, Itin A, et al. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1995;1:1024–1028.

# 第4章　バイオ・生物製剤への応用に向けた DDS 技術の動向と実用化の可能性

## 第2節バイオ分野に期待される技術の動向

### ［1］細胞製剤技術の現状と実用化の課題

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# 第２節　バイオ分野に期待される技術の動向
## ［1］　細胞製剤技術の現状と実用化の課題

はじめに

理想的な薬の投与方法は，当然ながら薬物を必要な場所に必要な量だけ必要なときに送り込むことであり，DDSはこれらを達成し薬物治療の効率を向上させることを目標に発展してきた。材料化学の進歩によって，薬剤を持続的に放出（徐放）したり，体内半減期の短い薬剤の寿命を延長したり，薬剤の吸収促進，目的部位へのターゲティングなど，さまざまな方法が可能になってきている。しかし，生体内で起こっている現象は単純ではなく，治療の過程において薬剤が必要な場所や量，種類は常にダイナミックに変化しているはずである。このような生体内の現象に迅速に対応できるDDSは現状では１つしか考えられない。それは「細胞」である。生体の基本単位である細胞は，生体の維持のために必要なところに必要な量だけの分化した細胞を生み，必要なときに必要な量だけ生理活性物質を分泌（徐放）して，生体の諸機能を調節しているのであり，まさにDDSを実践していると言える。従って，生きている細胞を薬として扱う「細胞製剤」は理想的な究極の薬物療法と言っても過言ではない。

細胞を用いる方法として，生体から取り出した細胞を元の機能を維持したまま利用する方法や，個々の病態に対応した生理活性物質の分泌機能を遺伝子導入によって新たに付与した機能化細胞を用いる方法がある。さらに，これらの細胞を目的の場所に投与する方法として，単純に細胞懸濁液を注入する方法や細胞を高分子カプセルで免疫隔離する方法，細胞シート化してから移植する方法などがある。ここではこのような細胞を薬の運び屋として利用する「細胞製剤」の現状を紹介する。

### 1.　細胞の機能化

細胞をDDSとして用いるとき，移植した細胞が周囲の環境と調和して生理活性物質を持続的に徐放し，標的部位の治癒に積極的に関与することが期待できる。細胞は生きている限り薬（生理活性物質）を徐放し，さらに１種類ではなく複数の薬を環境に応じて徐放するため，１つや２つの薬を包含した従来の材料ベースのDDSよりもはるかに高性能でインテリジェントな効果が期待できる。さらに遺伝子導入によって人工的に新たな機能を付与された細胞を用いる方法も検討されている。ここでは，眼科領域における細胞製剤（細胞治療）を中心に述べてみたい。

#### 1.1　眼科医療における細胞治療

視覚は我々が外から得る情報の約８割を占めると言われている。視覚の中枢となる網膜は，眼球の後ろ側の内壁を覆う薄い膜状の組織であり，視細胞や神経節細胞等の神経細胞から構成され，視覚的な映像（光の情報）を神経信号（電気信号）に変換し，視神経を通して脳中枢へ伝達する働きを持つ。そのため，加齢黄斑変性症や網膜色素変性症などに代表される網膜変性疾患によって網膜に障害が起こると著しい視覚障害を引き起こし，我々の生活の質を著しく低下させる。一部の網膜変性疾患に遺伝子異常が判明している以外，病態については不明なものが多く，一旦発症すると重篤になるものが多く存在し，有効な治療法がないのが現状である。

網膜色素上皮細胞（Retinal pigment epithelium；RPE）は網膜を構成する細胞の中でも，上皮細胞の性質を持つことから神経細胞よりも取扱いが容易であるため，RPEを網膜下に移植して網膜疾患を治療する試みが行われてきた。RPEは視細胞と脈絡膜（血管組織）の間に位置する１層の上皮組織であり，視細胞への酸素や栄養供給など神経網膜の代謝と維持に重要な役割を果たしている。１日に視細胞外節の約10％を貪食し視細胞をリフレッシュしていると言われており，人体の中でも酸素消費量の多い組織である。加齢に伴うRPEの異常によって脈絡膜より新生血管が出現して視細胞を障害する加齢黄斑変性やRPEの遺伝子異常によって発症する網膜色素変性症など難治性網膜疾患と深く関わる組織である。

RPE移植の臨床応用は，1991年以降複数の施設より報告されている。RPE移植は，障害された，もしくは失われ