Macular Pigment Lutein Is Antiinflammatory in Preventing Choroidal Neovascularization

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Background—Choroidal neovascularization (CNV) is a critical pathogenesis in age-related macular degeneration, the most common cause of blindness in the developed countries. The aim of the current study was to investigate the effect of lutein supplementation on the development of the murine model of laser-induced CNV together with underlying molecular mechanisms.

Methods and Results—Mice were orally pretreated with lutein daily from 3 days before laser photocoagulation till the end of the study. The index of CNV volume was significantly suppressed by the treatment with lutein, compared with vehicle-treated animals. Lutein treatment led to significant inhibition of macrophage infiltration into CNV and of the in vivo and in vitro expression of inflammation-related molecules including vascular endothelial growth factor, monocyte chemotactic protein−1, and intercellular adhesion molecule-1. Importantly, lutein suppressed IkB-α degradation and nuclear translocation of NF-κB p65 both in vivo and in vitro. Additionally, the development of CNV was significantly suppressed by inhibiting NF-κB p65 nuclear translocation, to the levels seen in the lutein treatment.

Conclusions—Lutein treatment led to significant suppression of CNV development together with inflammatory processes including nuclear factor-κB activation and subsequent upregulation of inflammatory molecules, providing molecular evidence of potential validity of lutein supplementation as a therapeutic strategy to suppress CNV.

Key Words: choroidal neovascularization ■ lutein ■ inflammation ■ nuclear factor-κB ■ age-related macular degeneration

Age-related macular degeneration (AMD) is the most common cause of blindness in the developed countries. The macula is located at the center of the retina, and the visual acuity depends on the function of the macula. AMD is complicated by choroidal neovascularization (CNV), leading to severe vision loss and blindness. During CNV generation, new vessels from the choroid invade the subretinal space through Bruch’s membrane, resulting in the formation of the neovascular tissue including vascular endothelial cells, retinal pigment epithelial (RPE) cells, fibroblasts, and macrophages. Subretinal hemorrhage and lipid exudation develop from the immature vessels, causing injury to the photoreceptors.

Molecular and cellular mechanisms underlying CNV are not fully elucidated. CNV seen in AMD develops with oxidative stress and chronic inflammation adjacent to RPE, Bruch’s membrane, and choriocapillaris. Recent experimental and clinical studies have indicated vascular endothelial growth factor (VEGF) as a critical factor for promoting CNV. CNV formation is associated with the influx of inflammatory cells including macrophages, which are the rich source of VEGF. Pharmacological depletion of macrophages resulted in significant suppression of murine CNV. Genetic ablation of intercellular adhesion molecule (ICAM)-1 or C-C chemokine receptor (CCR)-2, a receptor for monocyte chemotactic protein (MCP)-1/CCL-2, inhibited CNV in the murine model. We have also highlighted the inflammatory mechanisms mediated by the renin–angiotensin system and interleukin (IL)-6 receptor signaling, demonstrating the regulation of inflammation as an important therapeutic strategy to suppress CNV.

Lutein is one of the most prevalent carotenoids, a group of fat-soluble yellow pigments, and is abundantly present in fruits and leafy green vegetables. Humans are not capable of synthesizing carotenoids de novo, and thus their presence in human tissues is entirely attributable to dietary intake. Chemically, lutein and its structural isomer zeaxanthin differ from other carotenoids in having 2 hydroxyl groups, 1 on each side of the molecule. Lutein and zeaxanthin are highly concen-
trated in the macula and are likely to function as a filter protecting the macula from blue light. In addition to its role as the yellow filter, lutein is a potent antioxidant. As various observational and interventional studies showed the possibility of lutein consumption for reducing the risk of AMD, the protective effect of lutein supplementation has recently been attracting attention. No data have been reported, however, that show the direct molecular evidence of the anti-pathogenic role of lutein in CNV generation. Here we report the first evidence of the in vivo effect of lutein on CNV, together with underlying molecular and cellular mechanisms.

Materials and Methods

Animals
Male C57BL/6J mice (CLEA, Tokyo, Japan) at the age of 6 to 8 weeks were used. All animal experiments were conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. Totally, ~200 mice were used throughout the study.

Induction of CNV
Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of inflammation-related CNV seen in AMD. In this model, new vessels from the choroid invade the subretinal space after photocoagulation. Laser photocoagulation was performed at 5 spots per eye around the optic disc using a slit-lamp delivery system (NOVUS spectra; Lumenis), as described previously. Treatment with Lutein
Animals were pretreated with lutein (Kemin Industries; 99.9% purified) or phosphate-buffered saline (PBS) containing 0.1% di-methyl sulfoxide (DMSO) as vehicle daily for 3 days before photocoagulation and the treatment was continued daily till the end of the study. Lutein was orally administered to mice with the dose of 1, 10, or 100 mg/kg body weight (BW).

High Performance Liquid Chromatography for Lutein
The RPE-choroid complex was collected from mice treated with lutein for 6 days and homogenized in 150 μL of PBS. Concentration of lutein and its isomer zeaxanthin in the RPE-choroid was measured by high performance liquid chromatography, as described previously. Individual samples were corrected for total protein concentration.

Inhibition of Nuclear Factor-κB
Animals were pretreated with an nuclear factor (NF)-κB inhibitor (DHMEQ; dehydroxymethylpoxyquinomicin) or vehicle (0.25% DMSO in Dulbecco’s modified Eagle’s Medium [DMEM; Sigma]) 1 day before photocoagulation, and the treatment was continued daily till the end of the study. Mice received intraperitoneal injections of DHMEQ with the dose of 0.5, 1, or 5 mg/kg BW. DHMEQ is a novel NF-κB inhibitor, based on the structure of epoxyquinomicin C, which was originally isolated from Amycolatopsis. DHMEQ has been shown to inhibit nuclear translocation of NF-κB without affecting phosphorylation and degradation of IκB-α. Optically active (+)-DHMEQ was used throughout the experiments.

Quantification of Laser-Induced CNV
One week after laser injury, eye cups were incubated with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Vector). CNV was visualized using a scanning laser confocal microscope (FV1000; Olympus). The area of CNV-related fluorescence was measured by NIH (National Institutes of Health) image. The summation of whole fluorescent area was used as the index of CNV volume, as described previously.

ELISA for ICAM-1, MCP-1, and VEGF
Protein extracts were obtained from the homogenized RPE-choroid complex 3 days after photocoagulation. The levels of ICAM-1, MCP-1, and VEGF were determined with the mouse ICAM-1, MCP-1, and VEGF ELISA kits (R&D Systems), as described previously. The VEGF ELISA kit recognizes all the isoforms of VEGF.

Western Blot Analyses for IκB-α
Protein extracts were obtained from the homogenized RPE-choroid complex at 2, 4, 6, and 8 hours after photocoagulation. Western blot analyses for IκB-α was performed with the same procedures using a rabbit or mouse monoclonal antibody against IκB-α (Cell Signaling Technology), as described previously.

Immunohistochemistry for Infiltrating Macrophages
Whole-mount choroid-sclera complex obtained 3 days after photocoagulation were incubated with a goat polyclonal antibody against mouse PECAM-1 (CD31; Santa Cruz Biotechnology) and a rat polyclonal antibody against F4/80 (ABD Serotec). Avidin-Alexa 488- and Avidin-Alexa 546-tagged secondary antibodies (Invitrogen) were then applied, as described previously.

Quantification of Infiltrating Macrophages
Total RNA was isolated from the RPE-choroid complex 3 days after photocoagulation and reverse-transcribed. Quantitative polymerase chain reaction (PCR) analyses for F4/80 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were performed in an ABI 7500 Fast real-time PCR system (Applied Biosystems) in combination with TaqMan chemistry, as described previously.

In Vitro Assays
We examined the in vitro effect of lutein on inflammatory responses from 3 major cell types associated with CNV formation, ie, microvascular endothelial cells, macrophages, and RPE cells, using the murine cell lines b-End3 and RAW264.7 and the human cell line ARPE-19, respectively. Cells were pretreated with lutein (10, 50, or 100 μg/mL) or vehicle in serum-starved DMEM for b-End3 and RAW264.7 and the human cell line ARPE-19, respectively. Cells were treated with TNF (10 ng/mL) with or without lutein (100 μg/mL) or vehicle. After 30-minute incubation, the cell lysates from b-End3 and ARPE-19 cells were processed for Western blot analyses for IκB-α. After 6-hour incubation, the cell lysate from b-End3 cells and the supernatants from b-End3 and RAW264.7 cells were processed for ELISA for ICAM-1, MCP-1, and VEGF, respectively. After 24-hour incubation, the supernatant from ARPE-19 cells was processed for ELISA for VEGF and MCP-1.

Immunohistochemistry for NF-κB p65 in Murine CNV
Paraffin sections of murine eyes 6 hours after photocoagulation were incubated with a rabbit polyclonal antibody against human NF-κB p65 (Santa Cruz Biotechnology) and an Avidin-Alexa 488-tagged secondary antibody. The samples were then treated with TOTO-3 (Invitrogen) for nuclear staining.

Immunocytochemistry for NF-κB p65 in b-End3 and ARPE-19 Cells
After 24-hour pretreatment with lutein (100 μg/mL), DHMEQ (10 μg/mL), or vehicle in serum-starved DMEM for b-End3 cells and DMEM/F12 for ARPE-19 cells, cells were incubated with TNF-α (10 ng/mL) with or without lutein (100 μg/mL) or DHMEQ (10 μg/mL).
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Results

Suppression of CNV in Mice Receiving Lutein
Development of CNV was significantly suppressed by the treatment with lutein in a dose-dependent fashion. Lutein-treated mice at the dose of 10 or 100 mg/kg BW showed a significant (P<0.001) decrease in the index of CNV volume (401 ± 159 252 μm³ for 10 mg/kg BW, 348 ± 162 775 μm³ for 100 mg/kg BW) compared with vehicle-treated mice (553 ± 200,135 μm³; Figure 1). To confirm whether orally applied lutein reaches the eye, we measured the tissue concentration of lutein with high performance liquid chromatography. Lutein concentration in the RPE-choroid was significantly higher in mice treated with lutein at the dose of 10 or 100 mg/kg BW than in the vehicle-treated mice (Figure 1A). In the quantitative analyses using the real-time PCR, lutein-treated mice at the dose of 10 or 100 mg/kg BW showed a significant decrease in the expression of F4/80 in the RPE-choroid complex, compared with vehicle-treated animals (P<0.001, Figure 2B).

In Vivo Inhibition of Inflammatory and Angiogenic Molecules by the Treatment with Lutein
To determine whether lutein treatment affects inflammatory and angiogenic molecules related to the pathogenesis of CNV, proteins of ICAM-1, MCP-1, and VEGF in the RPE-choroid complex were ELISA. Protein levels of ICAM-1, MCP-1, and VEGF in the RPE-choroid complex were higher in mice with CNV than in age-matched normal controls (Figure 2C-2E). Lutein treatment significantly suppressed protein levels of ICAM-1, MCP-1, and VEGF (P<0.05 for all).

In Vitro Inhibition of Inflammatory and Angiogenic Molecules by the Treatment With Lutein
To confirm in vivo effects of lutein on choroidal inflammation and neovascularization, we further performed in vitro analyses. In b-End3 cells, lutein treatment significantly (P<0.05) reduced protein levels of ICAM-1, MCP-1, and VEGF in the RPE-choroid complex, both of which were induced by TNF-α. In ARPE-19 cells, lutein application led to a significant (P<0.001) decrease in the protein levels of TNF-α-induced VEGF (Figure 3C) and MCP-1 (Figure 3D). Similarly in RAW264.7 macrophages, lutein treatment significantly (P<0.05) reduced the protein level of LPS-induced VEGF (Figure 3E).

In Vivo Inhibition of NF-κB Activation by the Treatment With Lutein and Suppression of CNV by NF-κB Inhibition
To define the signaling pathway involved in the treatment with lutein, we focused on NF-κB as an upstream transcriptional factor of inflammatory mediators and analyzed nuclear translocation of NF-κB p65 and the protein level of IκB-α in vivo. In the murine RPE-choroid tissues, photocoagulation induced NF-κB activation including nuclear translocation of NF-κB p65 (Figure 4A) and IκB-α degradation (Figure 4B and 4C). Nuclear translocation of NF-κB p65 was detected in choroidal vascular endothelial cells and RPE cells (Figure 4A). Protein levels of IκB-α in the RPE-choroid complex were significantly (P<0.05) reduced 2 to 6 hours after photocoagulation compared with age-matched normal controls, and were returned to the normal level after 8 hours (data not shown). Lutein significantly (P<0.05) inhibited IκB-α degradation at 4 hours in the murine RPE-choroid complex (Figure 4B and 4C) in a dose-dependent fashion. In contrast, lutein application to normal mice for 4 days did not affect IκB-α levels in the RPE-choroid (data not shown). Importantly, mice treated with DHMEQ, a potent inhibitor of NF-κB nuclear translocation, at the dose of 0.5, 1, or 5 mg/kg showed a significant and dose-dependent decrease in the index of CNV volume (402 ± 159 032 μm³ for 0.5 mg/kg BW, 346 ± 153 867 μm³ for 1 mg/kg BW, 201 ± 67 614 μm³ for 5 mg/kg BW) compared with vehicle-treated mice (627 ± 146 665 μm³; Figure 4D and 4E).

Statistical Analyses
All results were expressed as mean ± SD. The values were processed for statistical analyses (Mann–Whitney test). Differences were considered statistically significant at P<0.05.
In Vitro Inhibition of NF-κB Activation by the Treatment With Lutein

To further confirm in vivo effects of lutein on NF-κB inhibition, we performed in vitro experiments using b-End3 vascular endothelial cells and ARPE-19 cells stimulated by TNF-α. Lutein significantly (P<0.01) inhibited IκB-α degradation enhanced by TNF-α in b-End3 cells (Figure 5A and 5B). Nuclear translocation of NF-κB p65, enhanced by TNF-α, was significantly (P<0.001) suppressed by application of lutein to the level seen in treatment with DHMEQ in b-End3 cells (Figure 5C and 5D). In RPE cells as well, lutein significantly inhibited both IκB-α degradation (P<0.01, Figure 5E and 5F) and nuclear translocation of NF-κB p65 (P<0.001, Figure 5G and 5H).

Discussion

The present study reveals several important findings concerning the antipathogenic role of lutein in the development of CNV. First, treatment with lutein led to significant suppression of CNV (Figure 1). Second, the molecular and cellular mechanisms in the lutein treatment included the inhibitory effects on macrophage infiltration into CNV (Figure 2) and...
on inflammation-related molecules in the RPE-choroid complex (Figure 2) and in cultured microvascular endothelial cells, RPE cells, and macrophages (Figure 3). Third, CNV generation was accompanied by NF-κB activation in the RPE-choroid, and lutein treatment resulted in inhibition of NF-κB activation in vivo (Figure 4) and in vitro (Figure 5). Importantly, pharmacological blockade of NF-κB suppressed CNV (Figure 4).

Lutein is reported to function in two ways to protect the ocular tissue: first as a filter of high-energy blue light, and second as an antioxidant that scavenges light-induced reactive oxygen species (ROS). Depending on these effects, lutein is hypothesized to slow the incidence and development of AMD. We have demonstrated for the first time that lutein supplementation led to suppression of CNV in vivo (Figure 1). This effect is not likely to result from its role as the yellow
filter because mice lack the lutein-rich macula. Accordingly, it is reasonable to use mice in the present study to clarify the inhibitory effect of lutein as the ROS scavenger on NF-κB-mediated inflammation in CNV.

As molecular and cellular mechanisms for suppressing CNV by the treatment with lutein, the present data showed that lutein application led to significant suppression of macrophage infiltration (Figure 2) and of CNV-related molecules including ICAM-1, MCP-1 and VEGF in vivo (Figure 2) and in vitro (Figure 3). Previous reports concerning the molecular mechanisms underlying CNV generation showed VEGF as a critical angiogenic factor. Macrophages, the rich source of VEGF, facilitate the development of CNV.27 More recently, several in vivo experiments with genetically altered mice demonstrated significant contribution of ICAM-1 and MCP-1 both of which are required for macrophage infiltration. Collectively, the currently observed suppression of CNV by the treatment with lutein is likely attributable to the inhibition of multiple inflammatory steps including MCP-1–induced migration and ICAM-1–dependent adhesion of macrophages and subsequent macrophage-derived VEGF secretion.

Because NF-κB is known to induce the expression of inflammation-related molecules, we investigated the role of NF-κB in the development of CNV. After IκB phosphorylation and degradation attributable to various stimuli, NF-κB p65/p50, capable of entering the nucleus and binding the κB sequence, promotes the transcription of target genes including VEGF, ICAM-1, and MCP-1.29 Our current data demonstrated the critical role of NF-κB activation in the RPE-choroid after CNV induction. Lutein inhibited activation of NF-κB by suppressing degradation of IκB in the RPE-choroid of the murine model (Figure 4) and in vascular endothelial cells and RPE cells (Figure 5). In macrophages as well, lutein has shown to inhibit IκB degradation and subsequent NF-κB activation.30

Importantly, the inhibition of NF-κB activation with DHMEQ (Figure 4) exhibited equivalent effects on CNV development compared with lutein application (Figure 1). Recent studies have established the inhibitory effects of DHMEQ on tumor growth and angiogenesis and diabetic retinal inflammation.31 The present data on the critical role of NF-κB in CNV (Figure 4) are supported by a previous study showing the NF-κB inhibition with the gene transfer of a double-stranded phosphorothioate oligonucleotides against the NF-κB binding site led to significant suppression of vascular leakage in experimental CNV.32 Collectively, the regulation of NF-κB is a novel therapeutic strategy for inflammatory neovascularization such as CNV, showing the importance of lutein capable of inhibiting NF-κB activation.

Because the current therapeutic intervention for blocking VEGF tends to be limited to the advanced stage, an alterna-
tive early treatment is desirable targeting inflammation as an antecedent event leading to neovascularization. Epidemiologic risk factors for AMD include age, smoking, cardiovascular diseases such as atherosclerosis, and nutrient status. It is reasonable to intervene modifiable risk factors such as nutrient status for prevention of AMD. Interestingly, lutein has proved to be protective against early atherosclerosis. Additionally, human sample studies have revealed that AMD shares several inflammatory mechanisms with atherosclerosis. Extracellular deposits associated with AMD, known as drusen, contain multiple components common to atherosclerotic lesions including apolipoprotein E, complement factors, immunoglobulins, and lipids. Macrophages expressing scavenger receptors accumulate to human CNV lesions with oxidized lipoproteins, as is demonstrated in atherosclerosis. Reasonably, lutein application, which may inhibit not only inflammatory neovascularization in the eye but also improve the systemic background predisposing to AMD, is likely to be a novel therapeutic strategy as a preventive additive treatment for AMD. To determine whether oral supplementation with lutein and zeaxanthin decreases the risk of progression to advanced AMD, a randomized controlled clinical trial is currently in progress.

Sources of Funding
This work was supported by grant-in-aid for Scientific Research of Japanese Ministry of Education, Culture, Sports, Science and Technology (No.19592039 to S.I.).
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Arterioscler Thromb Vasc Biol. published online October 11, 2007;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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