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Pathophysiological Function of Endogenous Calcitonin Gene–Related Peptide in Ocular Vascular Diseases

Yuichi Toriyama,*† Yasuhiro Iesato,*† Akira Imai,*† Takayuki Sakurai,* Akiko Kamiyoshi,* Yuka Ichikawa-Shindo,* Hisaka Kawate,* Akihiro Yamauchi,* Kyoko Igarashi,* Megumu Tanaka,* Tian Liu,* Xian Xian,* Liuyu Zhai,* Shinji Owa,* Toshinori Murata,† and Takayuki Shindo*

From the Department of Cardiovascular Research, Shinshu University Graduate School of Medicine, Nagano; and the Department of Ophthalmology,† Shinshu University School of Medicine, Nagano, Japan*

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Address correspondence to Takayuki Shindo, M.D., Ph.D., Department of Cardiovascular Disease, Shinshu University Graduate School of Medicine, Asahi 3-1-1, Matsumoto, Nagano 390-8621, Japan. E-mail: tshindo@shinshu-u.ac.jp.

Calcitonin gene–related peptide (CGRP; official name CALCA) has a variety of functions and exhibits both angiogenic and anti-inflammatory properties. We previously reported the angiogenic effects of the CGRP family peptide adrenomedullin in oxygen-induced retinopathy; however, the effects of CGRP on ocular angiogenesis remain unknown. Herein, we used CGRP knockout ($CGRP^{-/-}$) mice to investigate the roles of CGRP in ocular vascular disease. Observation of pathological retinal angiogenesis in the oxygen-induced retinopathy model revealed no difference between $CGRP^{-/-}$ and wild-type mice. However, much higher levels of the CGRP receptor were present in the choroid than the retina. Laser-induced choroidal neovascularization (CNV), a model of exudative age-related macular degeneration, revealed more severe CNV lesions in $CGRP^{-/-}$ than wild-type mice, and fluorescein angiography showed greater leakage from CNV in $CGRP^{-/-}$. In addition, macrophage infiltration and tumor necrosis factor (TNF)-α production were enhanced within the CNV lesions in $CGRP^{-/-}$ mice, and the TNF-α, in turn, suppressed the barrier formation of retinal pigment epithelial cells. *In vivo*, CGRP administration suppressed CNV formation, and CGRP also dose dependently suppressed TNF-α production by isolated macrophages. From these data, we conclude that CGRP suppresses the development of leaky CNV through negative regulation of inflammation. CGRP may thus be a promising therapeutic agent for the treatment of ocular vascular diseases associated with inflammation. *(Am J Pathol 2015, 185: 1783–1794; http://dx.doi.org/10.1016/j.ajpath.2015.02.017)*

In acquired blindness, retinal or choroidal neovascularization (CNV) is the main causative factor. Retinal neovascularization is induced by retinal hypoxia and is observed in diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. CNV occurs as a result of abnormalities in Bruch membrane and the retinal pigment epithelium (RPE)[1] and is observed in exudative age-related macular degeneration (AMD), angioid streaks, and high myopia.

Calcitonin gene–related peptide (CGRP; official symbol CALCA) is a 37–amino acid peptide produced by alternative splicing of the primary transcript of the calcitonin gene (*CALCA*). CGRP was originally identified as a sensory neurotransmitter,[2] and is now known to be structurally similar to the angiogenic factor adrenomedullin (AM).[3] Moreover, CGRP and AM share the same receptor, calcitonin receptor-like

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receptor (CLR), a seven transmembrane G protein–coupled receptor.[4] The affinity of CLR for CGRP or AM is determined by three accessory proteins called receptor activity–modifying proteins (RAMPs 1 to 3).[4] When associated with RAMP1, CLR has high affinity for CGRP; association with RAMP2 or RAMP3 gives CLR a high affinity for AM. Through analysis of knockout mice, we were able to demonstrate the novel angiogenic functions of AM and RAMP2.[5–8] Recently, CGRP was also reported to possess angiogenic functionality.[9–12] However, the precise roles of CGRP in ocular vascular development and disease remain totally unknown.

To investigate pathological angiogenesis in the eye, two major animal models are frequently applied: the oxygen-induced retinopathy (OIR) model, which is designed to investigate retinal neovascularization under hypoxic exposure, and the laser-induced CNV model, which is recognized as a model of exudative AMD. Both models are characterized by uncontrolled production of fragile and leaky capillaries in the retina. In OIR, the transition from hyperoxia to normoxia causes relative ischemia within the retina, which may be the precursor of pathological angiogenesis. On the other hand, both angiogenic and inflammatory processes play important roles in the pathophysiology of CNV. Interestingly, evidence now suggests that CGRP may be involved in the regulation of inflammatory responses[13,14] and, thus, could be involved in the ocular neovascularization associated with inflammation.

In the present study, therefore, we investigated the pathophysiological activities of endogenous CGRP in retinal and choroidal neovascularization. For this purpose, we induced OIR or CNV in CGRP knockout ($CGRP^{-/-}$) mice.

Materials and Methods

Experimental Animals

$CGRP^{-/-}$ mice were generated in our group using a targeting DNA construct that replaced exon 5 encoding a CGRP-specific region.[15] The $CGRP^{-/-}$ strain was on a pure C57BL/6J background and had undergone backcross breeding to C57BL/6J using the speed congenic method.

All animal handling procedures were in accordance with a protocol approved by the ethics committee of Shinshu University School of Medicine (Nagano, Japan). All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research and our institutional guidelines.

Physiological Angiogenesis in Neonates

On postnatal day 7 (P7) and P10, mice were evaluated using flat-mounted specimens of retina stained with isolectin B4 (isolectin GS-IB4 from Griffonia simplicifolia, Alexa Fluor 594 conjugate I21413; 1:200 dilution; Invitrogen, Carlsbad, CA). Superficial vascular development in the retina was quantified on P7, and deep vascular development was evaluated on P10. Vascular progression was measured by defining a straight line from the angiogenic front to the center of the retina for each retinal quadrant under low magnification. The number of vessel branches and the vessel density in the area near the developing vascular front on P7 were quantified in 400 × 400-μm^2 fields.

OIR Model

Ischemic retinopathy and pathological neovascularization were induced using the OIR model established by Smith et al.[16] Beginning on P7, mice were exposed to 75% oxygen for 5 days (P12) and then allowed to recover in room air to induce retinal neovascularization. Retinal angiogenesis was quantified, as described previously, with slight modification.[17] Briefly, on P17, the eyes were fixed in 4% paraformaldehyde for 1 hour at 4°C and then washed with phosphate-buffered saline (PBS). Retinas were then isolated and stained overnight at 4°C with isolectin B4 in PBS with 0.3% Triton X-100. After washing three times in PBS, the retinas were whole mounted onto microscope slides with the photoreceptor side down and then embedded in fluorescent mounting medium (Dako, Glostrup, Denmark). Images of whole-mounted retinas were taken at ×40 magnification using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan). Avascular zones and neovascular tuft formation (regarded as pathological angiogenesis) were quantified using digital imaging/photoediting software (Adobe Photoshop CS5; Adobe Systems, San Jose, CA).

Laser-Induced CNV Model

Male mice between 9 and 12 weeks of age were used. After mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol (240 mg/kg; Wako, Osaka, Japan), the pupil was dilated with 1 drop of 0.5% tropicamide and 0.5% phenylephrine (Mydrine P; Santen, Osaka, Japan). CNV was then induced, as described previously, with some modification.[18] Laser injury was induced using a slit-lamp delivery system (model GYC-1000; NIDEK, Gamagori, Japan) with a coverslip serving as a contact lens. The wavelength was 532 nm, the power was 200 mW, the duration was 50 milliseconds, and the spot size was 50 μm. In each eye, four laser spots were placed around the optic disc. Only eyes that exhibited subretinal bubbles, which indicated rupture of the Bruch membrane, were used for studies.

At 1, 3, or 7 days after laser injury, RPE-choroid-sclera complexes (choroidal complexes) were isolated for real-time RT-PCR.

Fluorescein Isothiocyanate–Dextran Perfusion and Choroidal Flat Mount

The sizes of the CNV lesions were measured in RPE-choroid-sclera flat mounts (choroidal flat mounts), as previously described.[19] On day 14 after laser application, mice were anesthetized and perfused with 1 mL of PBS containing 50 mg/mL fluorescein-labeled dextran (molecular weight,