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Article

Suppression of Laser-Induced Choroidal Neovascularization by the Oral Medicine Targeting Histamine Receptor H4 in Mice

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Purpose: This study aimed to examine relationship of histamine receptor H4 (HRH4) and the pathogenesis of laser-induced choroidal neovascularization (laser-CNV) and to determine whether oral administration of HRH4 antagonists suppressed laser-CNV in mice.

Methods: Laser photocoagulation was performed in mice to induce the laser-CNV. Histamine was administered intravitreously, and CNV volume was measured. Laser photocoagulation and intravitreous injection of HRH4 antagonist JNJ7777120 were performed after intraperitoneal injection of clodronate liposome, which depletes circulating monocyte-derived macrophages; CNV volume was compared with that in mice injected with control (dimethyl sulfoxide [DMSO]/PBS). Three days after laser-CNV, the $F4/80^{+}CD11b^{+}$ macrophage population in retinal pigment epithelium (RPE)/choroid complex was quantified with flow cytometry in wild-type and *Hrh4*$^{-/-}$ mice. The long-acting HRH4 antagonist JNJ28307474 was then administrated periorally, and the laser-CNV volume was compared with controls.

Results: Intravitreous injection of histamine did not affect laser-CNV volume. The laser-CNV from the eye injected with JNJ7777120 was equivalent to that injected with the DMSO/PBS in mice that had intraperitoneally received clodronate liposome. Flow cytometry after laser-CNV induction revealed a smaller $F4/80^{+}CD11b^{+}$ macrophage population in the RPE/choroid complex of *Hrh4*$^{-/-}$ mice than in wild-type mice. Oral administration of JNJ28307474 significantly reduced laser-CNV volume in wild-type mice.

Conclusions: Our results suggested that HRH4-positive macrophages played an important role in the pathogenesis of laser-CNV and that they require a different ligand from that of histamine. The oral administration of an HRH4 antagonist successfully reduced laser-CNV.

Translational Relevance: Our results indicate that drugs targeting HRH4 are potentially a novel oral treatment for age-related macular degeneration.

Introduction

Neovascular age-related macular degeneration (AMD), also known as wet-AMD, is one of the most common causes of blindness in developed countries.[1–3] Wet-AMD is characterized by choroidal neovascularization (CNV) that develops through Bruch's membrane and into the subretinal space with subsequent damage to the central retina.[4–6] Currently, the standard treatment for wet-AMD targets VEGF, a key regulator of CNV in patients with wet-AMD. Although anti-VEGF therapy has dramatically changed the therapeutic strategies for wet-AMD,[7–9] treatments targeting VEGF alone are insufficient as

they require the repeated injections of anti-VEGF drugs.[10]

Histamine receptor H4 (HRH4), the most recently discovered histamine receptor, is expressed in bone marrow and peripheral hematopoietic cells as well as in neurons and endothelial cells in the central nervous system.[11–15] In normal tissue, HRH4 expression is extremely low and is induced or altered in response to inflammatory stimuli.[11,16] HRH4 is of particular importance for regulating immune cell functions, including chemotaxis and cytokine secretion, whereas HRH4 antagonists have shown anti-inflammatory, antihyperalgesic, and anti-allergic effects in several acute and chronic experimental rodent models.[17–20] We have previously reported that genetic depletion of *Hrh4* and the anti-HRH4 chemicals in mice suppressed laser-induced CNV (laser-CNV).[21]

Macrophages play a major role in ocular angiogenesis, including CNV in wet-AMD.[22] Monocyte chemotactic protein (MCP) is highly expressed in patients with AMD,[23] and macrophage depletion diminishes laser-CNV in rodent models.[24] In our previous report, we described the infiltration of macrophages into laser-CNV sites that expressed HRH4; however, normal retinas did not generally express HRH4.[21] In the present study, we investigated HRH4-positive cells to determine their role in the pathogenesis of laser-CNV. In addition, we examined whether the oral administration of an HRH4 antagonist suppressed laser-CNV to assess its therapeutic potential as an oral treatment for patients with wet-AMD.

Methods

Animals

Transgenic mice lacking the *Hrh4* gene (C57BL/6.129 tm1 [Histamine 4 Receptor] Lex) were gifted by Janssen Research & Development, LLC (Raritan, NJ). Male $Hrh4^{-/-}$ and wild-type C57BL/6J mice (CLEA Japan, Tokyo, Japan) aged 6–8 weeks were used in the experiments. Mice were randomly assigned to standard cages (4–6 mice per cage) in a temperature-controlled room (25°C) under a 12-hour light/dark cycle and with ad libitum access to food (CE-2; CLEA Japan) and water. For all procedures, the animals were anesthetized with an intraperitoneal (i.p.) injection of 400 mg/kg Avertin (2.5% 2,2,2-tribromoethyl and tertiary amyl alcohol; Sigma-Aldrich, St. Louis, MO), and the pupils were dilated with a combination of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen, Osaka, Japan). The experimental protocol was approved by the Nagoya University Animal Care Committee, and all animal experiments were performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Laser-CNV Volume Analysis

To generate laser-CNV, we applied laser photocoagulation (532 nm, 180 mW, 100 ms, 75 μm; Novus Verdi; Coherent, Inc., Santa Clara, CA) at four sites in the fundus of each eye. This procedure was performed on day 0 by an individual who was masked to the group assignments.[21,25] The laser spots, which were created with a slit lamp and a coverslip as a contact lens, were placed at equal distances around the optic nerve. The laser-CNV volume was measured using a previously described method.[21,25] In brief, the eyes were enucleated 1 week after the laser injury and fixed with 4% paraformaldehyde (PFA) for 2 hours. The eyecups were obtained by removing the anterior segments and were incubated overnight at 4°C with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Sigma-Aldrich). The eyecups were washed and radially dissected to create flat mounts. The CNV in the retinal pigment epithelium (RPE) flat mount was visualized using a blue argon laser wavelength (488 nm) and a scanning laser confocal microscope (Eclips C1 confocal microscope; Nikon, Tokyo, Japan). Horizontal optical sections were obtained at 1-μm intervals from the top of the CNV to the RPE surface. The images of each layer were stored digitally and measured with ImageJ software to determine the area of CNV-related fluorescence. The total fluorescent area in each horizontal section was used as an index for CNV volume. Finally, we calculated the average volume of all laser spots in each eye (n = number of eyes). The imaging analysis was conducted by an operator masked to the group assignments.

Intravitreous Histamine Injections

We examined whether histamine stimulated HRH4 in laser-CNV by intravitreously injecting different doses of histamine (5, 10, and 50 μg/d; Sigma-Aldrich) dissolved in PBS immediately after the laser injury on day 0, and on days 1, 2, and 3. The intravitreous injection was performed using a 33-gauge needle (Ito Corp., Tokyo, Japan) under a surgical microscope.

Clodronate Liposome and JNJ7777120

As previously described, we used clodronate liposome (Katayama Kagaku, Osaka, Japan) to examine whether the HRH4 antagonist JNJ7777120 (Sigma-Aldrich) affected macrophage depletion.[22,24,26–28] Clodronate liposome has been reported to deplete only the circulating monocyte-derived macrophages but not the resident retinal microglias.[29] Wild-type mice received i.p. injections of clodronate liposome (100 μL) on days −3 (3 days before injury) and 0. Next, JNJ7777120 was dissolved in a dimethyl sulfoxide (DMSO)/PBS solution, and 1 μg of it was injected intravitreously after laser injury on days 0 and 3. The control, an equal volume of vehicle (DMSO/PBS), was injected using the same procedure.

The Oral Administration of JNJ28307474

JNJ28307474 is an HRH4 antagonist that has a longer half-life than JNJ7777120 and has been administered periorally (p.o.) in other studies.[30,31] Therefore, we expected that JNJ28307474 would have more potency as an oral drug for AMD than would JNJ7777120. The JNJ28307474 was gifted by Janssen Research & Development, LLC. Wild-type mice that had undergone laser photocoagulation on day 0 were administered JNJ28307474 (20 mg/kg/day) p.o. on days −1, 0, 1, 2, and 3. On day 7, the eyes were enucleated to measure the laser-CNV volume.

Flow Cytometry

Flow cytometry was performed for the RPE/choroid complex using a previously described method with slight modifications.[32,33] In brief, RPE/choroid complex tissues were harvested from both eyes, incubated with collagenase D (20 U/I; Roche Diagnostics, Mannheim, Germany), and treated with Fc-block (10 μg/mL; BD Biosciences, San Diego, CA) in a tube placed on ice for 15 minutes. After single cell suspension, the RPE/choroid cells (1×10^6) were incubated with FITC-conjugated anti-mouse CD11b antibody (1:50, clone M1/70; BD Biosciences) and allophycocyanin (APC)-conjugated anti-mouse F4/80 antibody (1:20, AbD; Serotec, Oxford, UK). Propidium iodide (20 μg/mL; Sigma-Aldrich) was used to detect dead cells, which were excluded from the analysis. The cells were analyzed with a minimum of 50,000 events on a FACS Canto II flow cytometer (BD Biosciences) using FlowJo software (Treestar, Inc., Ashland, OR).

The Immunostaining of Mouse RPE Flat Mounts with Laser-CNV

Seven days after inducing laser-CNV in wild-type and *Hrh4*$^{-/-}$ mice, the eyes were fixed in 4% PFA. The RPE flat mount was prepared as previously described with some modifications.[22] In brief, after five freeze/thaw cycles, the eyecups were radially incised and the vitreous was removed thoroughly. The RPE/choroid samples were permeabilized for 2 hours, blocked with 5% goat serum in PBS, and incubated with a primary antibody for 12 hours and secondary antibodies for 9 hours. The primary antibodies were rabbit antibody against mouse HRH4 (1:100; Abcam, Cambridge, MA) and rat antibody against mouse F4/80 (1:100; AbD Serotec, Kidlington, UK), and the secondary antibodies were the Alexa-488 and Alexa-594 (1:500; Invitrogen Corp., Carlsbad, CA). Images were taken using a BZ-9000 fluorescence microscope (Keyence Corp., Osaka, Japan) or Eclips C1 confocal scanning laser confocal microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

The mouse MCP-1 levels were measured using an ELISA as previously described.[34] In brief, 3 days after the laser photocoagulation, the protein lysates were prepared using the RPE/choroid complex from the wild-type or *Hrh4*$^{-/-}$ mice with a radioimmunoprecipitation assay buffer (Sigma-Aldrich) and a protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). The lysate was centrifuged, and the supernatant was collected. Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA), and the level of MCP-1 was measured (MJE-00; R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Duplicate evaluations were performed for each sample.

Statistical Analysis

The data were expressed as mean ± SEM (n = number of samples). The result for the control sample was defined as 100%, and the percent difference relative to the control was calculated for each sample. The data from the laser-CNV volume after the injection of different volumes of histamine were analyzed with the Kruskal-Wallis test, and if significance was detected ($P < 0.05$), Steel's test was applied for comparison with the control. For the data

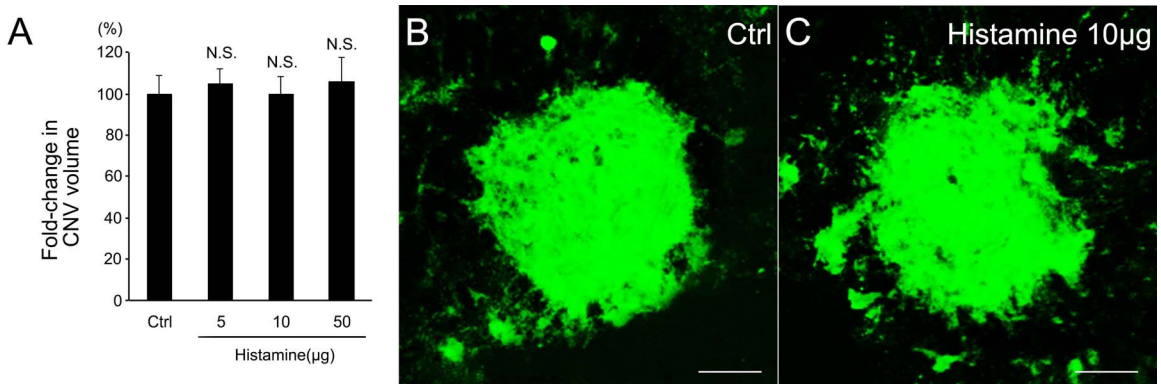

Figure 1. Histamine had no effect on laser-induced choroidal neovascularizations (laser-CNVs). (A) The laser-CNVs volume in wild-type mice that received the multiple injections of different doses (5, 10, and 50 μg) of histamine were unaffected as compared with that in mice injected with control (Ctrl) PBS ($P = 0.91$; Kruskal-Wallis test). (B, C) Representative images of laser-CNV in wild-type mice that received multiple injections of histamine (10 μg) (C) or the control (Ctrl) PBS (B). *Scale bar*: 50 μm. N.S., no significant difference.

from the laser-CNV volume with clodronate liposome and JNJ7777120, the Kruskal-Wallis test and Steel-Dwass test were applied. The data from other examinations were analyzed using the Mann-Whitney U test (unpaired samples). Differences were considered statistically significant at $P < 0.05$.

Results

The Effect of Histamine on Laser-CNV

A previous study revealed that laser-CNVs in *Hrh4*$^{-/-}$ mice are significantly smaller than those in wild-type mice.[21] Therefore, we first examined whether histamine promoted laser-CNV progression in wild-type mice. Compared to the CNV volume from the eye injected with control PBS (1.00 ± 0.09, n = 9), the volumes from the eyes injected with 5, 10, and 50 μg of histamine were 1.05 ± 0.07 (n = 9), 1.00 ± 0.09 (n = 9), and 1.06 ± 0.12 (n = 9), respectively. The CNV volumes in the eyes injected with different doses of histamine did not show significant differences compared to that in the control eyes ($P = 0.91$, Kruskal–Wallis test; Fig. 1). These results suggested that the HRH4-positive cells in laser-CNV had ligands other than histamine.

The Relationship between Macrophage Depletion and HRH4 in Laser-CNV

We have previously shown that the intravitreous injections of HRH4 antagonist JNJ7777120 reduced laser-CNV by 47% in wild-type mice, and some of the HRH4-positive cells were costained with the macrophage marker F4/80 in laser-CNV.[21] Clodronate liposome has been reported to reduce laser-CNV by depleting macrophages.[24,27] Therefore, we hypothesized that clodronate liposome could cancel the HRH4 antagonist-induced laser-CNV reduction by depleting macrophages. To evaluate our hypothesis, we intraperitoneally injected clodronate liposome and intravitreously injected JNJ7777120 or control DMSO/PBS and then compared the laser-CNV volumes (Fig. 2). For comparison, we measured the laser-CNV from mice without clodronate liposome (1.00 ± 0.07, n = 12). After confirming that there was a statistical difference with a Kruskal-Wallis test ($P = 0.0094$), we compared each group. There was a significant difference in the eyes with intravitreous injections of JNJ7777120 and intraperitoneal injections of clodronate liposome (0.75 ± 0.03, n = 10) as compared with the eyes without clodronate liposome ($P = 0.012$, Steel-Dwass test). More importantly, we observed that the CNV volume of the eyes with intravitreous injections of JNJ7777120 and intraperitoneal injections of clodronate liposome did not show a significant difference as compared with those with intravitreous injections of control DMSO/PBS and intraperitoneal injections of clodronate liposome (0.78 ± 0.03, n = 10; $P = 0.68$, Steel-Dwass test). Our results indicated that the laser-CNV suppression by the HRH4 antagonist was aborted during the macrophage-depleted condition by clodronate liposome.

The Phenotype of *Hrh4*$^{-/-}$ Mouse with Laser-CNV

We further examined the characteristics of *Hrh4*$^{-/-}$ mice in the pathogenesis of laser-CNV. We measured the MCP-1 expression in the RPE/choroids with

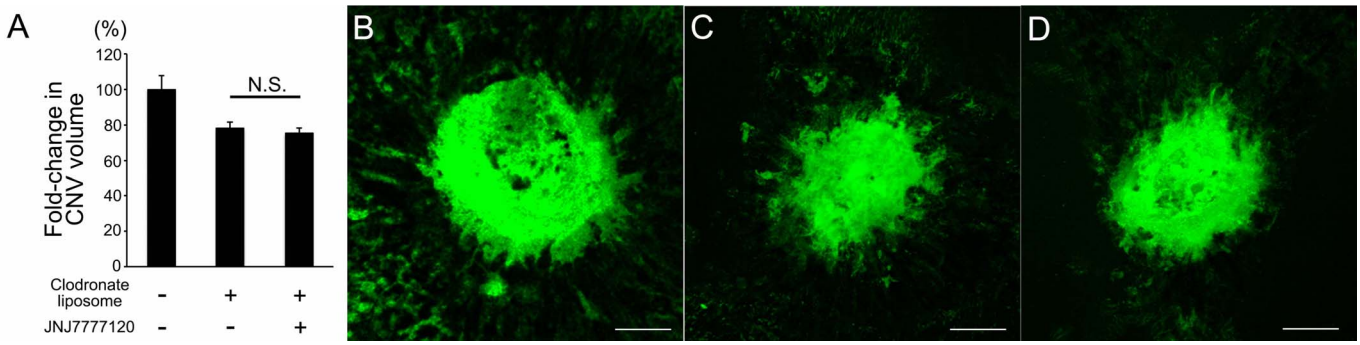

Figure 2. The anti-angiogenic effect of HRH4 blockade was suppressed by macrophage depletion. (A) The volumes of laser-CNVs from the mice with intravitreous injection (IVT) of JNJ7777120 and i.p. injection of clodronate liposome were unchanged compared with those injected with control DMSO/PBS (IVT) and clodronate liposome (i.p.) ($P = 0.68$). (B-D) Representative images of laser-CNV in wild-type mice that did not receive clodronate liposome or JNJ7777120 (B), mice that received injections of JNJ7777120 (1 μg, days 0 and 3 (D), and mice that received control DMSO/PBS (C) following i.p. injections of clodronate liposome. *Scale bar*: 50 μm. N.S., no significant difference.

laser-CNVs (Fig. 3A). The MCP-1 level in *Hrh4*$^{-/-}$ mice (1.42 ± 0.08, n = 8) was significantly higher than that in wild-type mice (1.00 ± 0.07, n = 8, $P = 0.005$). The flat-mount images from the RPE flat mount after inducing laser-CNV showed cells with costaining for F4/80 and HRH4 in wild-type mice (Fig. 3B). We further examined the macrophage population with flow cytometry. Because macrophages accounted for only a small part of the overall cell population in the RPE/choroid, we used gating with two independent macrophage-specific antibodies to increase the specificity. Three days after the laser-CNV was induced, the percentage of F4/80^{+}CD11b^{+} macrophages in the *Hrh4*$^{-/-}$ mouse RPE/choroid was significantly smaller than that in the wild-type mouse (Figs. 3C, D, 100 ± 10% vs. 51 ± 5%, $P = 0.002$, n = 7). These results suggested that, in the laser-CNV model, HRH4-positive macrophages played an important role in the pathogenesis of laser-CNV.

The Oral Administration of HRH4 Antagonist

Currently, the most efficient treatment for CNV is an anti-VEGF drug, and all FDA-approved anti-VEGF drugs are designed for intravitreous injections. However, repeated intravitreous injections are stressful for both patients and physicians. Therefore, we examined whether the potency of an orally administered HRH4 antagonist was sufficient to prevent CNV. After the repeated oral administration of JNJ28307474, laser-CNV was significantly reduced when compared with the controls (Figs 4A-C; 1.00 ± 0.12 vs. 0.71 ± 0.07, $P = 0.018$, n = 23). Thus, an oral drug targeting HRH4 successfully reduced CNV in the eye.

Discussion and Conclusions

In this study, we demonstrated that the oral administration of an HRH4 antagonist could prevent laser-CNV. Developing an oral medicine for AMD is

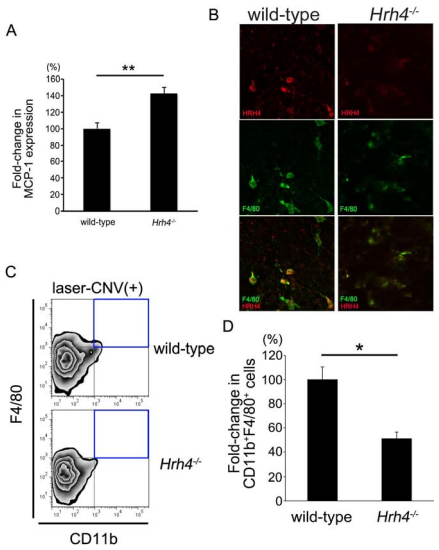

Figure 3. The characteristics of macrophages in *Hrh4*$^{-/-}$ mice in the pathogenesis of laser-CNV. (A) The comparison of MCP-1 levels in RPE/choroid complex with laser-CNV. MCP-1 in *Hrh4*$^{-/-}$ mice was significantly higher than that in wild-type mice. (B) Flat mounts stained with the macrophage marker F4/80 and HRH4 antibodies revealed the costaining of F4/80 and HRH4 in wild-type mice but not in *Hrh4*$^{-/-}$ mice. (C) Flow cytometry detected fluorescence emitted from the F4/80^{+}/CD11b^{+} cells (*blue square*) isolated from the RPE/choroid complex 3 days after laser photocoagulation in wild-type (*upper*) and *Hrh4*$^{-/-}$ mice (*lower*). (D) The percentage of F4/80+/CD11b+ macrophages isolated from the *Hrh4*$^{-/-}$ mice RPE/choroid complex were significantly lower than that in wild-type mice. $^{**}P < 0.01$; $^{*}P < 0.05$.

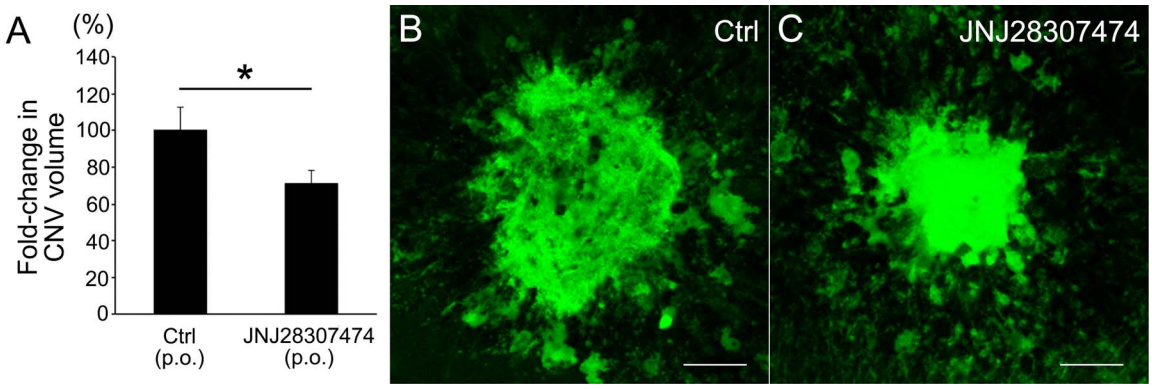

Figure 4. The oral administration of HRH4-antagonist reduced ocular angiogenesis in wild-type mice. (A) The volumes of laser-CNVs in wild-type mice with the oral administration of JNJ28307474 showed significant reductions as compared with those in wild-type mice with the oral administration of the control ($P = 0.018$; n = 23). (B, C) The representative images of laser-CNV in wild-type mice with the oral administration of HRH4-antagonist JNJ28307474 (20 mg/kg/day) (C) and the control (B). *Scale bar*: 50 μm. *$P < 0.05$.

very challenging, but could be of great benefit to a number of people. The patients who receive intravitreous injections of anti-VEGF drugs are forced to receive multiple injections. It can cause mental and physical stress for patients, particularly the elderly. Oral medications possibly could reduce the number of intravitreous injections required. HRH4-targeted medicines have been suggested as a possible oral medicine for AMD. However, before we continue this research with medium-sized animals or, eventually, with humans, we have to elucidate precisely how these HRH4-positive cells interact in the pathogenesis of CNV and how HRH4-targeted medicines work to suppress CNV.

We had previously revealed that the HRH4 antagonist JNJ7777120 inhibited laser-CNV and that HRH4 was expressed on macrophages that infiltrated CNV sites.[21] Many studies have validated the strong relationship that exists between CNV and macrophages. MCP-1 is highly expressed in the CNV specimens of patients with AMD,[23] and the depletion of macrophages decreased CNV growth in mice models.[24,27,28] On the other hand, Apte et al.[26] showed that macrophages were protective against laser-CNV growth. There still are some contradictions in the data regarding the function of macrophages in the pathogenesis of laser-CNV. In our current study, the HRH4 antagonist did not reduce laser-CNV after macrophage depletion by clodronate liposome.

HRH4 mediates the chemotaxis of murine mast cells, eosinophils, neutrophils, dendritic cells, and macrophages.[35] In contrast, HRH4 has been reported to downregulate MCP-1 (also known as CCL2) in monocytes and dendritic cells, subsequent to suppressing monocyte migration.[36] In our study, we confirmed that MCP-1 was up-regulated in the RPE in *Hrh4*$^{-/-}$ mice as compared with wild-type mice after laser-CNV induction. Nevertheless, we also demonstrated that HRH4 deficiency reduced macrophage infiltration into the RPE/choroid after laser-CNV induction. Moreover, the RPE flat mounts demonstrated the existence of not only HRH4-positive but also HRH4-negative macrophages that had infiltrated into the RPE after laser-CNV induction. HRH4-related macrophage chemotaxis and its real function in the pathogenesis of laser-CNV are still not fully understood; therefore, further assessments are needed.

In spite of the ineluctable problems that will need to be solved before oral administration of HRH4 antagonists for AMD treatment in humans, there were limitations to our study. For instance, the categorization of HRH4-positive macrophages and HRH4-negative macrophages by flow cytometry and the comparison of their functions in the pathogenesis of laser-CNV are needed. In addition, the comparison of CCR2 and IL-10 expression in both HRH4-positive and HRH4-negative macrophages will be very helpful to clarify their function. Moreover, the examination of the percentages of HRH4-positive and HRH4-negative macrophages in the RPE/choroid of the wild-type mouse eyes injected with JNJ7777120 may show the precise mechanism of HRH4-targeted laser-CNV reduction. However, we unfortunately were unable to find the appropriate antibodies to detect HRH4 with flow cytometry. Even without using the appropriate HRH4 antibodies, another examination could be performed by generating enhanced green fluorescent protein

(EGFP) chimeric mice after the transplantation of EGFP bone marrow that lacked the *Hrh4* gene into wild-type mice.[37] Analyzing the migration ability of HRH4-negative EGFP-positive monocytes in the laser-CNV chimeric mice will enable better understanding.

Although histamine is a ligand for histamine receptors, we found no evidence that histamine exacerbated laser-CNV. There are several explanations that could account for this observation. One explanation is that histamine is a high-affinity ligand for human HRH4, but it has a lower affinity for rat and mouse HRH4.[38] CCL16 is a high-affinity ligand for HRH4 in mice.[39] IL-10 is related to CCL16 in monocytes, and IL-10 is reported to be an important factor in the pathogenesis of laser-CNV.[26] Additional assessments with laser-CNV volume and flow cytometry analyses need to be further explored with the stimulation of CCL16. The second possibility is that histamine has a short half-life, and the doses used in our study may have been insufficient. We may have needed additional injections to observe the effects of histamine. Further investigation is needed to confirm the mechanisms of HRH4 stimulation.

Current anti-VEGF therapies against wet-AMD require repeated intravitreal injections because of CNV recurrence; however, repeated injections increase the risk of endophthalmitis. In the present study, we demonstrated that the oral administration of the HRH4 antagonist JNJ28307474 decreased the development of laser-CNV in mice, therefore proposing a novel therapeutic strategy that may impose less stress on the patient and has a lower risk of endophthalmitis. Interestingly, a recent cohort study showed that patients with a history of allergies were less likely to have AMD than patients with no history of allergies.[40] Thus, it is possible that anti-allergy drugs suppressed the immune response through histamine receptors. Performing fluorescent immunostaining of F4/80 and HRH4 and, if possible, flow cytometry analysis after oral JNJ28307474 administration will enable us to better understand this mechanism. A more detailed understanding of the relationship between AMD and anti-allergy drugs is required.[41,42]

In summary, our study showed the involvement of HRH4 on macrophages infiltrating into laser-CNVs. Furthermore, we revealed a potential treatment against CNV with the oral administration of an HRH4 antagonist, suggesting a novel therapeutic strategy for wet-AMD.

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