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RESEARCH PAPER

Histamine H_4 receptor as a new therapeutic target for choroidal neovascularization in age-related macular degeneration

Hiroki Kaneko[1]*, Fuxiang Ye[1]*, Ryo Ijima[1], Shu Kachi[1], Seiichi Kato[2], Masatoshi Nagaya[1], Akiko Higuchi[1] and Hiroko Terasaki[1]

[1]*Department of Ophthalmology, Nagoya University Graduate School of Medicine, Nagoya, Japan, and* [2]*Department of Pathology and Laboratory Medicine, Nagoya University Hospital, Nagoya, Japan*

Correspondence
Hiroki Kaneko, Department of Ophthalmology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: h-kaneko@med.nagoya-u.ac.jp

*These authors contributed equally to this work.

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BACKGROUND AND PURPOSE

The present treatment for choroidal neovascularization (CNV) associated with age-related macular degeneration (AMD) is not sufficient. Hence, we examined the therapeutic efficacy of reducing histamine H_4 receptor expression on CNV in mice.

EXPERIMENTAL APPROACH

H_4 receptor expression was examined in CNVs from patients with AMD. In mice, laser photocoagulation was performed in the retina to induce experimental CNV (laser CNV). Protein and mRNA expression levels were determined and CNV volume measured in wild-type and *Hrh4*$^{-/-}$ mice with laser CNV. The effects of JNJ7777120, an H_4 receptor antagonist, administered intravitreously, on CNV volume and pathological vessel leakage were determined in mice with laser CNV and controls. Fundus imaging, retinal histology and electroretinography were performed on eyes injected with JNJ7777120 to evaluate retinal toxicity.

KEY RESULTS

Human H_4 receptors were only confirmed in CNV samples from AMD patients and not in the other subretinal tissues. Mouse H_4 receptors were expressed in retinal pigment epithelium only after inducing laser CNV in wild-type mice, and were co-localized with the macrophage marker F4/80. Laser CNV volume was reduced in *Hrh4*$^{-/-}$ mice compared with that in wild-type mice, and JNJ7777120 suppressed laser-induced CNV volume and pathological CNV leakage in wild-type mice. Also eyes injected with JNJ7777120 did not show retinal degeneration.

CONCLUSIONS AND IMPLICATIONS

H_4 receptors are expressed in macrophages that accumulate around CNVs. Suppressing H_4 receptor expression prevented the pathological vessel leakage without showing retinal toxicity, indicating that the H_4 receptor has potential as a novel therapeutic target in AMD.

Abbreviations

AMD, age-related macular degeneration; CNV, choroidal neovascularization; ERG; electroretinography; HREC; human retinal endothelial cell; IHC, immunohistochemistry; laser CNV, experimental CNV; PVR, proliferative vitreoretinopathy; RPE, retinal pigment epithelium

Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness in most industrialized nations (Ambati *et al.*, 2003a; Bird, 2010). AMD has two different forms: wet and dry. Whereas dry AMD shows atrophy of the retinal pigment epithelium (RPE; Kaneko *et al.*, 2011), wet AMD is characterized by the invasion of choroidal neovascularization (CNV) into the sensory retina. Advanced CNV growth causes severe visual damage (de Jong, 2006). Enhanced expression of the pro-angiogenic cytokine VEGF has been validated in patients with wet AMD, and anti-VEGF antibody treatment is the current standard treatment for wet AMD (Gragoudas *et al.*, 2004; Brown *et al.*, 2006; Rosenfeld *et al.*, 2006). However, continuous administration of anti-VEGF antibodies leads to a new problem; that is, over-suppression of VEGF possibly impairs regular tissue function (Yang *et al.*, 2013) including retinal function (Takeda *et al.*, 2009). Hence, a new therapeutic treatment for CNV other than anti-VEGF treatment is desired (Carmeliet and Jain, 2011).

The histamine H_4 receptor is, as are other histamine receptors, a member of the GPCR superfamily (Alexander *et al.*, 2013), and is the most recently identified (Nakamura *et al.*, 2000; Oda *et al.*, 2000). The H_4 receptor is one of four recently discovered histamine receptors, and it has a distinct pharmacological profile compared with the other histamine receptors (de Esch *et al.*, 2005). Recent evidence has shown that the H_4 receptor is expressed in rat brain endothelial cells, which indicated a potentially important role in vascular permeability (Karlstedt *et al.*, 2013). Based on this evidence, we hypothesized that the H_4 receptor plays an important role in ocular angiogenesis. We, therefore, investigated the expression of H_4 receptors in the pathogenesis of wet AMD. In addition, we further explored the therapeutic potential of H_4 receptor gene (*Hrh4*)-targeted AMD treatment without damaging the physiological condition of the eye. This is the first report that a treatment targeting the H_4 receptor potentially has anti-angiogenic properties in severe human ocular disease.

Methods

Human CNV and subretinal tissue

Human CNV specimens were surgically removed from a 78-year-old male patient and a 68-year-old male patient with severe wet AMD. Subretinal tissues that were used as controls were surgically removed from a 35-year-old female patient with retinal detachment-induced proliferative vitreoretinopathy (PVR) and a 17-year-old male patient with traumatic PVR. Tissues were fixed with 10% neutral buffered formalin after removal. After fixation for 24 h, tissues were embedded in paraffin and serially cut in 3 μm sections, which were used for immunohistochemistry (IHC). Immunohistochemical staining was performed with the rabbit antibody against human H_4 receptors (1:200; Abcam, Cambridge, MA, USA). Rabbit isotype IgG (1:200, Vector Lab, Burlingame, CA, USA) was substituted for the primary antibody to assess the specificity of staining. Bound antibody was detected with a Vectastain ABC-AP kit (Vector Lab) and the enzyme complex was visualized with an alkaline phosphatase blue substrate kit (Vector Lab). Levamisole (Vector Lab) was used to block endogenous alkaline phosphatase activity. The sections were counterstained with haematoxylin and mounted with mounting media (Mount-Quick; Daido Sangyo, Tokyo, Japan). The procedure was approved by the institutional review board of Nagoya University hospital and informed consent was obtained from each patient.

Human donor eyes

Donor eyes from patients with wet AMD were obtained from Minnesota Lions Eye Bank in the USA. The diagnoses were confirmed by dilated ophthalmic examination before acquisition of the tissues or eyes or upon examination of the eye globes *post mortem*. The study followed the guidelines of the Declaration of Helsinki.

Animals

Male wild-type C57BL/6J mice (CLEA, Tokyo, Japan) between 6 and 8 weeks of age were used. Transgenic mice lacking the *Hrh4* gene [C57BL/6.129 tm1 (histamine 4 receptor) Lex] were a gift from Janssen Research & Development, LLC (USA), and those between 6 and 8 weeks of age were used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Mice were randomly assigned to standard cages, with 4–5 animals per cage, and kept in standard housing conditions in a room with a temperature-controlled environment at 25°C under a 12 h light/dark cycle with *ad libitum* access to food (CE-2; CLEA) and water. For all procedures, the animals were anaesthetized with i.p. injection of 400 $mg \cdot kg^{-1}$ Avertin (2.5% 2,2,2-tribromoethyl and tertiary amyl alcohol; Sigma-Aldrich, St. Louis, MO, USA) and pupils were dilated with a combination of tropicamide 0.5% and phenylephrine 0.5% (Mydrin-P; Santen, Osaka, Japan). The experimental protocol was approved by the Nagoya University Animal Care Committee. All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse model of CNV

Four spots of laser photocoagulations (532 nm, 180 mW, 100 ms, 75 μm; Novus Verdi; Coherent Inc., Santa Clara, CA, USA) were placed in each fundus of the eye on day 0 by one individual blinded to the group assignment, as described previously (Tomida *et al.*, 2011). The laser spots were created around the optic nerve using a slit lamp delivery system and a coverslip was used as a contact lens. The morphological end point of the laser injury was the appearance of a cavitation bubble, which is the sign of Bruch's membrane disruption (Kleinman *et al.*, 2008).

Immunostaining of mouse laser CNV

Three days after laser photocoagulation, mouse eyes fixed in 4% paraformaldehyde were prepared as eyecups, cryoprotected in 30% sucrose, embedded in an optimal cutting temperature compound (Tissue-Tek OCT; Sakura Finetek, Torrance, CA, USA) and cryosectioned into 10 μm sections. Sections were stained with rabbit antibody against mouse H_4 receptor (1:100; Abcam) and rat antibody against mouse F4/80 (1:50; Serotec, Oxford, UK), and were visualized with Alexa-488 or Alexa-594 secondary antibodies (Invitrogen,

Carlsbad, CA, USA) and DAPI (Invitrogen). To confirm antibody specificity, IHC with anti-H_4 receptor antibodies was performed on the laser CNV tissues from *Hrh4*$^{-/-}$ mice. Images were taken with a bioimaging navigator fluorescence microscope (Olympus FSX100; Olympus, Tokyo, Japan).

Fundus imaging

Human and mouse ocular fundus images were obtained using a high-resolution digital fundus camera (TRC-50DX; Topcon, Tokyo, Japan, or CF-60DSi; Canon, Tokyo, Japan). For adjusting focus on the mouse fundus, a 20 diopter lens was placed in contact with the fundus camera lens (Tarallo *et al.*, 2012).

Fluorescein angiography

Fluorescein angiography was performed by an operator blinded to the group assignments of the animals. One week after laser photocoagulation, 0.1 mL of 1% fluorescein sodium (Alcon, Tokyo, Japan) diluted with saline was i.p. injected and images were captured using a fundus camera with a specific fluorescein angiography filter (TRC-50DX; Topcon). Fluorescent leakage from each laser CNV was evaluated. Each image was graded as described previously (Mizutani *et al.*, 2013). Briefly, the lesions were graded on an ordinal scale based on the spatial and temporal evolution of fluorescein leakage as follows: 0 (non-leaky) = no leakage, faint hyperfluorescence or mottled fluorescence without leakage; 1 (questionable leakage) = hyperfluorescent lesion with no progressive increase in size or intensity; 2 (leaky) = hyperfluorescence increasing in intensity but not in size, no definite leakage; 3 (pathologically significant leakage) = hyperfluorescence increasing in intensity and size, definite leakage. The percentages of grade 3 leakage at late phase (5–6 min) in each eye were calculated and the two groups were compared.

Laser CNV volume analysis

Laser CNV volume was measured by a method similar to that described previously (Sakurai *et al.*, 2003b; Kleinman *et al.*, 2011). Briefly, 1 week after laser injury, the eyes were enucleated and fixed with 4% paraformaldehyde. The eyecups obtained by removing the anterior segments were incubated with 0.5% FITC-isolectin B4 (Sigma-Aldrich). CNV was visualized using a blue argon laser (488 nm wavelength) and a scanning laser confocal microscope (Eclipse C1 confocal; Nikon, Tokyo, Japan). Horizontal optical sections were obtained at 1 μm intervals from the top of the CNV to the surface of the RPE. The images of each layer were stored digitally and the area sizes were measured. The area of CNV-related fluorescence was measured using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The summation of the whole fluorescent area in each horizontal section was used as an index for the volume of CNV. The average volume obtained from all laser spots (3–4 spots) per eye was generated (n = number of eyes). Imaging was performed by an operator blinded to the group assignments.

Intravitreous injections of JNJ7777120, JNJ10191584 and mouse VEGF antibodies

H_4 receptor antagonists JNJ7777120 and JNJ10191584 (Sigma-Aldrich) were dissolved in DMSO and PBS. To evaluate the effect of JNJ7777120 on CNV, 1 μg of JNJ7777120 or the same volume of vehicle (DMSO/PBS) was administered intravitreously at day 0 immediately after laser injury and at day 3 into the eyes of the wild-type mice. JNJ10191584 (3 μg) or the same volume of vehicle (DMSO/PBS) was administered intravitreously at day 0 immediately after laser injury and at days 1, 2 and 3 into the eyes of wild-type mice. For measuring fluorescein leakage, JNJ7777120 (1 μg) was administered intravitreously at day 0 after inducing laser photocoagulation. For evaluating retinal toxicity of JNJ777120, JNJ7777120 was administered at 5 μg. For blocking mouse VEGF, 1 μg of anti-mouse VEGF antibody (R&D Systems, Minneapolis, MN, USA) was injected as previously described (Ishida *et al.*, 2003; Takeda *et al.*, 2009). To investigate the biological co-relationship between H_4 receptors and VEGF, anti-mouse VEGF antibody (0.5 μg) was mixed with JNJ7777120 (1 μg) after laser CNV at day 0 followed by an additional injection of JNJ7777120 (1 μg) at day 3. CNV volumes were compared with those injected with anti-mouse VEGF antibody (0.5 μg) mixed with control solutions. Intravitreous injection was performed with a 33 G needle (Ito Corporation, Shizuoka, Japan).

H_4 receptor Western blot analysis

For the human donor eye, the retina to which CNV was adhered was cut only from the macular area and lysed in RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN, USA). For the mice samples, the RPE/choroid complex was carefully isolated from the eyes 3 days after inducing laser CNV and was then lysed in the same buffer. The lysate was centrifuged at 15 000× *g*. for 15 min at 4°C, and supernatant was collected. Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Richmond, CA, USA) with BSA as a standard. Proteins (70 μg) were run on 4–15% SDS precast gels (Bio-Rad) and transferred to PVDF membranes with an iBlot blotting system (Invitrogen). The transferred membranes were washed in TBS-T (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH = 8.0, 0.05% Tween 20; Sigma-Aldrich) and then blocked in 5% skimmed dried milk/TBS-T at 4°C overnight. Membranes were then incubated with the rabbit antibody against H_4 receptors (1:1000; Alpha Diagnostic, San Antonio, TX, USA) at 4°C overnight. Protein loading was assessed by immunoblotting using an anti-α/β tubulin antibody (1:2 500; Cell Signalling Technology, Beverly, MA, USA). The HRP-linked secondary antibody was used (1:5 000; Invitrogen) for 1 h at room temperature (RT). The signal was visualized with enhanced chemiluminescence (ECL plus; GE Healthcare, Waukesha, WI, USA) and captured by ImageQuant LAS-4000 (GE Healthcare).

ELISA

Mouse VEGF (mVEGF) levels were measured with an ELISA as described previously (Nishiguchi *et al.*, 2010; Tomida *et al.*, 2011). Briefly, 3 days after laser photocoagulation, protein lysates were prepared from the RPE/choroid complex with the same procedure as above, and the level of VEGF was measured with mVEGF ELISA (MMV-00; R&D Systems) according to the manufacturer's protocol. All procedures were conducted at 4°C until the final washing step was completed. The plates were analysed by measuring absorbance at 450 nm (reference at 570 nm) using a plate reader (Bio-Rad). Duplicate evaluations were performed for each sample.

RNA isolation and RT-PCR for Hrh4

Twenty-four hours after laser photocoagulation, the mouse RPE/choroid complex was stored in RNA later (Ambion Inc., Austin, TX, USA) and total RNA was purified using a Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA was reverse transcribed using a Transcriptor Universal cDNA master (Roche Applied Science, Indianapolis, IN, USA) starting with 2 μg of total RNA from each sample. RT-PCR was performed using the Thunderbird Probe qPCR mix (Toyobo Life Science, Osaka, Japan) and a Gene Expression Assay containing primers and FAM dye-labelled TaqMan probe for detecting mouse *Hrh4* (Mm00467634_m1; Applied Biosystems, Foster City, CA, USA) and eukaryotic 18S rRNA (Hs_99999901_s1; Applied Biosystems) that is available both for human and mouse 18S rRNA (Kingston *et al.*, 2009; Raffetseder *et al.*, 2011). PCR cycles consisted of a pre-denaturation step at 95°C for 2 min followed by 40 cycles of denaturing steps at 95°C for 15 s and annealing and extending steps at 60°C for 60 s. Because the control sample did not show *Hrh4* expression, quantitative RT-PCR was not thought to be correctly evaluated. Therefore, the PCR products were additionally run on a 1.5% agarose gel with ethidium bromide (10 μg·mL^{-1}; Sigma-Aldrich) and DNA bands were visualized with UV light.

Mouse electroretinography

Scotopic electroretinography (ERG) was recorded as previously described (Miyata *et al.*, 2007; Kleinman *et al.*, 2011). Briefly, animals were dark adapted overnight and anaesthetized with an i.p. injection of Avertin. After the pupils had been dilated, electroretinograms were recorded from the corneal surface with a coiled platinum wire that made contact through a thin layer of 1% methylcellulose. A similar wire was placed in the conjunctival sac, and a needle electrode that was inserted in the tail served as the reference and ground electrodes respectively. Electroretinograms were amplified by 1000× with a bandpass of 1–300 Hz. The ERG results were stored in a computer (V 3.5; Mac Laboratory, Gladstone, Australia). Strobe flash stimuli were presented in a Ganzfeld bowl (Full Field Ganzfeld Stimulator Model GS 2000; LACE Elettronica sel via Marmicciolo, Pisa, Italy). The maximum luminance was 1.0 log cd-s·m^{-2} (photopic unit) and neutral density filters were used to reduce the full-intensity stimulus.

Tube formation assay

To evaluate the effectiveness of JNJ7777120 on angiogenesis, a tube formation assay was performed as previously described (Ito *et al.*, 2012). Briefly, human retinal endothelial cells (HRECs) from Cell Systems (Kirkland, WA, USA) were cultured with EGM-2 medium (Lonza, Tokyo, Japan) in an incubator with 5% CO_2-enriched air. Extracellular matrix gels were prepared with a Chemicon *in vitro* angiogenesis assay kit (EMD Millipore, Billerica, MA, USA). Gels were solidified over a 96-well microplate. Using this kit, 1.5 × 10^4 HRECs were added to the surface of the gels and 0.1–10 μM JNJ7777120 was added to the medium. After 4 h of incubation, the tubes were labelled by Calcein-AM solution and photographed.

Statistical analysis

Results are expressed as mean ± SEM (n = number of samples). All examinations were analysed statistically using the Wilcoxon signed-rank test (paired samples) or the Mann–Whitney U-test (unpaired samples). Differences were considered to be statistically significant at $P < 0.05$.

Results

H_4 receptors were expressed in human CNV

First, we examined H_4 receptor expression in human ocular tissues. CNV was surgically removed from a 78-year-old male patient with AMD (Figure 1A shows his ocular fundus image). In the macular area, subretinal CNV was observed (white arrow). H_4 receptor-positive cells were detected in the CNV tissues (Figure 1B). IHC using isotype IgG on the same tissue revealed that H_4 receptor staining (blue) was not from a

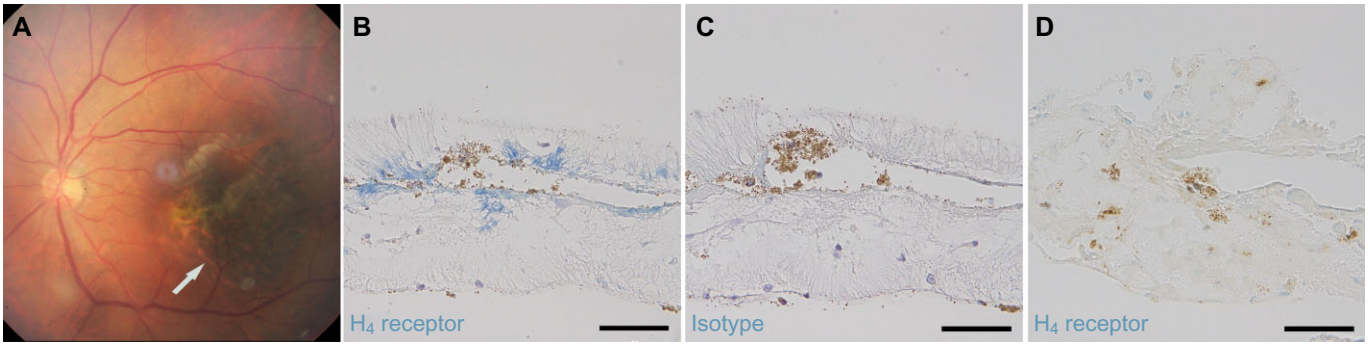

Figure 1

H_4 receptors were expressed in the CNV of a patient with AMD. (A) Colour fundus image showing CNV in a 78-year-old male patient with AMD (arrow). (B) IHC showed H_4 receptor-positive cells (blue) in the CNV specimen surgically removed from this patient (A). (C) Specificity of H_4 receptor staining was confirmed by absence of reaction production with an isotype control antibody. (D) The H_4 receptor was not expressed in the subretinal tissue from the control subject. IHC with H_4 receptor antibodies in the subretinal tissue that was surgically removed from a 35-year-old female patient with severe retinal detachment. Scale bar = 50 μm. Slides were counterstained with haematoxylin (B and C).

non-specific reaction (Figure 1C). Furthermore, IHC using the same H_4 receptor antibody on the subretinal tissue from a 35-year-old female patient with severe retinal detachment-induced PVR showed no H_4 receptor staining, and a 17-year-old male patient with traumatic PVR showed no H_4 receptor staining (Figure 1D and Supporting Information Fig. S1b). Furthermore, Western blotting showed that the macular retina and CNV from the eye with wet AMD showed H_4 receptor expression (Supporting Information Fig. S1a). These results revealed that H_4 receptor expression in the retinas was specific only for CNVs in the AMD patients.

H_4 receptors were expressed in laser CNV in mice

Then we examined H_4 receptor expression in mouse tissues. Laser photocoagulation created experimental CNV, which enabled us to study protein and mRNA expression in CNV in more detail. Laser CNV was created in wild-type and *Hrh4*$^{-/-}$ mice. Interestingly, H_4 receptor protein (Figure 2A) and mRNA (Figure 2B) were expressed only after inducing laser CNV in wild-type mouse eyes. To confirm the accuracy of the bands, RPE/choroid lysates from *Hrh4*$^{-/-}$ mice with laser CNV were also prepared. As expected, the samples from *Hrh4*$^{-/-}$ mice did not show any band even after inducing laser CNV. Moreover, IHC with anti-H_4 receptor antibodies in the *Hrh4*$^{-/-}$ mouse tissues with laser CNV showed no staining (Supporting Information Fig. S2). We further determined whether cells expressed H_4 receptors in mouse laser CNV. We performed IHC with H_4 receptor antibodies and the macrophage marker F4/80. H_4 receptor-positive cells accumulated around CNV (Figure 2C) and co-localized with the macrophage marker F4/80 (Figure 2D,E). It has been reported that accumulated macrophages have an important role in the pathogenesis of AMD (Ambati *et al.*, 2003b; Sakurai *et al.*, 2003a). Our results indicated that macrophages recruited around laser CNV expressed H_4 receptors, whereas mouse retinas did not express H_4 receptors in a normal state.

No effect of JNJ7777120 on HREC tube formation

We also examined the effect of JNJ7777120 on retinal endothelial cells *in vitro*. In the tube formation assay, various concentrations of JNJ777120 were tested but none induced significant changes in vessel formation (Supporting Information Fig. S4).

Hrh4$^{-/-}$ *mice showed suppressed CNV*

Measuring laser CNV volumes is also a very useful tool for comparing two different groups; for example, checking drug effectiveness to promote or inhibit angiogenesis. Furthermore, by comparing CNV volumes in mice that lack a specific gene to that in wild-type mice, we could evaluate whether the specific gene is important for pro- or anti-angiogenesis. To evaluate the role of H_4 receptors in ocular angiogenesis, laser CNVs were performed in wild-type and *Hrh4*$^{-/-}$ mice. The CNV volume of *Hrh4*$^{-/-}$ mice was smaller by 25% compared with that of wild-type mice (Figure 3A–C, 1.00 ± 0.06 vs. 0.75 ± 0.05, $P = 0.0025$, $n = 10$). In finding new therapeutic factors to control CNV, it is important to investigate the relationship with VEGF, because VEGF is expressed not only in CNV but also in normal adult human retinas for maintaining regular conditions in the eye (Marneros *et al.*, 2005; Nishijima *et al.*,

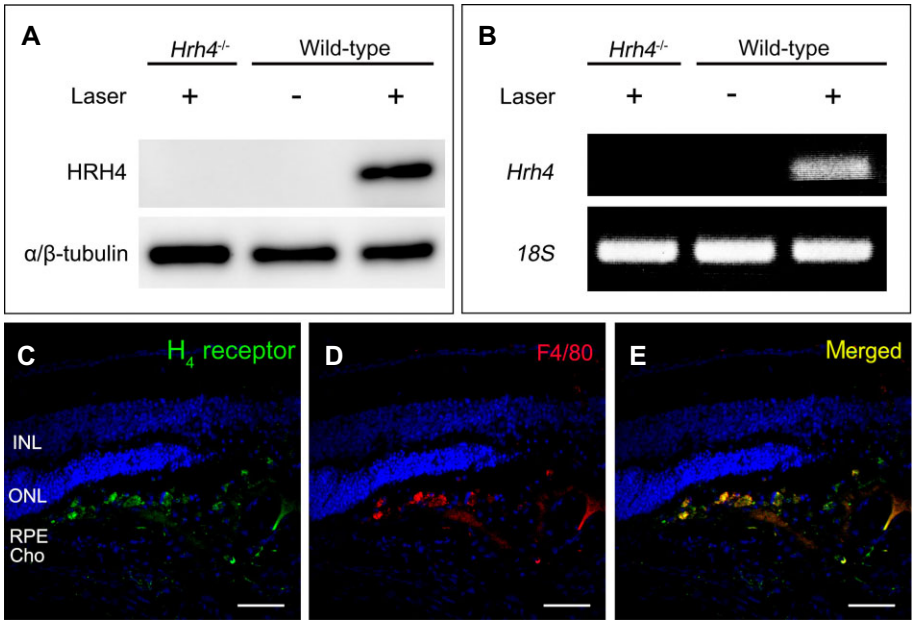

Figure 2

The H_4 receptor was expressed in the laser-induced CNVs in wild-type mice. (A,B) Protein and mRNA H_4 receptor were expressed from the RPE in wild-type mice with laser-induced CNV (laser CNV), but not from the RPE from wild-type mice RPE without laser CNV or *Hrh4*$^{-/-}$ mice with laser CNV. (C–E) IHC from wild-type mice retinal sections with laser CNV. H_4 receptors (green) were expressed in the RPE and choroid (Cho) space with laser CNV and co-localized with F4/80 (red). Scale bar = 50 μm.

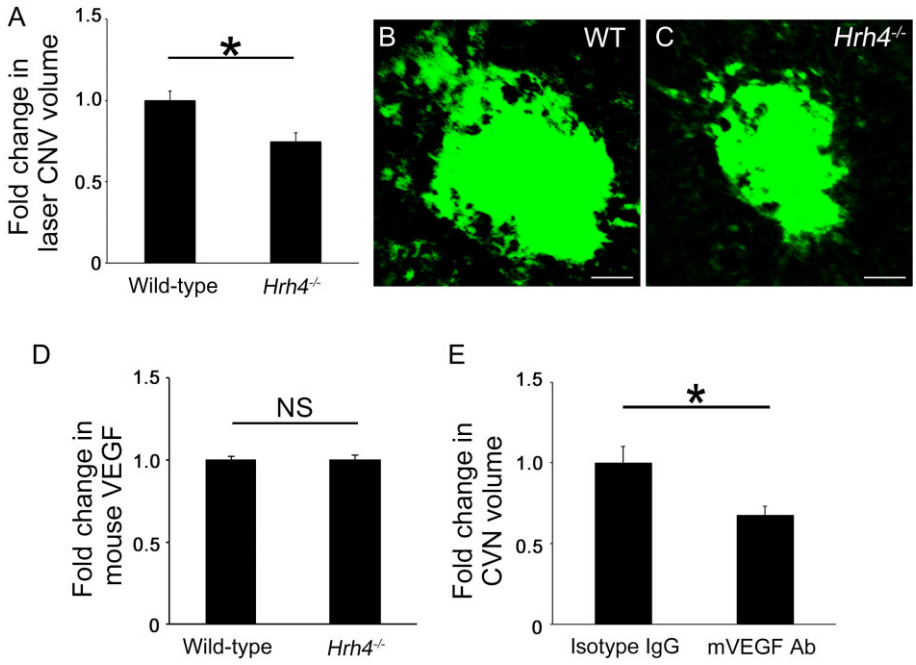

Figure 3

Laser-induced CNVs (laser CNVs) were reduced in *Hrh4*$^{-/-}$ mice. (A) The volume of laser CNVs in *Hrh4*$^{-/-}$ mice was reduced by 24% ($P = 0.0095$) compared with that in wild-type mice. (B,C) Representative images of laser CNV in wild-type (B) and *Hrh4*$^{-/-}$ mice (C). (D) mVEGF expression was not reduced in *Hrh4*$^{-/-}$ mice compared with wild-type mice ($P = 1.0$). (E) Laser CNV in *Hrh4*$^{-/-}$ mice was 35% reduced after mVEGF neutralizing antibody injection ($P = 0.0001$). Scale bar = 50 μm, *$P < 0.05$, NS, showed no significant difference.

2007; Saint-Geniez *et al.*, 2008; 2009). To examine the relationship between H_4 receptors and VEGF, we further compared mVEGF expression in the RPE/choroid with laser CNV in wild-type compared to *Hrh4*$^{-/-}$ mice. mVEGF levels were not significantly different (Figure 3D, 1.00 ± 0.02 vs. 1.00 ± 0.03, $P = 1.0$, $n = 14$). Moreover, we tested CNV volumes in *Hrh4*$^{-/-}$ mice injected with a neutralizing antibody against mVEGF (R&D Systems) compared with that injected with isotype IgG. Interestingly, CNV was reduced by 35% (1.00 ± 0.1 vs. 0.68 ± 0.06, $P = 0.007$, $n = 12$ and $n = 11$ respectively). These results indicate that deletion of *Hrh4* worked as an anti-angiogenesis factor in CNV, independent of VEGF production.

Intravitreous injections of H_4 receptor antagonists suppressed CNV and pathological vessel leakage

To pursue the therapeutic possibility of H_4 receptor-targeted treatment, we further examined the effectiveness of H_4 receptor antagonists on laser CNV. JNJ7777120 and JNJ10191584 are selective H_4 receptor antagonists and have been reported to be effective as H_4 receptor antagonists in mice as well as in humans (Jablonowski *et al.*, 2003; Thurmond *et al.*, 2004; Connelly *et al.*, 2009; Dunford and Holgate, 2011; Desmadryl *et al.*, 2012). Intravitreous injections of JNJ7777120 reduced the laser CNV volume by 47% compared with controls (Figure 4A, 1.00 ± 0.19 vs. 0.53 ± 0.06, $P = 0.028$, $n = 11$) and intravitreous injections of JNJ10191584 reduced the laser CNV volume by 28% compared with controls (Supporting Information Fig. S4, 1.00 ± 0.08 vs. 0.72 ± 0.06, $P = 0.014$, $n = 15$). We also examined the pathological vessel leakage from the CNV by fluorescein angiography of the mouse fundus. JNJ7777120 suppressed pathological leakage; that is, 42.5 ± 8.4% of grade 3 leakage in the eye injected with JNJ7777120 compared with 77.5 ± 6.9% in controls ($P = 0.035$, $n = 10$). Additionally, CNV volumes of the eyes injected with anti-mouse VEGF antibodies combined with JNJ7777120 were significantly smaller than those injected with anti-mouse VEGF only (Supporting Information Fig. S3).

JNJ7777120 did not induce retinal toxicity

We also evaluated the retinal toxicity of intravitreously injected JNJ7777120. Fundus imaging and retinal section analyses to identify retinal degeneration were used. In addition, ERG was used to evaluate functional changes after JNJ7777120 administration. All examinations were performed 7 days after injection of 5 μg of JNJ7777120. JNJ7777120 did not induce funduscopic changes; for example, retinal degeneration ($n = 6$). Haematoxylin and eosin staining of cryosections from the eyes showed no histological changes. Moreover, ERG showed that a-waves (photoreceptor function) from the eyes injected with JNJ7777120 were not reduced compared with controls (1.00 ± 0.09 vs. 1.04 ± 0.17, $P = 0.85$, $n = 11$ and $n = 9$; see Figure 5). These results indicate that intravitreous injection of JNJ7777120 can successfully reduce mouse CNV without causing retinal toxicity.

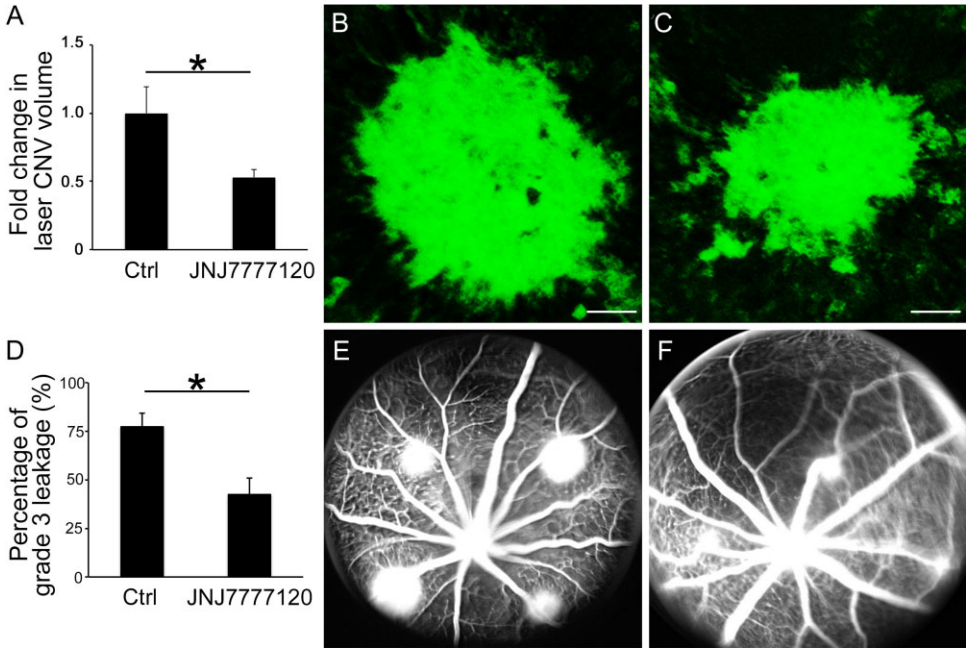

Figure 4

Laser-induced CNVs (laser CNVs) were reduced by the H_4 receptor antagonist JNJ7777120. (A) Intravitreous injection of JNJ7777120 significantly reduced laser CNV volume by 43% compared with controls (P = 0.0083). (B,C) Representative images of laser CNV in wild-type mouse eyes injected with JNJ7777120 (C) and controls (B). (D) JNJ7777120 reduced pathological vessel leakage in laser CNVs. The percentage of grade 3 (pathological) leakage was 42.5% in the eyes injected with JNJ7777120, which was significantly less than that of controls (77.5%, P = 0.003). (E,F) Representative late-phase images of fluorescent angiography 7 days after inducing laser CNV in wild-type mice injected with JNJ7777120 (F) and controls (E). Scale bar = 50 μm *<0.05.

Discussion and conclusions

In this study, we showed that the H_4 receptor is expressed in human and mouse CNVs and indicated the possibility of a H_4 receptor-targeted treatment for wet AMD. Because the H_4 receptor has a unique profile in terms of expression patterns, its molecular biology and pharmacology has been investigated as a new therapeutic target in many diseases (Leurs *et al.*, 2009; Zampeli and Tiligada, 2009). The H_4 receptor is primarily expressed in peripheral tissues such as spleen, thymus, colon, leukocytes and bone marrow (Oda *et al.*, 2000; Cogé *et al.*, 2001; Gantner *et al.*, 2002). A recent study showed that the H_4 receptor is also expressed in human and mouse brain neurons (Connelly *et al.*, 2009). Interestingly, the H_4 receptor expressed in the epithelium of the gastrointestinal tract, showed reduced expression in the case of advanced gastric carcinoma (Zhang *et al.*, 2012). Furthermore, H_4 receptors are expressed in human breast cancer cells, and a H_4 receptor antagonist decreased cell proliferation and decreased intratumoural vessels (Martinel Lamas *et al.*, 2013). These results indicate that the H_4 receptor is associated with carcinogenesis in some tissues. Because tumorigenesis is strongly associated with angiogenesis, we primarily hypothesized that the H_4 receptor has a strong relationship with angiogenesis; however, in our study, H_4 receptor-positive cells were not co-stained with CD31 antibodies, a vascular endothelial cell marker (data not shown). Instead, the cells showed co-staining with F4/80, a macrophage marker. Interestingly, when examining the influence of JNJ7777120 on vessel permeabilization in ocular angiogenesis, JNJ7777120 successfully reduced fluorescein leakage from immature neovascular cells. Although the H_4 receptor-positive cells were not identical, the result was similar in that vascular permeability was reduced by H_4 receptor blockade. However, when comparing mVEGF expression levels between wild-type and *Hrh4*$^{-/-}$ mice, there was no significant difference in the RPE with laser CNV. Moreover, when comparing the laser CNV volume of *Hrh4*$^{-/-}$ mice injected with anti-mVEGF neutralizing antibodies compared to isotype IgG, CNVs were reduced in the *Hrh4*$^{-/-}$ mice injected with anti-mVEGF antibodies. These results indicate that the anti-angiogenesis effect induced by H_4 receptor suppression occurred independently of mVEGF up-regulation caused by laser photocoagulation. Our results strongly indicate that H_4 receptor expression in CNV was in macrophages that were accumulating in the CNV. A previous study showed that H_4 receptors are expressed in chemotactic-induced human monocyte-derived dendritic cells (Gutzmer *et al.*, 2005). There was also a previous report showing that H_4 receptors suppressed CCL2 production in monocytes, presumably indicating a negative feedback mechanism to avoid persistent histamine release (Dijkstra *et al.*, 2007). Therefore, it is possible to hypothesize that specific macrophages accumulating around the CNV express H_4 receptors, and are pro-angiogenesis independently of mVEGF. Previous reports demonstrated that H_4 receptors have different ligands from histamine (Nakayama *et al.*, 2004). Because H_4 receptors can

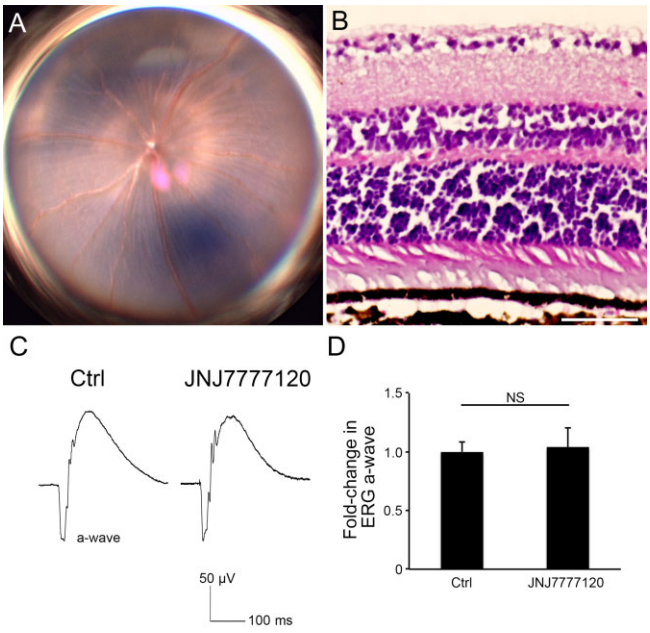

Figure 5

JNJ7777120 did not show retinal toxicity. Colour fundus image (A) and haematoxylin and eosin staining of a cryosection (B) from the wild-type mouse eye injected with 5 μg of JNJ7777120 showing no retinal degeneration. (C) Representative electroretinogram (ERG) from the eye injected with JNJ7777120. (D) The electroretinogram showed no significant reduction in the a-wave amplitude from the eye injected with JNJ7777120 compared with the controls ($P = 0.85$). Scale bar = 50 μm. NS, no significant difference.

show different characteristics dependent on cell type, further investigations are required to elucidate the interactions between monocytes, macrophages and H_4 receptors.

We used JNJ7777120, one of the most common H_4 receptor antagonists, and found that single intravitreous injections of 0.1 and 1 μg of JNJ7777120 did not reduce laser CNV volume (data not shown). This was presumably because of its short half-life. Hence, we examined double injections of JNJ7777120 (1 μg) and this reduced CNV. We also examined the effect of JNJ10191584, another selective H_4 receptor antagonist, on laser CNV in mice, and this also reduced CNV. However, these compounds required us to inject repeatedly to suppress CNV, probably due to the short half-lives of these compounds (Zhang *et al.*, 2007). More long-acting antagonists are required for clinical use to suppress human CNV. However, 5 μg JNJ7777120 did not induce retinal degeneration in mice. It has been reported that JNJ7777120 inhibits 5-HT_{2A} receptors and the NA transporter, but had no cross-reactivity against 50 other targets (Thurmond *et al.*, 2004). These results suggest a strong advantage when thinking about pharmacological applications. H_4 receptors were not expressed in the normal mouse RPE, and we therefore do not have to consider negative side effects as with anti-VEGF treatments. However, it has been reported that suppressing H_4 receptors with its antagonist worsened mouse autoimmune encephalomyelitis, indicating a detrimental aspect of H_4 receptor blockade (Ballerini *et al.*, 2013). In summary, JNJ7777120 shows some potential as a treatment for AMD; however, more investigations are needed for before it can be applied to humans.

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Contributions

Study conception and design: HK, FY. Acquisition of data: FY, RI, SeK, MN. Analysis and interpretation of data: ShK, AH, HT. Drafting of manuscript: HK. Critical revision: ShK, AH, HT.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 The H_4 receptor was expressed in the macular retina with choroidal neovascularization (CNV) from a patient with age-related macular degeneration (AMD). (a) A Western blot of the macular retina with CNV showed an abundance of H_4 receptors. Isotype IgG was used to exclude non-specific reactions. (b) H_4 receptors were not expressed in the subretinal tissue from a 17-year-old male patient with proliferative vitreoretinopathy. Scale bar = 50 μm. A slide was counterstained with haematoxylin (b).

Figure S2 Specificity of anti- H_4 receptor antibody on mice tissues. (a,b) H_4 receptor-positive cells were detected in the laser-induced choroidal neovascularizations (CNVs) from wild-type mice (b), but not from *Hrh4*$^{-/-}$ mice (a). (c) Specificity of H_4 receptor staining was confirmed by the absence of reaction production with an isotype control IgG in laser-induced CNV from wild-type mice. H_4 receptor or isotype IgG (green), 4′,6-diamidino-2-phenylindole (blue).

Figure S3 The H_4 receptor antagonist JNJ7777120 did not induce or suppress tube formation of human retinal endothelial cells (HRECs). (a) Compared with the area size of HREC vessels with control medium (control: 1.0 ± 0.06, n = 8), those with 0.1 μM (1.07 ± 0.06, P = 0.50, n = 9), with 1 μM (1.03 ± 0.06, P = 0.70) and with 10 μM (1.10 ± 0.06, P = 0.21, n = 9) of JNJ7777120 did not induce significant changes in vessel formation. (b–e) Representative images of tube formation of HRECs with each concentration of JNJ7777120. Scale bar = 100 μm. NS = they did not show a significant difference.

Figure S4 Laser-induced choroidal neovascularizations (laser CNVs) were reduced by the H_4 receptor antagonist JNJ10191584. (a) Intravitreous injections of JNJ10191584 significantly reduced laser CNV volume by 28% compared with controls (P = 0.014). (b,c) Representative images of laser CNV in wild-type mouse eyes injected with JNJ10191584 (c) and controls (b). Scale bar = 50 μm, *<0.05.

Figure S5 Laser-induced choroidal neovascularizations (laser CNVs) were reduced by anti-mouse vascular endothelial growth factor (VEGF) antibody combined with H_4 receptor antagonist JNJ7777120. The volume of laser CNVs in wild-type mice injected with mouse VEGF neutralizing antibody (day 0) combined with JNJ7777120 (days 0 and 3) was reduced by 41% compared with those with anti-mouse VEGF antibody only (1.00 ± 0.10 vs. 0.59 ± 0.10, P = 0.019, n = 7) *<0.05.