Adrenomedullin Inhibits Choroidal Neovascularization via CCL2 in the Retinal Pigment Epithelium

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The molecular mechanism that leads to age-related macular degeneration (AMD) is poorly understood. Gene expression profiling identified adrenomedullin (ADM) as a possible molecular target for the treatment of AMD and expression of ADM was upregulated in eyes with laser-induced choroidal neovascularization (CNV). *In vivo* experiments strongly indicated that ADM inhibits laser-induced CNV. *In vitro* tube formation assay demonstrated that neither ADM nor conditioned medium from the retinal pigment epithelial (RPE) cells, D407 cells, treated with ADM affected the capillary-formation of human umbilical vein endothelial cells. In contrast, *in vitro* macrophage migration assay clearly demonstrated that the conditioned medium of D407 inhibited macrophage migration. Furthermore, the expression of C-C motif chemokine 2 (CCL2) was significantly inhibited in D407 cells after ADM treatment. *In vivo* experiments using a laser-induced CNV model in ADM^{+/-} mice demonstrated that CCL2 expression was upregulated in ADM^{+/-} mice with concomitant increase in macrophage migration in the subretinal space. Additionally, the effect of ADM was abrogated in CCL2 knockout mice. These results suggest that administration of ADM inhibits macrophage migration in the subretinal space and leads to the suppression of laser-induced CNV in an animal model. The inhibition of macrophage migration occurred through the CCL2 from RPE. This study provides a novel potential therapeutic target for AMD which does not substantially disrupt VEGF-A signaling

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Choroidal neovascularization (CNV) in age-related macular degeneration (AMD) is the leading cause of blindness in people over 50 years of age.^{1,2} AMD is characterized by progressive degeneration of the retina, retinal pigment epithelium (RPE), and underlying choroid. The earliest clinical hallmark of AMD is the appearance of drusen, localized lipoproteinaceous deposits between the RPE, and Bruch membrane.³ Drusen and RPE dysfunction are implicated in CNV formation.⁴ A body of evidence suggests that injury to the RPE plays a role in drusen biogenesis.^{5,6} This injury is considered to occur through gene mutations, light damage, oxidative stress, and lipofuscin accumulation, and results in the release of cytokines into the Bruch membrane.^{7–13} Some of the chemokines diffuse into the choroid and work for monocytes, and these inflammatory cells amplify the local inflammatory cycle by mechanisms including immune complex formation and complement activation.^{14–18} Thus, chronic inflammation is an important process in drusen biogenesis. Previous studies have shown that C3 and membrane attack complex (MAC) deposit in CNV lesions. It has also been reported that the accumulation of complement component can induce vascular endothelial growth factor (VEGF) in RPE and contribute to CNV formation.^{17,19} The factors involved in this chronic inflammatory process are therefore potential therapeutic targets of AMD.

Early epidemiological studies have established that the disease is heritable and clearly modulated by nongenetic environmental risk factors^{20,21}, whereas biochemical and histological studies have demonstrated that the complement system was involved in drusen biogenesis

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and the etiology of AMD.¹⁸ One of these genes, *complement factor H (CFH)* on chromosome 1q32 has been identified by a number of studies as a major AMD-susceptible gene polymorphism.^{22–26} In addition, biochemical and pathological studies have shown that a CFH Y402H polymorphism may be involved in AMD.^{27,28} Some studies have suggested that RPE and choroidal cells may have the capacity to synthesize a number of these complement-regulatory molecules.^{12,29–32} If these cells synthesize significant quantities of complement component molecules and the associating factors, they could make a substantial contribution to the pathogenesis of AMD.

Evidence supports a role for VEGF in CNV.³³ Of the subtypes of VEGF, VEGF-A has become a major therapeutic target for CNV.³⁴ Blockage of VEGF-A signaling led to the remarkable recovery of visual acuity in neovascular AMD patients^{35–37}; however, it should be noted that receptors for VEGF-A are present in normal retinal neuronal cells and that VEGF-A provides neuroprotection in the retina.³⁸ Under certain pathological conditions, such as the development of CNV and retinal neovascularization in the eye or in cancerous tumor development, angiogenesis is considered to be an adverse phenomenon.^{39–41} It is therefore a concern that even if blocking VEGF-A signaling is effective in suppressing CNV development, the continuous blocking of VEGF-A signaling in these agents may in fact be dangerous to patients, particularly those of advanced age, such as AMD patients.^{38,42–48} Moreover, some AMD patients are refractory to current anti-VEGF therapies.^{37,49} Thus, treatment strategies based on more specific targeting of CNV are desirable.

In the present study, using aged mice and mice with laser-induced CNV, the expression of complement components and their associating factors in the RPE/choroid were examined, especially focusing on factors associated with CFH using microarray analysis. Among these factors, adrenomedullin (ADM) was found to be remarkably upregulated in the RPE/choroidal layer after laser treatments. This result led us to investigate the effects of ADM on the development of CNV.

Materials and Methods

Animals

Male 6- to 8-week old mice or 18-month old mice as the aged mice were used in all experiments. ADM homozygous knockout have an embryonic lethal phenotype with abnormal vascular development; the study used ADM knockout (*ADM*^{+/−}) mice on a C57BL/6 background kept as a heterozygous line, in which decreased expression of ADM has been confirmed.⁵⁰ C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan), and *CCL2* knockout (*CCL2*^{−/−}) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All experiments were conducted in accordance with the Animal Care and Use Committee, and the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research.

Cell Culture

Human retinal pigment epithelial cells, D407, and RAW264.7 macrophage cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100U/mL of penicillin, and 10 μg/mL of streptomycin. Human umbilical vein endothelial cells (HUVECs) were maintained in Medium 200 supplemented with Low Serum Growth Supplement kit. These cells were incubated at 37°C in a humidified 5% CO₂ incubator.

ADM was delivered at concentrations of 10^{−7}, 10^{−8}, and 10^{−9}M into the culture media, and confluent cultures of the RPE cells were incubated with ADM. After 12 hours, cells were collected and used for mRNA quantification by real-time reverse transcription-PCR (RT-PCR). The conditioned medium was collected for *in vitro* tube formation assay and macrophage migration assay.

Experimental CNV

General anesthesia was induced with an intraperitoneal injection of a mixture (5:1) of ketamine hydrochloride (Ketalar, Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celctal, Bayer, Tokyo, Japan). The pupil was dilated with 1 drop of 0.5% tropicamide and 0.5% phenylephrine (Mydrine P, Santen Pharmaceutical, Osaka, Japan) for photocoagulation. Experimental CNV was created as has been described elsewhere.⁵¹ Laser photocoagulations were applied to each eye between the major retinal vessels around the optic disk with a diode laser photocoagulator (DC-3000, NIDEK, Osaka, Japan) and a slit lamp delivery system (SL 150, Topcon, Tokyo, Japan) at a spot size of 75 μm, duration of 0.05 seconds, and intensity of 200 mW. An attempt was made to break the Bruch membrane, as evidenced by the central bubble formation. Immediately after the photocoagulation, 1 μL of ADM (10^{−7} M) or PBS was injected into the vitreous cavity.

Microarray Analysis

Male 6- to 8-week-old mice and 18-month-old mice were used for microarray analysis. Seven days after laser treatment, mRNA from RPE/choroidal was isolated using RNeasy Mini kit (Qiagen, Valencia, CA). Target RNA was hybridized on a GeneChip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA) at the research Center for Advanced Science and Technology, University of Tokyo. The experimental procedures for the GeneChip were performed according to the Affymetrix technical manual. Two paired sets of samples from individual eyes were used for analysis.

Fluorescein Angiography

Seven days after the laser treatment, the mice were intraperitoneally anesthetized. Fluorescein angiography was performed to quantify the amount of leakage from the CNV, as previously described.⁵¹ Four to 6 minutes after the injection, three angiograms were taken using a retinal camera (TRC-50IX, Topcon), with the built-in filter for fluorescein. The images were captured with a 3CCD color video camera (640 × 480 pixels) (DXC970MD, SONY, Tokyo, Japan),

imported into a Windows personal computer, and analyzed using ImageJ software version 1.46 (NIH, Bethesda, MD). The signal intensities (brightness) within the leakage from CNV were measured and integrated at each lasered site.

Fluorescein Isothiocyanate Dextran Angiography and Flat Mount

Mice were perfused with 1 mL of PBS containing 50 mg/mL of fluorescein-labeled dextran from the left ventricle for 1 minute, followed by removal of the eyes. After the eyes were enucleated and briefly fixed in 4% PFA, the anterior segment and neural retina were carefully removed. Four to six radial cuts were made from the edge to the equator, and the eye cup was flat mounted with the sclera facing down. Using the appropriate FITC filters, the flat mounts were viewed under an epifluorescent microscope. A computer-operated digital camera with image capture (Olympus BX51, Olympus, Tokyo, Japan) was used to photograph the areas of choroidal neovascularization. The total area of hyperfluorescence was measured using ImageJ software.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Label Staining

Apoptotic cell death was judged by terminal deoxynucleotidyl transferase-mediated dUTP nick end-label (TUNEL) staining, which was performed using a kit (DeadEnd Fluorometric TUNEL System), according to the manufacturer's instructions (Promega, Madison, WI). The apoptotic cells were visualized with fluorescein-12-dUTP, and nuclei were stained with propidium iodide (PI). For positive controls, sections were processed with DNase I (Takara Bio, Tokyo, Japan).

Immunostaining and Transmission Electron Microscopy

Immunostaining was performed as has been described previously.⁵² Briefly, after the RPE-choroidal flat mount was made, the sample was incubated with rat anti-mouse F4/80 antibody. Alexa 488-conjugated secondary antibody was used to visualize the immunostaining. The immunostained flat mounts were viewed in five fields at $\times 200$ magnification, and F4/80-positive cells around CNV were counted for investigating the macrophage infiltration toward CNV.

Transmission electron microscopy was performed by Hanaichi Ultrastructure Research Institute Co. (Okazaki, Japan) with reference to the previous report.⁵³ Eyes were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS at pH 7.4, and semithin sections were analyzed using an electron microscope (JEOL JEM-200EX, Akishima, Japan).

Histological Analysis

Eyes were enucleated and embedded in paraffin blocks, and 7- μ m semithin sections were made. The sections were mounted on glass slides and stained with H&E.

In Situ RT-PCR

Immediately after enucleation, eyes were fixed in 4% paraformaldehyde in PBS for 1 hour. The eyes were embedded in optimum cutting temperature compound (Tissue-Tek, Torrance, CA) and cut into 7- μ m sections. *In situ* RT-PCR was performed with the sections covered by a slide seal (Takara Bio, Tokyo, Japan) and using a thermal cycler equipped with a unit for *in situ* PCR (DNAEngine; BioRad). After reverse transcription (RT), PCR was performed in the presence of 1 mM DIG-11-dUTP (Roche Diagnostics, Basel, Switzerland) using ADM-specific primers. Development of the sections was performed following the protocols of the manufacturer and as has been previously described.⁵⁴

mRNA Quantification by Real-Time RT-PCR

mRNA for RT-PCR was isolated using SV Total RNA Isolation Kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. cDNA was prepared using Superscript III for RT-PCR (Invitrogen, Carlsbad, CA). Each PCR was carried out in a 20- μ L volume using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) for 30 seconds at 95°C denature, followed by 55 cycles at 95°C for 5 seconds, 55°C for 5 seconds, and 72°C for 10 seconds in a Roche LightCycler. Values for each gene were normalized to expression levels of GAPDH. The sequences of the primers used for RT-PCR were as follows:

Human GAPDH, left, 5'-GAGTCAACGGATTGGTCGT-3', right, 5'-TTGATTTGGAGGGATCTCG-3'; mouse GAPDH, left, 5'-CACATTGGGGGTAGGAACAC-3', right, 5'-AACT-TTGGCATTGTGGAAGG-3'; human VEGF-A, left, 5'-ATGTGCATGGTGATGTTGGA-3, right, 5'-GCTTGCTGCTGTACCTCCAC-3'; human VEGF-B, left, 5'-CCCTTGACTGTGAGCTCAT-3', right, 5'-CACTGGCTGTGTTCTTCCAG-3'; human CCL2, left, 5'-CAGCCAGATGCAATCAATGC-3', right, 5'-GTGGTCCATGGAATCCTGAA-3'; mouse CCL2, left, 5'-CAGGTCCCTGTCATGCTTCT-3', right 5'-TCTG-GACCCATTCTCTTCTTG-3'; human CCL11, left, 5'-CCCTTCAGCGACTAGAGAGC-3', right, 5'-TGGCTTTGGAGT-TGGAGATT-3'; human CCL24, left, 5'-GCCTTCTGTTCTT-TGGTGTC-3', right, 5'-TGTACCTCTGGACCCACTCC-3'; human CCL26, left, 5'-CTGCTTCCAATACAGCCACA-3', right, 5'-CACAATTGTTTCGGAGTTTCA-3'; mouse Adrenomedullin, left, 5'-TTCGCAGTTCCGAAAGAAGT-3', and right 5'-CCATGGGTGTTCTGCTCGTC-3'.

In Vitro Tube Formation Assay

In vitro angiogenesis activity was evaluated with the *in vitro* tube formation assay. Human umbilical vein endothelial cells (HUVECs) starved of serum for 4 hours were seeded at a cell density of 40,000 cells per well in 24-well culture plates (Nalge Nunc International), precoated with 0.4 mL of low-growth factor synthetic matrix (Matrigel, BD Bioscience, San Jose, CA) and cultured with the conditioned medium (DMEM) collected from D407 cells or D407 cells exposed to ADM for 24 hours. Tube formation was determined 16 hours after cells were plated on Matrigel by counting the number of connected cells in five

randomly selected fields at $\times 200$ magnification, and dividing that number by the total number of cells in the same field. Micrographs were taken under a phase-contrast light microscope (Olympus, Tokyo, Japan).

In Vitro Migration Assay

Transwell migration assays were performed using a modification of the 96-well microchemotaxis assay, as has been described.^{55,56} RAW264.7 cells (5×10^4 cells) were loaded onto the top wells of the chamber (Corning Costar, Cambridge, MA) with conditioned medium (DMEM), collected from D407 cells or D407 cells exposed to ADM for 24 hours in the bottom chamber and incubated for 6 hours. Cells on the lower surface of the filter were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and stained with H&E staining. The transmigrated cells were photographed in triplicate at $\times 40$ magnification. Cell migration was determined as the number of cells that had migrated across the filter in the photographed area.

Statistical Analysis

All results are presented as the mean ($\pm$ SEM). The nested analysis of variance test was performed to assess the CNV area. Tube formation assay, macrophage migration assay, and RT-PCR were assessed using the Student *t*-test. Values of $P < 0.05$ were taken as statistically significant. Statistical analysis was performed with the SPSS 17.0 program for Windows (SPSS Inc., Chicago, IL).

Results

Increased ADM Expression in RPE/Choroid and Retina with Laser-Induced CNV

To investigate whether there was any role of complement pathway molecules in the pathogenesis of AMD, aged mice and laser-induced CNV model mice, the most widely used animal model of this disease, were used. The results demonstrated that at the mRNA (mRNA) level, the complement pathway proteins such as C1q, C3, and C4 are upregulated in RPE/choroid in CNV model mice, and these results are consistent with previous reports (see Supplemental Table S1 at <http://ajp.amjpathol.org>).^{17,19} It was confirmed that C3 is also upregulated in aged mice. The relative abundances of these mRNAs in the RPE/choroid support the theory that local cells have the potential to make a substantial contribution to CNV.

Expression of factors that regulate CFH or bind with CFH and have coactivity was also investigated. It is known that C-reactive protein (CRP), fibromodulin, and ADM bind with CFH.⁵⁷ Of these genes, interest was focused on ADM because the expression level was increased in the RPE/choroidal layer of laser-induced CNV model mice (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Real-time RT-PCR demonstrated that the expression of ADM was increased 1.57-fold in the RPE/choroid layer and 1.87-fold in the retina after laser treatment, in comparison to

Figure 1. Expression pattern of ADM mRNA in the retina and the RPE/choroid layer. Real-time RT-PCR demonstrated that after laser treatment, the expression of ADM was increased 1.87-fold in the retina (**A**) and 1.57-fold in the RPE/choroid layer (**B**), respectively, in comparison to non-photocoagulation mice. $n = 8$ for all. * $P < 0.05$. **C** and **D**: *In situ* RT-PCR analysis in albino mouse and laser-induced CNV model mouse. Normal retina expressed detectable levels of ADM in the ganglion cell layer, inner and outer nuclear layer, endothelial cells, and retinal pigment epithelial cells (**C**). ADM mRNA was also detected in the laser-induced CNVs in mice, most prominently in highly vascularized lesions and also in stromal cells and retinal pigment epithelial cells (**D**).

non-photocoagulation mice (Figure 1, A and B). The expression pattern of ADM mRNA was examined with *in situ* RT-PCR in normal retina and 7 days after laser treatment. Under physiological conditions, normal retina expressed detectable levels of ADM in the ganglion cell layer, inner and outer nuclear layer, endothelial cells, and retinal pigment epithelial cells (Figure 1C). ADM mRNA was also detected in the laser-induced CNVs in mice, most prominently in highly vascularized lesions and also in stromal cells and retinal pigment epithelial cells (Figure 1D).

Increased CNV Lesions in $ADM^{+/-}$ Mice

To evaluate the effect of ADM on CNV, eyes from $ADM^{+/-}$ mice and age-matched wild-type mice were subjected to laser-induced CNV. The retinal structure, retinal vessels, and structure of RPE/choroid of $ADM^{+/-}$ mice were normal as examined by light microscopic analysis, TUNEL staining, flat mount analysis, and transmission electron microscopic analysis (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Interestingly, $ADM^{+/-}$ mice showed significant increase in leakage from CNV compared with control mice (Figure 2, A and B). To confirm whether the increase in leakage in fluorescein angiography is actually a consequence of the CNV lesions, the CNV lesions were analyzed by choroidal flat mounts, which demonstrated that the CNV lesions in $ADM^{+/-}$ mice were significantly larger than those in wild-type mice (Figure 2, C and D). In addition, the effects of intraocular ADM administration were examined after laser treatment in wild-type mice;

Figure 2. Pathological leakage representing CNV formation 7 days after laser photocoagulation. Large and diffuse areas of leakage were observed in *ADM*^{+/-} mice in comparison with wild-type mice. **A** and **B**: Fluorescein isothiocyanate dextran angiography. **C** and **D**: Flat mount. *n* = 12 for all. **P* < 0.05. **E** and **F**: ADM significantly reduced the CNV volume, and this effect was almost equal to VEGF-A antibody. *n* = 8 for all. **P* < 0.05.

and the results showed that the areas of CNV were smaller in mice that received intraocular ADM administration in comparison with controls. It should be noted that the inhibitory effect of ADM was almost equal to that of VEGF-A antibody (Figure 2, E and F).

Taken together, these data demonstrate that ADM acts to suppress laser-induced CNV.

Effects of ADM on Angiogenesis

The pathogenesis of CNV consists of inflammation, angiogenesis, and subsequently neovascularization. Several studies have suggested the role of ADM on both angiogenesis and inflammation,^{58,59} and it is possible that the anti-CNV effect of ADM might derive from various components of this cascade. Thus, the expression of VEGF-A, VEGF-B, pro-angiogenic CCR3 ligands including CCL11, CCL24, and CCL26 were first examined in an RPE cell line, D407, stimulated with ADM for 24 hours by real-time RT-PCR. Specifically, the real-time RT-PCR measurements indicated that VEGF-A and -B showed no change in the expression in comparison with vehicle-treated cells, under both normoxic and hypoxic conditions (Figure 3, A and B); however, expression of CCL11, CCL24, and CCL26 were not detectable in D407.

Furthermore, *in vitro* tube formation assay was performed to examine whether HUVECs form capillary-like

structures in response to secreted factors from the D407 cells exposed to ADM. The results demonstrated that HUVECs cultured with the conditioned medium of ADM-treated D407 did not inhibit capillary-like tube formation. To further examine whether ADM directly affects tube formation, HUVECs were cultured with conditioned medium, which was collected from non-ADM-treated D407, and ADM was administered immediately before culturing HUVECs. These results also demonstrated that ADM does not directly affect capillary formation. To summarize, the above-described results demonstrated that ADM not only does not affect capillary-like tube formation, but also neither directly acts on vascular endothelial cells nor acts indirectly to affect pro-angiogenic factors through RPE cells (Figure 3, C and D).

ADM Inhibits Macrophage Migration by Modulating CCL2 from RPE

Another major function of ADM is to modulate immune reaction at inflammation sites. Consequently, the next experiments were performed to investigate the effects of ADM on inflammation. First, we quantified macrophage migration *in vivo*. In this assay, the number of subretinal

Figure 3. Effect of ADM in RPE and vascular endothelial cells. Expression of VEGF-A (**A**) and VEGF-B (**B**) were examined in RPE cells (D407) stimulated with ADM for 24 hours by real-time RT-PCR. The real-time RT-PCR measurements indicated that VEGF-A and -B showed no change in the expression in comparison with vehicle-treated cells. **C**: *In vitro* tube formation assay demonstrated that HUVECs cultured with conditioned medium (CM) of ADM treated RPE cells (D407) did not inhibit capillary-like tube formation. Capillary-like tube formation was inhibited by coinubation with human VEGF-A antibody but not with ADM immediately before culturing HUVECs. *n* = 6 for all. **P* < 0.05. **D**: Representative examples of micrographs. Original magnification, ×200. **Top left**: Cultured with the conditioned medium collected from D407 cells. **Top middle**: Cultured with the conditioned medium collected from D407 cells exposed to ADM. Cultured with the conditioned medium collected from D407 cells and ADM (**top right**), VEGF-A (**bottom left**), or VEGF-A antibody (**bottom right**) just before assay.

Figure 4. ADM modulates macrophage migration. **A:** Subretinal macrophages were counted 7 days after CNV induction. Macrophages were stained with F4/80. Increased accumulation with F4/80-positive cells was observed especially in $ADM^{+/-}$ mice in comparison with wild-type mice. $n = 6$. $*P < 0.05$. **Arrowheads** indicate F4/80-stained macrophages. **B:** *In vitro* macrophage migration assay. Number of macrophages decreased when RPE cells (D407) were cultured with ADM and used as conditioned medium in comparison without ADM. $n = 4$ for all. $*P < 0.05$. CCL2 was used as positive control. **C:** RT-PCR measurements indicated CCL2 was significantly inhibited after ADM treatment in a dose-dependent manner in comparison with vehicle-treated cells in two types of RPE cell lines: D407 and ARPE 19. $n = 4$ for all. $*P < 0.05$ and $**P < 0.01$. **D:** Enzyme-linked immunosorbent assay analysis also demonstrated the decreased expression of CCL2 protein from RPE cells. $n = 8$. $*P < 0.05$. **E** and **F:** Expression of CCL2 was increased in $ADM^{+/-}$ mice at both mRNA (**E**) and protein levels (**F**). $n = 8$ for all. $*P < 0.05$.

F4/80-positive macrophages that were concentrated within the laser burns and around the borders of the laser scars was counted 7 days after laser treatment. Increased infiltration with F4/80-positive cells was observed in $ADM^{+/-}$ mice in comparison with wild-type mice (Figure 4A).

Macrophage migration was investigated using *in vitro* macrophage migration assay in response to ADM or to secreted factors from D407. The results demonstrated that ADM does not directly inhibit macrophage migration. On the other hand, however, when D407 was cultured with ADM and used as conditioned medium, macrophage migration was inhibited significantly, suggesting that ADM mediates indirect inhibition of macrophage migration through modulating the expression of factor(s) from RPE (Figure 4B).

Previous studies have suggested a link between complement cascade and an important chemoattractant in CNV, ie, C-C motif chemokines, especially CCL2.^{60–62} These results led to an investigation in the present study as to whether CCL2 secreted from RPE is modulated by ADM. Real-time PCR measurements demonstrated that CCL2 mRNA was significantly inhibited after ADM treatment in a dose-dependent manner, in both D407 and ARPE19 cells (Figure 4C). Enzyme-linked immunosorbent assay analysis also demonstrated the decreased expression of CCL2 protein from RPE cells (Figure 4D). In addition, the expression of CCL2 mRNA in RPE/choroid from $ADM^{+/-}$ mice and wild-type mice was examined. Expression of CCL2 was increased in $ADM^{+/-}$ mice at both the mRNA and protein levels (Figures 4, E and F), corroborating the results from *in vitro* experiments. No difference in VEGF-A expression between $ADM^{+/-}$ mice and wild-type mice (data not shown) was detected. In addition, the CNV lesion was evaluated in $CCL2^{-/-}$ mice after intravitreal ADM administration (Figure 5) that demonstrated that inhibitory effects of ADM on CNV formation were abrogated in $CCL2^{-/-}$ mice, suggesting that ADM inhibits choroidal neovascularization mainly by modulating the expression of CCL2 from RPE/choroid.

Discussion

The current study demonstrated that expression of ADM was significantly increased after laser treatment in wild-

type mice. Human ADM is a 52-amino acid peptide originally isolated from human pheochromocytoma.^{63,64} ADM has been detected in plasma and other biological fluids from healthy humans and may behave as a circulating hormone.^{65–67} ADM belongs to the calcitonin gene peptide superfamily based on its slight homology with calcitonin gene-related peptide (CGRP) and amylin.^{68,69} ADM has important effects in vascular tone and permeability and promotes vasodilation.^{70,71} Previous studies clarified that ADM is upregulated in retinal neovascularization and hypoxic retina in mice^{72,73} and in *Ndph*-knockout mice, a knockout mouse model for Norrie disease.⁷⁴ Upregulation was also noted in RPE cells in a clinically relevant *in vitro* model of substrate advanced glycation-end product (AGE) accumulation and hypoxic condition.^{75,76} These studies, together with the current results, support a role for ADM on retinal and choroidal neovascularization and possibly on the pathogenesis of AMD. Thus, an important issue for future clinical research is to address whether ADM is upregulated in AMD patients.

The present data clearly demonstrate that reduced expression of ADM leads to the aggravation of CNV, whereas exogenous administration of ADM suppresses laser-induced CNV. Some studies demonstrated that ADM is also a potent angiogenic molecule. These studies described the angiogenic effects of ADM to direct stimulation of endothelial cell proliferation and protection of endothelial cells from apoptosis.^{77–80} The current study, however, failed to demonstrate any direct angiogenic function of ADM. Thus, this angiogenic effect of ADM needs to be clarified in further studies. Recent investigations have determined that ADM signaling is necessary for murine lymphatic vascular development. Mice in

Figure 5. Effect of CCL2 in ADM-mediated CNV inhibition. Inhibitory effects of ADM on CNV formation were abrogated in $CCL2^{-/-}$ mice. $n = 8$. $P = 0.304$.

which ADM has been genetically deleted develop cutaneous edema midgestational lethality due to a defect in lymphatic vessel growth and a cardiovascular defect.^{81,82} Thus, ADM also affects lymphatic vasculature, and the conflicting results of the current study and the previous studies may be reconciled by the fact that choroidal vasculatures lack lymph vessels and most previous studies used models in which both lympho- and angiogenesis was ongoing.^{82–85} Further studies are needed to clarify to the molecular mechanism underlying these conflicting results.

The present study results suggest that the main mechanism of CNV inhibition with administration of ADM is mediated through CCL2. A previous study demonstrated that ADM enhanced CFH-mediated cleavage of C3b via factor I.^{86,87} Several lines of evidence support that the inactive derivative iC3b binds to CR3 and modulates the production of CCL2. It is well known that C3 has been found in drusen and choroidal tissue, and some reports have shown that C3 was also expressed in RPE cells.⁸⁸ These data lead us to analyze whether ADM modulates iC3b expression. In this study, iC3b was not detected in the RPE cell line (D407) (data not shown),⁶⁰ suggesting that it is unlikely that ADM regulates the expression of CCL2 through acting on the complement pathway.

Accumulating evidence suggests that there is a relationship between macrophage accumulation and neovascular AMD; however, previous studies have presented conflicting results regarding the role of macrophage on CNV. Although a previous study¹⁴ observed spontaneous CNV in *CCL2*^{−/−} and *CCR2*^{−/−} mice, AMD-like lesion is observed only in senescence. One study⁸⁹ showed reduced laser-induced CNV lesion size in *CCL2*^{−/−} mice, and another study⁹⁰ reported that the size of laser-induced CNV were reduced with concomitant reduction in the number of ocular-infiltrating macrophages in *CCR2*^{−/−} mice. In contrast, other studies have demonstrated that macrophage inhibits laser-induced CNV, and have suggested that one possible explanation for these conflicting findings is a dual role of macrophages.^{91,92} The current results support the pro-angiogenic function of macrophages in CNV; however, diverse subsets of macrophages may have different roles in the pathogenesis of CNV.

The current *in vitro* experiments demonstrated that ADM did not inhibit VEGF-A expression directly, and showed that there is no difference between VEGF-A expression in *ADM*^{+/−} mice and wild-type mice. However, it is generally accepted that macrophages themselves are a source of VEGF-A and act to stimulate angiogenesis.^{90,93,94} Thus, current data do not completely rule out the possibility that intravitreal administration of ADM may result in the reduction of locally produced VEGF-A by macrophage.

In conclusion, the present study demonstrates that blocking CCL2 with the administration of ADM inhibits macrophage migration and leads to the suppression of laser-induced CNV in an animal model. Although anti-VEGF therapy is the main choice of treatment for neovascular AMD, there is concern about some of the side effects, and some cases are refractory to the current anti-VEGF therapy.^{37,38,42–44,49} Our study provides evi-

dence that demonstrates the potential of specific targeting of ADM for future therapies of CNV due to AMD. Studies are needed to further characterize the role of ADM in reducing CNV. In addition, the safety of intraocular administration of ADM remains to be determined. However, ADM hopefully will become an alternative treatment target for CNV treatment.

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