別紙３

厚生労働科学研究費補助金（障害者対策総合研究事業）
分担研究報告書

新規薬剤の生体内スクリーニングシステムの確立と
網膜保護用デバイスの開発に関する研究

研究分担者　永井　展裕　東北大学大学院医学系研究科 助教

剤についても同様の検討を行う。動物モデルは、網膜光障害モデルや網膜変性ラット（RCSラット）、トランスジェニック網膜色素変性ウサギなどを使用する。

Ｅ．結論
眼内注射に代わる眼内への安全な薬物投与方法として我々のデバイスが使用できる可能性を示した。

Ｆ．健康危険情報
該当なし

Ｇ．研究発表
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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３

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新規薬剤の生体内スクリーニングシステムの確立と網膜保護用デバイスの開発に関する研究

研究分担者　西澤松彦　東北大学大学院工学研究科　教授

研究要旨：
本研究は、比較的短期間で実現可能な既存薬や安全性が担保された薬剤ライブラリーを用いた神経保護薬剤スクリーニングとドラックデリバリーシステム（DDS）を確立することが目的である。分担研究として H23 年度は、将来的に人に応用するための検討として、DDS の形状を微細加工によって最適化し、サル眼に移植可能なデバイスを作製することを検討した。デバイスは眼球強膜上に移植するため、眼球面に密着する形状、強膜上に固定するための縫合用穴・溝、後眼まで挿入するためのデバイスの長さ、の検討を行った。その結果、直径 2cm のボールにフィットするように湾曲させ、かつデバイス長さは約 15 ㎜、縫合用にデバイス側面に 4 つの溝を設けた形状が現状では最適という結果を得た。

A．研究目的

本研究は、比較的短期間で実現可能な既存薬や安全性が担保された薬剤ライブラリーを用いた神経保護薬剤スクリーニングとドラックデリバリーシステム（DDS）を確立することが目的である。将来的に人に応用するための検討として、DDSの形状を微細加工によって最適化し、サル眼に移植可能なデバイスを作製することを検討した。デバイスは眼球強膜上に移植するため、眼球面に密着する形状、強膜上に固定するための縫合用穴・溝、後眼部まで挿入するためのデバイスの長さ、の検討を行った。

微細加工は切削装置のMicroMC-2 (PMT Co.) を使用した。これはマイクロ単位でアクリル板上にCAD (Computer aided design) でデザインした設計図を切削することができる。デバイスの形状をCADで作製し、アクリル板に掘って鋳型を作製し、これをもとにPDMS（ポリジメチルシロキサン）に鋳型を転写し、この2次鋳型を用いて、DDSの基材であるPEGDM(ポリエチレングリコールジメタクリレート)を光重合し、デバイスを作製している。

今回は眼科医（共同研究者）の意見を機器ながらデバイスを試作し、サル眼への移植でデバイス形状を微修正しながら、ヒト眼に移植できるようなデバイスデザインを検討した。

B．研究方法

１．デバイス作製用PDMS鋳型の作製

アクリル板にデバイスのリザーバー形状を切削した。このアクリル板にPDMSを乗せ、60℃でPDMSを硬化し、リザーバー形状をPDMSに転写した。このPDMSをシラン化処理した。以下、シラン化処理を示す。PDMSをエタノール、蒸留水の順で10分間ずつ超音波洗浄し、オーブンで乾燥した。プラズマアッシャー（YHS-R）で30秒間酸素プラズマ処理を施した。プラズマ処理したPDMSをシャーレに置き、ドラフト内でシラン（1H,1H,2H,2H-PERFLUOROOCTYL TRICHLOROSILANE、WAKO）を2ヶ所に2 μlずつPDMSに付かないように垂らし、ふたをして１時間以上静置した。

シラン化処理したPDMS上に別のPDMSを乗せて、60℃で硬化した。このPDMS鋳型が最終形である。

２．デバイス（リザーバー）の作製

PDMS鋳型に、TEGDM 1mlに2-Hydroxy-2-methyl-propiophenone 10μlを混合したプレポリマーを流し、UV架橋(25mW/cm2 - 3 min [SEN LIGHTS CORP])した。

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研究分担者　西澤松彦　東北大学大学院工学研究科　教授

３．移植用デバイスの作製
TEGDMリザーバーにモデルドラッグフルオレセイン（50mg／ml）を充填し、PEGDM/TEGDMプレポリマーで蓋をした。

４．サル眼への移植
ニホンサルの強膜上にデバイスを移植し縫合した。定期的に眼底検査を行い、眼内への副作用を検討した。

（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。

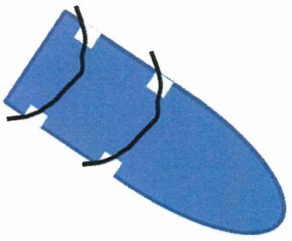
Ｃ．研究結果
１．デバイス鋳型の作製
CADを利用して、サル眼用のデバイス（リザーバー）鋳型を作製した（図１）。直径2センチの球にフィットするようにデザインした。リザーバーは20μLの薬剤ペレットが詰めることができるようにデザインした。また、強膜上に挿入する際に周囲の組織に傷をつけないように、デバイス先端は角のないサークル形状にデザインした。プレポリマーをPDMS鋳型①に流したあと、PDMS鋳型②で蓋をしてUV照射することでリザーバーを作製することができる。

図１．サル眼用デバイス（リザーバー）のPDMS鋳型。プレポリマーを①に流した後、②を①の上に乗せてUV硬化する。

２．デバイス形状の最適化
デバイス内のリザーバー（薬物搭載部分）が後眼部へ届く形状を目指した。また、様座な眼球の局面に対応できるように、デバイスの湾曲角度の変更を検討した。

３．サル眼への移植検討
サル眼へ移植した結果、後眼部に届く長さは約15㎜（図２の長さ②）、角度は直径2cmの球にフィットする角度が現状で最適と判断した。
縫合用の形状として、当初は穴を１つ設けたが、1点で縫合した場合、デバイス先端が動いたり、浮いて強膜に密着しない、という課題があった。そこで、穴ではなく、デバイスの側面に４つの溝を掘り、4点で縫合する形状を採用した（図４）。これによって強膜に密着させることができた。密着によって、薬剤が周囲へ漏れることや、Fibrosisが徐放面に侵入することを防ぐ効果があると考えられる。

図２．サル眼用デバイスの最終プロトタイプ



移植4か月目のサル眼結膜周囲の写真を示す（図３）。デバイス移植直後は軽い炎症を伴うが、約1か月の移植では、眼底にも問題はなく、副作用はないと考えている。

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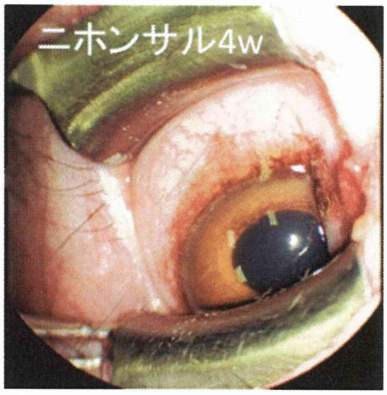

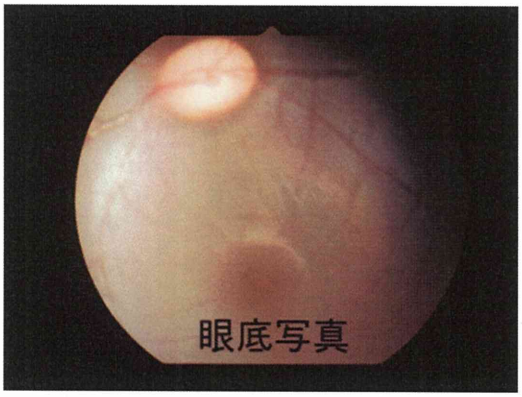

図３．サル眼移植4週目の移植部位周辺の写真と眼底写真。

D．考察

加齢黄斑変性症では、黄斑部周囲に薬剤を届ける必要があるため、できるだけ後眼部へデバイスのリザーバー部位を挿入する必要がある。また、徐放面が強膜に密着しなければ、Fibrosisが徐放面に侵入し薬剤が吸収されたり、デバイスと強膜の隙間から薬剤が逃げて結膜へ吸収され、薬剤送達効率が悪くなる可能性がある。最終プロトタイプでは、強膜への密着が強化され、徐放面が後眼部付近まで届くように設計されており、加齢黄斑変性症の治療に対して有効に働く可能性がある。

今後はモデルドラッグで眼内への移行性、薬物分布を評価し、移行が確認できたら、実際の薬物でレーザー誘発脈絡膜新生血管モデルサルに対する治療効果を評価する予定である。

E．結論

サル眼の後眼部に薬剤をデリバリーできる形状のデバイスを開発した。実際の薬物で前臨床試験を評価する準備ができたと考えている。

F．健康危険情報

該当なし

G．研究発表

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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４

研究成果の刊行に関する一覧表（阿部俊明）

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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-μm 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 μg of vasohibin-1/50 μL 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 μg of vasohibin-1/50 μL of vehicle 4 days after the laser burn. The third group of 3 eyes had 3 injections of 0.1 μg of vasohibin-1/50 μL of vehicle in the right eyes and 50 μL 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 bandpass 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-μm 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 μL 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

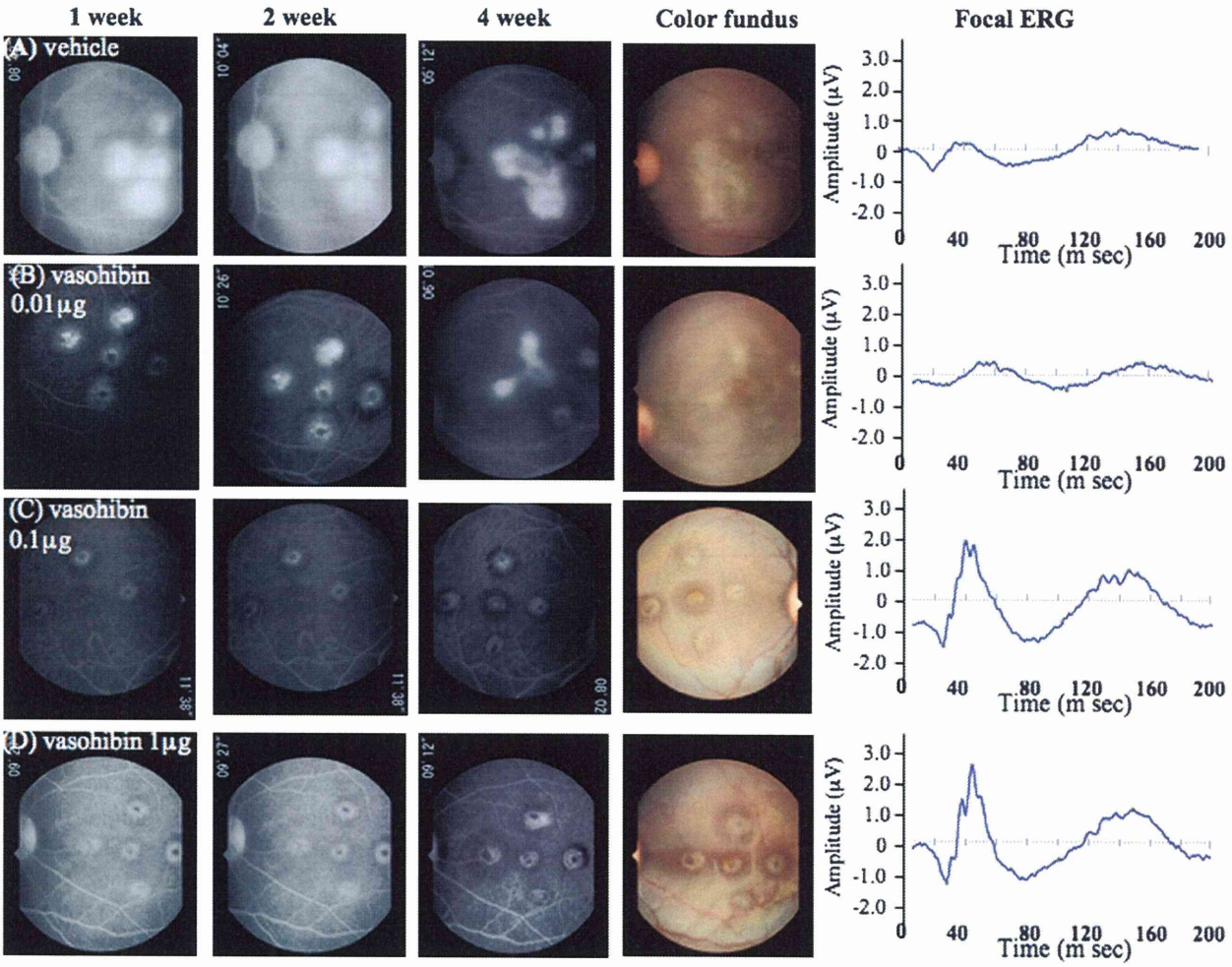
Safety Evaluations and Outcomes

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 μg of vasohibin-1/50 μL. After 0.01, 0.1, and 1 μg of vasohibin-1, the appearance of the retina and choroid did not differ from that of the vehicle-injected eyes. When 10 μg or 100 μg/50 μL 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 μg than with 100 μg of vasohibin, and the inflammation was resolved in 2 days after 10 μg and in 1 week after 100 μg (Table 1). When we injected 1 μg/50 μL 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 μg of vasohibin-1 was injected even after 3 injections. When we injected 50 μL of vehicle with almost the same amount of endotoxin (400 U/mL) as that of 100 μg of vasohibin-1, no inflammation was detected. These results indicated that mild inflammation can develop with $\geq$10 μg 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 μg to 100 μg of vasohibin-1 did not differ significantly from the vehicle-injected eyes. The a-wave amplitudes ranged from 87.3 μV to 180.3 μV (average, 119.3 ± 36.6 μV) before and from 100.7 μV to 195.8 μV (average, 131.3 ± 53.7 μV; $P = 0.444$) after the vasohibin-1 injection. The b-wave amplitudes ranged from 219.6 μV to 340.6 μV (average 250.6 ± 54.7 μV) before and from 240.8 μV to 345.2 μV (average 274.4 ± 82.0 μV, $P = 0.801$) 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 μg of vasohibin-1/50 μL 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 μg of vasohibin-1/50 μL 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.



COLOR

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.

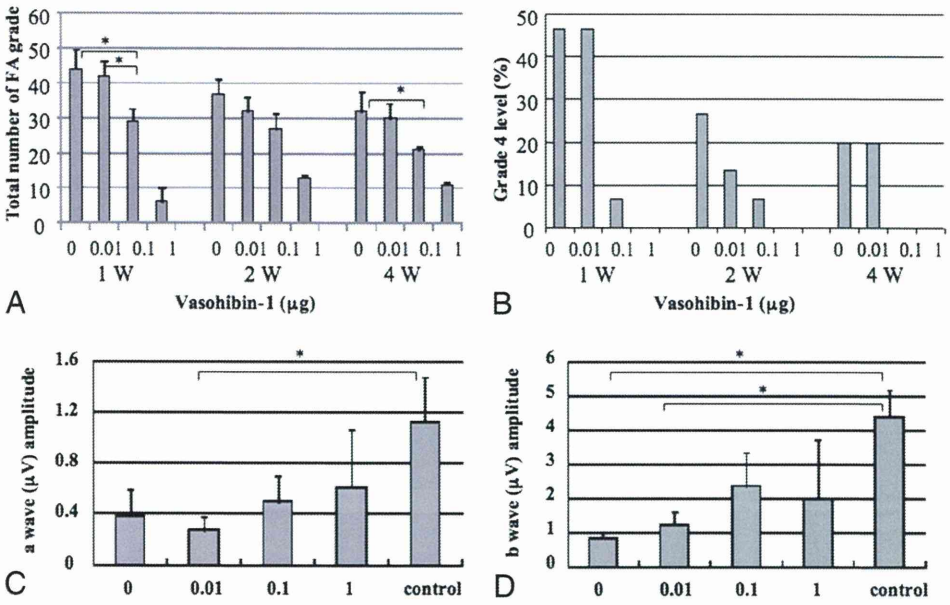


Fig. 2. Fluorescein angiographic scores for each of the 5 laser spots in each eye are plotted for each group, and the amplitudes of the a- and b-waves of the focal ERGs. **A.** Fluorescein angiographic scores for each of the five laser spots in each eye are plotted for each group. Statistically significant 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 μg 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 μg of vasohibin-1/50 μL 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 μg of vasohibin-1/50 μL 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 μg of vasohibin-1/50 μL 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 μg of vasohibin-1/50 μL 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