がコントロールと比較して維持されていた。また、TUNEL染色の結果、UNOデバイス移植群では、TUNEL-positive（アポトーシス）細胞数が少なかった。
以上より、UNO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UNOの放出条件と関係があることが示唆された。

（５）網膜色素変性モデル実験

P40カバーのUNO徐放デバイスをラット強膜上に移植し、1週間後と4週間後にERG検査を行った。1週後においては、未処置群と比較してUNOデバイス移植群は有意にERG振幅値が維持されていた。また、UNO硝子体注射群も維持されていた。一方、点眼群では保護効果は認めなかった。4週後においては、未処置群と比較してUNOデバイス移植群はERG振幅値が維持されていたが、他の群では維持が認められなかった。
以上よりUNO徐放デバイスは持続的に網膜変性を抑制する可能性が示唆された。

D．考察

本研究は、デバイス中の薬物ペレットおよびカバー（徐放膜）のPEGDM/TEGDM比率を変えることによって、UNOを異なる任意の速度でリリースできることを示した。また、UNO徐放デバイスは網膜光障害ラットおよび網膜色素変性ラットに対して網膜保護効果を示すことが示唆された。このUNOの網膜保護効果はIn vitro網膜細胞培養でも確認することができた。

UNOの薬理作用は不明な点が多かったが、最近になってイオンチャネル開口の作用が報告され、Caイオンの細胞内濃度を下げることによって細胞死を抑制することが示唆されている。本研究において、UNOは細胞死マーカーであるp38のリン酸化を抑制した。今回の研究において網膜細胞内のCaイオン濃度については不明であったが、イオンチャネル開口による細胞関連シグナルの抑制が網膜保護効果に寄与している可能性がある。Caイオン濃度の測定は今後の課題の1つである。また、本研究において、UNOによってROS産生が抑制されることが示唆された。ROSは酸化ストレスの1つであり、細胞障害性を有するため、ROS産生抑制が網膜保護に寄与している可能性がある。

S334terラットの研究では、UNO徐放デバイスの薬効持続性が示唆された。従来の点眼では網膜へ十分なUNOが届いていない可能性があり、本デバイスによる経強膜投与は効果的な網膜保護投与方法として期待できる。また、硝子体注射では1週間の薬効を示したが、4週後には効果が認められず、再注射が必要と考えられる。しかし、頻回の眼内注射は眼内感染症等の重篤な眼内副作用を惹起する可能性があるため、本デバイスによる経強膜投与は安全で持続的な投与方法として期待ができる。

E．結論

UNO徐放デバイスを作製し、網膜変性モデル動物でその薬効を評価した。また、UNOの細胞保護作用として、細胞死関連シグナルとROS産生の抑制が示唆された。また、点

眼や硝子体注射よりも持続的に網膜変性を抑制する可能性が示唆された。

Ｆ．健康危険情報
該当なし

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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

研究報告書

厚生労働科学研究費補助金（難治性疾患等克服研究事業（難治性疾患克服研究事業）研究事業）
（分担）研究報告書

動物実験によるデバイスの評価に関する研究

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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、ウノプロストン（UNO）を徐放することである。本分担研究は、ウノプロストン徐放デバイスの眼局所毒性についてSDラットおよび白色ウサギを用いて検討した。また、ウノプロストンの眼内移行量を白色ウサギを用いて評価した。眼毒性評価として、SDラットに移植後4週目まで定期的に網膜電図を評価した結果、未処理群やプラセボ群（PBS含有デバイス）と比較してUNO徐放デバイスによる網膜機能の変化はなかった。また、ウサギに移植後5か月目まで定期的に網膜電図を評価した結果、ラットと同様に未処理群、プラセボ群と比較して網膜機能に変化はなかった。白色ウサギにデバイス移植後1、４、７日の網膜、血漿中のUNO濃度をLC/MS/MSで測定を試みたが、UNO活性体（M1）の網膜内量が測定でき、点眼より多い量が持続的に網膜で確認され、また血漿中の最大量はむしろ点眼より低い傾向であった。

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、ウノプロストン（UNO）を任意の速度で徐放できる徐放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、動物実験によるUNO徐放デバイスの眼局所毒性評価およびUNO眼内移行性を評価した。

視覚はすべての情報の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。網膜疾患は加齢に伴い増えるため、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、まず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視覚中枢へ情報が伝えられる。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他覚的網膜機能評価方法である。
本分担研究では、ERG と組織学的評価と同等な評価ができる光干渉断層計（OCT)を用いてデバイスの眼局所毒性を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。また、眼内への UNO 移行量を LC/MS/MS 法によって網膜組織中の UNO の代謝産物を定量することによって評価した。

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 分加熱した硬化させた。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（25mW/cm2、3min [SEN LIGHTS CORP]）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、ウサギ用は幅 4.4mm×長さ 12mm×高さ 1.6mm、薬剤充填部容量は 20μl、ラット用は幅 2mm×長さ 2mm×高さ 0.6mm、薬剤充填部容量は 1.2μl である。
UNO を PEGDM と TEGDM の混合プレポリマー（PEGDM 40%/TEGDM 60% : P40）に混合し、リザーバーにキャストして UV 硬化（10mW/cm^2、0.5min）して作成した。
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 UNO を充填したリザーバー上にプレポリマーを滴下し、ガラス板でカバーした後、UV 硬化して作成した。
プラセボデバイスとして、Phosphate-buffered saline (PBS)を充填したデバイスを作製した。

（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。200-250g の雄の SD ラット、1.5-2kg の日本白色ウサギを使用した。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）の筋肉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、ラットおよびウサギ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縫合固定した。結膜を縫合し、タリビッド眼軟膏を点入し終了とした。

（４）ERG
コンタクトレンズ電極（ラット：2mm ベースカーブ、ウサギ：7.8mm 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/m2, 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。

（５）UNO 眼内量測定
UNO 徐放デバイスとして、3 種類の徐放膜（P60、P40、P20）でカバーしたデバイスを移植し、UNO 徐放性と UNO 眼内移行性の関係を評価した。移植 1、4、8 日目に動物を過剰麻酔で安楽死後、血液と眼球を摘出した。血液は遠心して血漿をサンプリングした。眼球は前房水を採取後、角膜、水晶体、硝子体、網膜、脈絡膜、強膜に分離し、網膜のホモジネートと血漿を LC/MS/MS で UNO の活性体（M1)測定に供した。

（６）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
デバイスの移植操作に問題はなかった。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見られなかった。デバイスの形状が眼球に Fit しないものなど、特に早期に作製したデバイスの一部は脱落もみられたが、デバイスの形状改善で脱落は無くなった。

（２）ERG
SD ラットへの移植では移植 4 週間目まで定期的に ERG を評価した。その結果、未処理群や UNO 非徐放デバイス移植群と比較して、UNO 徐放デバイス群では ERG の a,b 波振幅値や潜時に変化はなく、網膜機能の変化はないと推定された。
白色ウサギへの移植では移植 5 か月目まで定

別紙３

期的に ERG を評価した。その結果、ラットと同様に未処理群やプラセボデバイス移植群と比較して、UNO 徐放デバイス群では ERG の a,b 波振幅値や潜時に変化はなく、網膜機能の変化はないと推定された。

（３）UNO の眼内移行

３種類の徐放性の異なるデバイスを移植し、UNO 眼内移行性を評価した結果、放出が多いデバイスほど、網膜へ UNO が移行していることがわかった。また、過去の点眼による UNO 眼内移行データと比較した。点眼では 30 分後が最大であったが、P40 カバーデバイスでは、持続的に点眼と同等量以上が網膜へ移行していることがわかった。この移行は移植 8 日目にも観察され、本デバイスは点眼と同じ薬効濃度を持続的に維持できることが示唆された。
また、血漿中の UNO 量は点眼と比較して低いことがわかった。さらに、前房水への UNO 移行はほとんどなく、反対眼への移行もほとんど確認できなかった。すなわち、投与部位周辺に局所的に UNO を持続的投与できる可能性が示唆された。

D．考察

本研究は UNO 徐放デバイスの眼局所毒性評価として ERG による評価を行った。プラセボデバイスおよび UNO 徐放デバイスいずれにおいても網膜機能の低下は認められず、デバイス自体の毒性および UNO の持続徐放の毒性はないことが示唆された。デバイス中には PEGDM および TEGDM モノマーがわずかに残留することがわかっているが、その量は別研究の In vitro 細胞培養毒性実験の結果、毒性を示す濃度の 1 万分の 1 であり、さらに 1 週間後には残留モノマーは完全に溶出するため、長期の移植おいて毒性を示す可能性は低かったと考えられる。実際に 5 か月間移植していたウサギの ERG において、デバイスの毒性は認められなかった。また、UNO は点眼おいて結膜の発赤が見られることがあるが、デバイス移植群ではこれは見られないか、見られても極経度であった。また、UNO に伴う眼圧の変化も認められなかった。UNO 眼内移行のデータから、本デバイスは移植部位への局所移行性が高く、前眼部への移行や血漿、対眼への移行がほとんどなかったことから、結膜など周辺組織への影響が小さかったと推定される。これはデバイスのリザーバーが UNO を透過しない仕組みになっており、結膜側への放出はなく、強膜側一方向性の徐放を示すことが寄与していると考えられる。

E．結論

本研究は UNO 徐放デバイスの眼毒性評価を ERG によって評価した。SD ラットおよび白色ウサギのいずれにおいても、デバイス移植に伴う網膜機能の低下は認められず、また移植部位周辺に炎症や眼内への副作用はなく、局所毒性は低いことが示唆された。また、本デバイスは点眼と同程度の薬効濃度を持続的に網膜へ投与できる可能性が示された。

F．健康危険情報

別紙3

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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別紙４

研究成果の刊行に関する一覧表（大浪　英之）

雑誌

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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

Transscleral Sustained Vasohibin-1 Delivery by a Novel Device Suppressed Experimentally-Induced Choroidal Neovascularization

Hideyuki Onami[1,2], Nobuhiro Nagai[1], Hirokazu Kaji[3], Matsuhiko Nishizawa[3], Yasufumi Sato[4], Noriko Osumi[5], Toru Nakazawa[2], Toshiaki Abe[1]*

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Abstract

We established a sustained vasohibin-1 (a 42-kDa protein), delivery device by a novel method using photopolymerization of a mixture of polyethylene glycol dimethacrylate, triethylene glycol dimethacrylate, and collagen microparticles. We evaluated its effects in a model of rat laser-induced choroidal neovascularization (CNV) using a transscleral approach. We used variable concentrations of vasohibin-1 in the devices, and used an enzyme-linked immunosorbent assay and Western blotting to measure the released vasohibin-1 (0.31 nM/day when using the 10 μM vasohibin-1 delivery device [10VDD]). The released vasohibin-1 showed suppression activity comparable to native effects when evaluated using endothelial tube formation. We also used pelletized vasohibin-1 and fluorescein isothiocyanate-labeled 40 kDa dextran as controls. Strong fluorescein staining was observed on the sclera when the device was used for drug delivery, whereas pellet use produced strong staining in the conjunctiva and surrounding tissue, but not on the sclera. Vasohibin-1 was found in the sclera, choroid, retinal pigment epithelium (RPE), and neural retina after device implantation. Stronger immunoreactivity at the RPE and ganglion cell layers was observed than in other retinal regions. Significantly lower fluorescein angiography (FA) scores and smaller CNV areas in the flat mounts of RPE-choroid-sclera were observed for the 10VDD, VDD (1 μM vasohibin-1 delivery device), and vasohibin-1 intravitreal direct injection (0.24 μM) groups when compared to the pellet, non-vasohibin-1 delivery device, and intravitreal vehicle injection groups. Choroidal neovascularization can be treated with transscleral sustained protein delivery using our novel device. We offer a safer sustained protein release for treatment of retinal disease using the transscleral approach.

Citation: Onami H, Nagai N, Kaji H, Nishizawa M, Sato Y, et al. (2013) Transscleral Sustained Vasohibin-1 Delivery by a Novel Device Suppressed Experimentally-Induced Choroidal Neovascularization. PLoS ONE 8(3): e58580. doi:10.1371/journal.pone.0058580

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Competing Interests: The authors have declared that no competing interests exist.

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These authors contributed equally to this work.

Introduction

Age-related macular degeneration (AMD) is a well-known sight-threatening disease in developed countries [1]. Although many treatment regimens have been used to treat AMD [2–6], intravitreal injection of anti-vascular endothelial growth factor (VEGF) produced lesion improvement and better visual acuity in some patients [7,8]. However, intra-vitreal injection of anti-VEGF also produced irritation, infection, and other adverse side effects [9]. Further, that treatment required repeated injections, usually occurring once a month [7,8]. Thus, other types of drugs or drug delivery systems (DDSs) need to be developed to treat AMD.

Eye drops and systemic drug administration are unsuitable for retinal diseases if the physician is looking for effective drug penetration into the eye, especially for macular diseases such as AMD [10,11]. Although drug delivery device implantation into the vitreous showed effective delivery of drug to the retina, these treatments may cause severe side effects, such as infection, vitreous hemorrhage, or retinal detachment [12–14]. Drug delivery using viral vectors has been attempted for treatment of devastating retinal diseases [15]; however, this method may induce immune cell or humoral responses [16,17].

Subconjunctival drug delivery is less invasive than intravitreal drug injection and can deliver more drug than seen with eye drops or systemic administration [10,11]. There are published data investigating clinical use of subconjunctival drug administration [18,19]. Thus, the subconjunctival route may be an attractive method for drug delivery to the retina. The major difficulties with subconjunctival DDS are uncontrollable release of the target drug [20], as well as an unknown drug delivery route and mechanism to

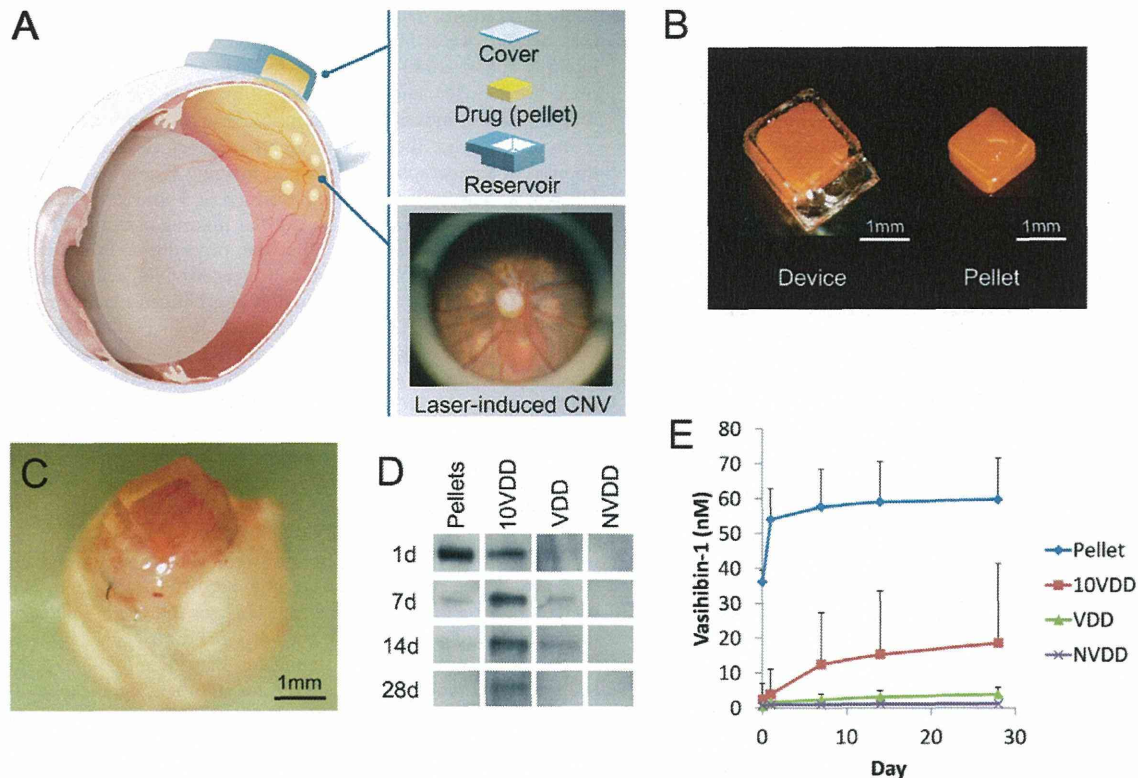

Figure 1. Device and vasohibin-1 release. (A) Schematic image of transscleral sustained vasohibin-1 delivery. We evaluated its effects via transscleral approach for rat laser-induced choroidal neovascularization (CNV). The device consists of a drug pelletized with PEGDM, a reservoir made of TEGDM, and a controlled-release membrane made of PEGDM that contains collagen microparticles. (B) Photograph showing a drug pellet and the delivery device containing a drug pellet. (C) Image of a device placed on the sclera of a rat eye at 3 days after implantation. The amount of vasohibin-1 in the PBS was measured at 1, 7, 14, and 28 days after starting incubation. The representative results of western blotting and the result of ELISA are shown in (D) and (E), respectively. We collected the samples at only the given time points and replaced only the equal volume of PBS. The released vasohibin-1 amounts accumulated for 6, 7, and 14 days. [The pellet samples collected at Day 1 (shown as 1d) were diluted five times due to their concentration before they were evaluated by western blotting]. NVDD: non-vasohibin-1 (vehicle) delivery device, VDD: 1 μM vasohibin-1 delivery device, 10VDD: 10 μM vasohibin-1 delivery device, Pellets: vasohibin-1 pelletized at the same concentration of 10VDD (without reservoir and cover).
doi:10.1371/journal.pone.0058580.g001

injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), and the animals' pupils were dilated with topical 2.5% phenylephrine and 1% tropicamide. Oxybuprocaine hydrochloride (0.4%) was also used for local anesthesia. In all *in vivo* experiments, the animal's left eye was used as a control.

2 Implantation of VDDs, Pellets, and Intravitreal Vasohibin-1 Injection. Devices were implanted subconjunctively in the right eyes of the rats (Table 1). A 4-mm long conjunctival incision was made along the limbus in the upper temporal position. The devices were inserted into the subconjunctival space using forceps, with the drug-releasing surface facing the sclera. The device was placed between the optic disc and the equator, in the posterior quadrant, using no suture to anchor it into place. The conjunctival incision was closed with 9–0 silk and antibiotic ointment was applied to the eyes. Vasohibin-1 protein (0.24 μM) was injected using a 10-μL glass syringe (Hamilton; Reno, NV) 4 days after the experimental CNV procedure. The left eyes were used as untreated controls.

The rats were anesthetized, pupils were dilated, and a fundus examination was performed immediately after the surgery.

Experiment 1: Monitoring the Implanted Devices and Pellets

To monitor the device and drug release, fluorescein isothiocyanate (FITC) dextran (FD40; Sigma-Aldrich) pelletized with PEGDM was prepared and used as a control drug. The FD40 was dissolved in PBS at a concentration of 250 mg/mL and loaded in the device in the same way as vasohibin-1. Eight SD rats were included in this experiment; 4 rats received the FD40 delivery device (FD40DD) and 4 rats received only pelletized FD40.

Experiment 2: Immunohistochemistry after Device Implantation

Immunostaining for vasohibin-1was performed 2 weeks after device implantation. Twelve SD rats were used as follows (Table 1): 4 rats received vehicle (non-vasohibin-1) in the delivery device on the sclera (NVDD), 4 rats received 1.5 μL of 10 μM vasohibin-1 in the delivery device (10VDD), and 4 rats received 1.5 μL of 10 μM vasohibin-1 pellets implanted on the sclera. Immunohistochemistry was performed as reported previously [25].

Animals were euthanized using overdoses of ketamine hydrochloride and xylazine hydrochloride. The eyes were enucleated

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Transscleral Sustained Vasohibin-1 Delivery by a Novel Device Suppressed Experimentally-Induced Choroidal Neovascularization

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Abstract

We established a sustained vasohibin-1 (a 42-kDa protein), delivery device by a novel method using photopolymerization of a mixture of polyethylene glycol dimethacrylate, triethylene glycol dimethacrylate, and collagen microparticles. We evaluated its effects in a model of rat laser-induced choroidal neovascularization (CNV) using a transscleral approach. We used variable concentrations of vasohibin-1 in the devices, and used an enzyme-linked immunosorbent assay and Western blotting to measure the released vasohibin-1 (0.31 nM/day when using the 10 μM vasohibin-1 delivery device [10VDD]). The released vasohibin-1 showed suppression activity comparable to native effects when evaluated using endothelial tube formation. We also used pelletized vasohibin-1 and fluorescein isothiocyanate-labeled 40 kDa dextran as controls. Strong fluorescein staining was observed on the sclera when the device was used for drug delivery, whereas pellet use produced strong staining in the conjunctiva and surrounding tissue, but not on the sclera. Vasohibin-1 was found in the sclera, choroid, retinal pigment epithelium (RPE), and neural retina after device implantation. Stronger immunoreactivity at the RPE and ganglion cell layers was observed than in other retinal regions. Significantly lower fluorescein angiography (FA) scores and smaller CNV areas in the flat mounts of RPE-choroid-sclera were observed for the 10VDD, VDD (1 μM vasohibin-1 delivery device), and vasohibin-1 intravitreal direct injection (0.24 μM) groups when compared to the pellet, non-vasohibin-1 delivery device, and intravitreal vehicle injection groups. Choroidal neovascularization can be treated with transscleral sustained protein delivery using our novel device. We offer a safer sustained protein release for treatment of retinal disease using the transscleral approach.

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Introduction

Age-related macular degeneration (AMD) is a well-known sight-threatening disease in developed countries [1]. Although many treatment regimens have been used to treat AMD [2–6], intravitreal injection of anti-vascular endothelial growth factor (VEGF) produced lesion improvement and better visual acuity in some patients [7,8]. However, intra-vitreal injection of anti-VEGF also produced irritation, infection, and other adverse side effects [9]. Further, that treatment required repeated injections, usually occurring once a month [7,8]. Thus, other types of drugs or drug delivery systems (DDSs) need to be developed to treat AMD.

Eye drops and systemic drug administration are unsuitable for retinal diseases if the physician is looking for effective drug penetration into the eye, especially for macular diseases such as AMD [10,11]. Although drug delivery device implantation into the vitreous showed effective delivery of drug to the retina, these treatments may cause severe side effects, such as infection, vitreous hemorrhage, or retinal detachment [12–14]. Drug delivery using viral vectors has been attempted for treatment of devastating retinal diseases [15]; however, this method may induce immune cell or humoral responses [16,17].

Subconjunctival drug delivery is less invasive than intravitreal drug injection and can deliver more drug than seen with eye drops or systemic administration [10,11]. There are published data investigating clinical use of subconjunctival drug administration [18,19]. Thus, the subconjunctival route may be an attractive method for drug delivery to the retina. The major difficulties with subconjunctival DDS are uncontrollable release of the target drug [20], as well as an unknown drug delivery route and mechanism to

reach the retina [20,21]. Sustained release, with no drug bolus effect, would be required to reduce side effects [22,23].

We previously reported our results of the use of a novel drug delivery device placed on the sclera that we thought would be an effective tool in treating retinal diseases [24]. The device consisted of a drug-releasing semi-permeable membrane and impermeable membranes acting as the drug reservoir. Because of the non-biodegradable and one-way release nature of the device, we could achieve sustained release of the drug to the retina. We examined the effects of this device using a laser-induced choroidal neovascularization (CNV) model in rats.

Anti-VEGF antibody is a well-known treatment agent in CNV therapy, but suppression of VEGF function may induce many harmful effects in physiological function [25]. We selected vasohibin-1 for the loading drug in the device in this study because of its well-known anti-angiogenic activity [26,27]. Vasohibin-1 is a 42-kDa polypeptide, a VEGF-inducible molecule expressed by cultured human endothelial cells (ECs) [26]. Vasohibin-1 inhibits the formation of EC networks *in vitro*, corneal neovascularization *in vitro* [26], retinal neovascularization in a mouse model of oxygen-induced ischemic retinopathy [27], and laser-induced mouse [25] and monkey CNV [28]. Each of the *in vivo* studies treated the tissue by direct intravitreal injection of vasohibin-1.

Here we shall show that continuous trans-scleral vasohibin-1 delivery by the device can suppress laser-induced CNV in rat eyes (Fig. 1A) as well as that by intravitreal injection. This technique and device may hold promise for safer and more effective treatment of patients with AMD.

Methods

Vasohibin-1 and Device Preparation

Vasohibin-1 was purified as reported previously [25]. For the preparation of the vasohibin-1 formulation, an 80-μL volume of vasohibin-1 (either 1.25 or 12.5 μM) in vehicle (phosphate buffered saline [PBS] control) was mixed with 20 μL of polyethylene glycol dimethacrylate (PEGDM), then underwent UV curing at an intensity of 11.5 mJ/cm^2 (Lightningcure LCS; Hamamatsu Photonics, Hamamatsu City, Japan) for 3 minutes.

The devices consisted of a semi-permeable drug-releasing membrane and an impermeable reservoir (Fig. 1A, 1B), as we reported previously [24]. The loaded vasohibin-1 doses included vehicle only (identified as NVDD), 1 μM vasohibin-1 (VDD), and 10 μM vasohibin-1 (10VDD), with a total volume of 1.5 μL in each device. The size of the device was 2 mm×2 mm wide ×1 mm high (drug-releasing surface area; 1.5 mm×1.5 mm = 2.25 mm^2) for the rat experiments (Fig. 1B, device) and 4 mm×4 mm×1.5 mm (drug-releasing surface area; 3.5 mm×3.5 mm = 12.25 mm^2) for the vasohibin-1 releasing *in vitro* assay. The release amount from the transplanted device was small and it was very difficult to detect released vasohibin-1 by the standard ELISA technique, so we decided to use a larger device for the ELISA procedure. As a control, we used pelletized vasohibin-1 without the reservoir and permeable membrane (Fig. 1B, pellet). The concentration of pelletized vasohibin-1 was adjusted to be the same concentration as that of the 10VDD (10 μM vasohibin-1). The total amount of vasohibin-1 released from the 10VDD device during the 2-week *in vivo* experiment was aimed to be equivalent to that of the intravitreal vasohibin-1 injection. A FITC-labeled 40 kDa dextran-loaded device (FD40DD) was also used for monitoring the position of the implanted device.

In Vitro Experiments

***1 In Vitro* Release Assay, Enzyme-linked Immunosorbent Assay, and Western Blotting.** The devices loaded with vasohibin-1 were placed in the wells of a 24-well culture plate filled with 200 μL PBS at 37°C. Aliquots (200 μL) of the buffer in each well were collected at Days 1, 7, 14, and 28 during change-out of old buffer for new buffer solution. The collected samples were considered to include only protein for vasohibin-1. We then determined the amount of vasohibin-1 in the buffer using an enzyme-linked immunosorbent assay (ELISA) [29] and western blotting [30]. The intensity of the color of the ELISA reaction products was measured with a microplate reader (MAXline; Molecular Devices Corporation, Sunnyvale, CA, USA). The measurements were made in duplicate, and the mean value was used for comparisons. The 50-μL collected samples and 100 fmol of recombinant vasohibin-1 (positive control) were loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% separating gel, and transferred to nitrocellulose membranes for western blotting. The membranes were blocked for 1 hour at room temperature with 5% ECL blocking agent (GE Healthcare Biosciences, Pittsburgh, PA, USA), and then incubated overnight at 4°C in PBS containing 0.05% Tween 20 (T-PBS), 2.5% skim milk, and 1 μg/mL horseradish peroxidase-conjugated anti–vasohibin-1 monoclonal antibody. The membrane filters were washed 3 times with T-TBS and the blots were detected using an enhanced chemiluminescence method (ECL Western Blotting Detection Kit; Amersham Biosciences, Piscataway, NJ, USA). The results were visualized using an imaging system (ImageQuant LAS-1000; GE Healthcare Biosciences).

2 Endothelial Tube Formation. Endothelial tube formation was assessed with normal human umbilical vein endothelial cells (HUVECs) (Takara Bio; Otsu, Japan) co-cultured on neonatal normal human dermal fibroblasts (NHDF, Takara Bio) layer using anti-human CD31 immunostaining, as reported previously [28]. Two nM vascular endothelial growth factor (VEGF, Wako; Tokyo, Japan) was then added to the endothelial cell growth medium (EGM, Takara Bio) containing no vasohibin-1 (control), and 0.2, 2, or 10 nM vasohibin-1, respectively. VEGF (2 nM) and samples of vasohibin-1 released from the vasohibin-1-loaded device over 3 hours at 37°C were used to examine released vasohibin-1 activity. We collected the released vasohibin-1 from the pellet and used it at a concentration of 0.56 nM (as measured by ELISA). On Day 3, the cells were fixed and stained using an anti-human CD31 immunostaining kit (Kurabo; Tokyo, Japan) according to the manufacturer's instructions. The number of stained HUVECs was determined using a computerized system (Kurabo Angiogenesis Image Analyzer program; Kurabo).

In Vivo CNV Experiments

1 Animals. The procedures used in the animal experiments followed the guidelines of 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 (Permit Number: 2011–136). Twenty Sprague-Dawley (SD) rats (Experiments 1 and 2) and 36 Brown Norway (BN) rats (Experiment 3) weighing between 250 and 300 g were used (Table 1). All animals were followed up to 2 weeks after device transplantation and/or laser burn. We examined the effects of devices either at 1 week or 2 weeks for FA evaluation and 2 weeks for flat-mount evaluation. Macro examination was performed at 1 and 2 weeks after the device transplantation. For all procedures, the rats were anesthetized with an intramuscular

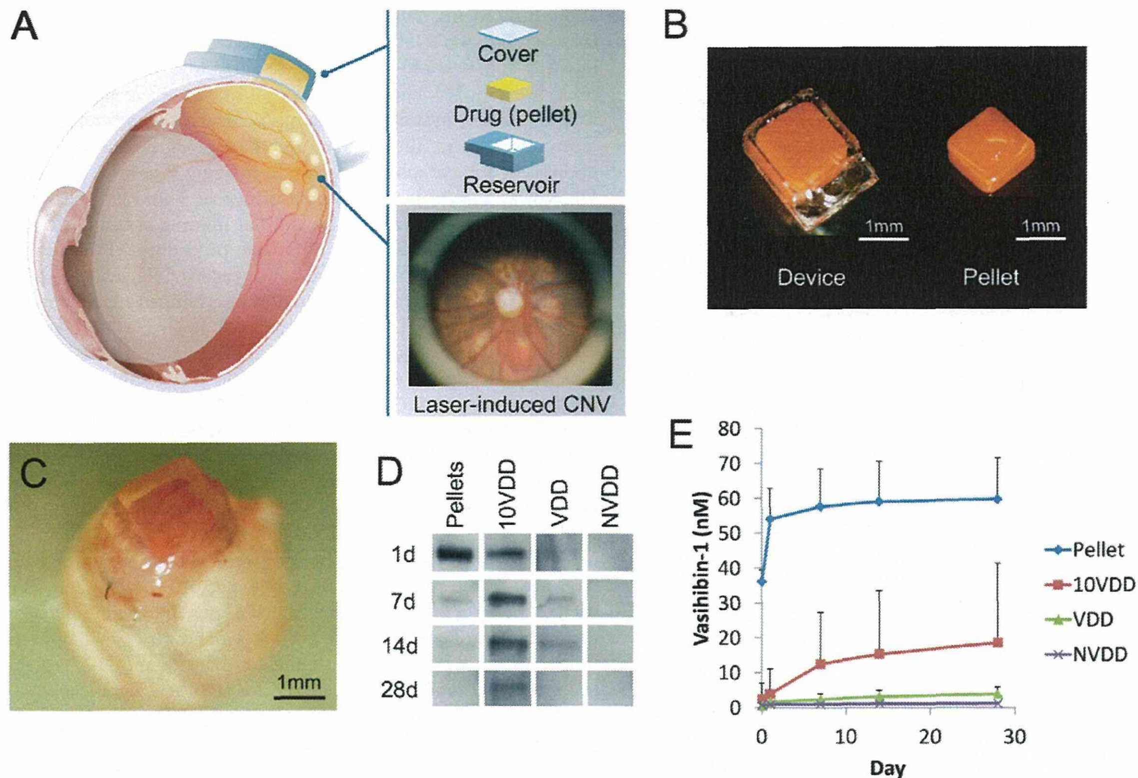

Figure 1. Device and vasohibin-1 release. (A) Schematic image of transscleral sustained vasohibin-1 delivery. We evaluated its effects via transscleral approach for rat laser-induced choroidal neovascularization (CNV). The device consists of a drug pelletized with PEGDM, a reservoir made of TEGDM, and a controlled-release membrane made of PEGDM that contains collagen microparticles. (B) Photograph showing a drug pellet and the delivery device containing a drug pellet. (C) Image of a device placed on the sclera of a rat eye at 3 days after implantation. The amount of vasohibin-1 in the PBS was measured at 1, 7, 14, and 28 days after starting incubation. The representative results of western blotting and the result of ELISA are shown in (D) and (E), respectively. We collected the samples at only the given time points and replaced only the equal volume of PBS. The released vasohibin-1 amounts accumulated for 6, 7, and 14 days. [The pellet samples collected at Day 1 (shown as 1d) were diluted five times due to their concentration before they were evaluated by western blotting]. NVDD: non-vasohibin-1 (vehicle) delivery device, VDD: 1 μM vasohibin-1 delivery device, 10VDD: 10 μM vasohibin-1 delivery device, Pellets: vasohibin-1 pelletized at the same concentration of 10VDD (without reservoir and cover).
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injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg), and the animals' pupils were dilated with topical 2.5% phenylephrine and 1% tropicamide. Oxybuprocaine hydrochloride (0.4%) was also used for local anesthesia. In all *in vivo* experiments, the animal's left eye was used as a control.

2 Implantation of VDDs, Pellets, and Intravitreal Vasohibin-1 Injection. Devices were implanted subconjunctively in the right eyes of the rats (Table 1). A 4-mm long conjunctival incision was made along the limbus in the upper temporal position. The devices were inserted into the subconjunctival space using forceps, with the drug-releasing surface facing the sclera. The device was placed between the optic disc and the equator, in the posterior quadrant, using no suture to anchor it into place. The conjunctival incision was closed with 9–0 silk and antibiotic ointment was applied to the eyes. Vasohibin-1 protein (0.24 μM) was injected using a 10-μL glass syringe (Hamilton; Reno, NV) 4 days after the experimental CNV procedure. The left eyes were used as untreated controls.

The rats were anesthetized, pupils were dilated, and a fundus examination was performed immediately after the surgery.

Experiment 1: Monitoring the Implanted Devices and Pellets

To monitor the device and drug release, fluorescein isothiocyanate (FITC) dextran (FD40; Sigma-Aldrich) pelletized with PEGDM was prepared and used as a control drug. The FD40 was dissolved in PBS at a concentration of 250 mg/mL and loaded in the device in the same way as vasohibin-1. Eight SD rats were included in this experiment; 4 rats received the FD40 delivery device (FD40DD) and 4 rats received only pelletized FD40.

Experiment 2: Immunohistochemistry after Device Implantation

Immunostaining for vasohibin-1was performed 2 weeks after device implantation. Twelve SD rats were used as follows (Table 1): 4 rats received vehicle (non-vasohibin-1) in the delivery device on the sclera (NVDD), 4 rats received 1.5 μL of 10 μM vasohibin-1 in the delivery device (10VDD), and 4 rats received 1.5 μL of 10 μM vasohibin-1 pellets implanted on the sclera. Immunohistochemistry was performed as reported previously [25].

Animals were euthanized using overdoses of ketamine hydrochloride and xylazine hydrochloride. The eyes were enucleated

Table 1. In Vivo Study Demographics.

Number of animals	Strain	Treatment	Methods	Position of implant
Experiment 1				
4	SD	Untreated	FD40DD	Sclera
4	SD	Untreated	FD40 Pellet	Sclera
Experiment 2				
4	SD	Untreated	NVDD	Sclera
4	SD	Untreated	10VDD	Sclera
4	SD	Untreated	Pellet	Sclera
Experiment 3				
6	BD	CNV	NVDD	Sclera
6	BD	CNV	VDD	Sclera
6	BD	CNV	10VDD	Sclera
6	BD	CNV	Pellet	Sclera
6	BD	CNV	Vehicle	Vitreous
6	BD	CNV	Vasohibin-1	Vitreous

SD: Sprague-Dawley rats, BN: Brown Norway rats, CNV: choroidal neovascularization, NVDD: non-vasohibin-1 delivery device, 10VDD: 10 μM vasohibin-1 delivery device.
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and fixed for 12 hours in 4% paraformaldehyde (PFA) at 4°C. The anterior segment and lens were removed from each eye. The posterior segment was cryoprotected at 4°C through successive 12-hour incubations in 10%, 20%, and 30% sucrose dissolved in saline. The tissues were immersed in OCT compound (Tissue-Tec; Sakura Finetec USA, Inc., Torrance, CA, USA) and frozen in acetone in a dry-ice bath. The frozen posterior segment was sectioned at the center of the implanted area at a thickness of 5 μm for each section, using a cryostat. We examined eight continuous sections per eye. The sections were incubated in rabbit polyclonal antibody against human vasohibin-1, followed by FITC-conjugated anti-rabbit IgG (1:200; Dako, Glostrup, Denmark) for 30 minutes. The sections were washed three times with PBS between each step. Negative controls (4 rats) incubated with just FITC-conjugated anti-rabbit IgG were also prepared. Slides were counterstained with 4, 6-diamino-1-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and photographed using a fluorescence microscope (Leica FW4000, Ver. 1.2.1; Leica Microsystems Japan, Tokyo, Japan).

Experiment 3: Choroidal Neovascularization Study

A total of 36 BN rats were used (Table 1). The devices and pellets were implanted on the same day as the CNV procedure. The rats were divided into six groups (6 rats in each group): rats with NVDD, rats with 1.5 μL of 1 μM vasohibin-1 in the delivery device (VDD), rats with 1.5 μL of 10 μM vasohibin-1 in the delivery device (10VDD), rats with 1.5 μL of 10 μM vasohibin-1 pellets implanted on the sclera, rats with intravitreal injection of 5 μL of vehicle, and rats with an intravitreal injection of 0.24 μM vasohibin-1 protein occurring 4 days after the experimental CNV procedure. The amount of intravitreal vasohibin-1 used and the day of the injection were determined based on our previous data [25]. The intravitreal injections were performed using a 10-μL glass syringe (Hamilton), and the needle was passed through the sclera just behind the limbus into the vitreous cavity.

3 CNV procedure. A green argon laser was used to rupture the choroidal membrane using a slit-lamp delivery system (Ultima 2000SE; Lumenis, Yokneam, Israel) with a contact lens [31]. The laser settings were: 50 μm diameter for 0.1 sec duration, at an intensity of 650 to 750 mW. Six laser burns were made around the optic disc (Fig. 1A). Each burn was confirmed to have induced sub-retinal bubbles, indicating a rupture of Bruch's membrane.

In addition to the routine ophthalmological examinations, fluorescein angiography (FA) with an imaging system (GENESIS-Df; Kowa, Tokyo, Japan) was performed at 1 and 2 weeks after the CNV laser burn, and choroidal flat mounts of the CNV site were performed at 2 weeks after the procedure. Two retinal specialists (HO and TA) and one non-specialist (NN) evaluated the angiograms for FA grading evaluation in a blinded manner using a grading system [32], where 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 the late phase beyond the treated areas. The camera was a handheld retinal camera for photographing humans, and the fact that rat eye optics differ from that of humans made the process somewhat difficult. Intense fluorescein leakage also made the results of photographs as faint. The laser burn sometimes made subretinal hemorrhages that were shown as fluorescein blockage. These results may have influenced the evaluation. We tried to focus on the laser burn as much as possible to not influence the evaluation. Further we also tried to synchronize evaluations as much as possible to avoid significant bias due to fluorescein leakage. Total grades were analyzed for statistical significance.

4 Fluorescein-Labeled Dextran Perfusion and Choroidal Flat-Mount Preparation. The size of the CNV lesion was measured on choroidal flat mounts to examine the effect of the vasohibin-1 delivery device (n = 6 eyes/group and each eye had 6 laser spots). Fourteen days after the CNV procedure, the rats were perfused with 5 mL PBS containing 50 mg/mL fluorescein-labeled dextran (FITC-dextran, MW: 2×10^6; Sigma-Aldrich). Results of mouse CNV experiments [25] indicated that laser-induced CNV lesions were most active at 14 days after laser application and gradually self-resolved more than 28 days after the laser burn. This data was supported by our previous study of laser-burned monkey eyes [28].

We enucleated the eyes in the current study at 14 days after the CNV laser procedure, after euthanizing the animals per the previously described method. The eyes were removed and fixed for 30 minutes in 4% phosphate-buffered PFA. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts (4 to 6) were made from the edge to the equator, and the eyecup of the RPE-choroid-sclera (R-C-S) complex was flat mounted in Permalfuor (Beckman Coulter; Fullerton, CA, USA) with the scleral side facing down. Flat mounts were examined by fluorescence microscopy (Leica FW4000, Leica Microsystems Japan), and the total area of each CNV zone associated with each burn was measured. The CNV lesions were identified by the presence of fluorescent blood vessels on the choroidal/retinal interface circumscribed by a region lacking fluorescence. This process duplicated past reported procedures [33,34]. Two retinal specialists (HO and TA) and one non-specialist (NN) evaluated the size of the dextran-fluorescein perfused CNVs in a blinded manner, as described above.

Statistical Analyses

Analysis of variance (ANOVA) with Tukey's test was used to examine differences in the leakage and severity of the CNVs in the fluorescein angiograms and the area of the choroidal flat mount. Endothelial tube formation was also evaluated by this method. P-values less than 0.05 were considered significant.