

# Eicosapentaenoic Acid Is Anti-Inflammatory in Preventing Choroidal Neovascularization in Mice

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**PURPOSE.** To investigate the role of eicosapentaenoic acid (EPA), the major  $\omega$ -3 polyunsaturated fatty acid (PUFA), in the development of choroidal neovascularization (CNV), together with underlying molecular mechanisms.

**METHODS.** Six-week-old C57BL/6 mice were fed with laboratory chow with 5% EPA or the  $\omega$ -6 PUFA linoleic acid (LA) for 4 weeks. Laser photocoagulation was performed to induce CNV, and the volume of CNV tissue was evaluated by volumetric measurements. The expression and production of intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF) and interleukin (IL)-6 in the retinal pigment epithelium (RPE)-choroid in vivo, and stimulated b-End3 endothelial cells and RAW264.7 macrophages in vitro were evaluated by RT-PCR and ELISA. Fatty acid composition in the serum and the RPE-choroid was analyzed by gas chromatography and high-performance liquid chromatography, respectively. Serum levels of C-reactive protein (CRP), IL-6, VEGF, MCP-1, and soluble ICAM-1 were examined by ELISA.

**RESULTS.** The CNV volume in EPA-fed animals was significantly suppressed compared with that in control mice, whereas the LA-rich diet did not affect CNV. The mRNA expression and protein levels of ICAM-1, MCP-1, VEGF, and IL-6 after CNV induction were significantly reduced in EPA-supplemented mice. In vitro, EPA application led to significant inhibition of mRNA and protein levels of ICAM-1 and MCP-1 in endothelial cells and VEGF and IL-6 in macrophages. EPA-fed mice exhibited significantly higher levels of EPA and lower levels of the  $\omega$ -6 PUFA arachidonic acid in the serum and the RPE-choroid than control animals. EPA supplementation also led to significant reduction of serum levels of IL-6 and CRP after CNV induction.

**CONCLUSIONS.** The present study demonstrates for the first time that an EPA-rich diet results in significant suppression of CNV

and CNV-related inflammatory molecules in vivo and in vitro. These results suggest that frequent consumption of  $\omega$ -3 PUFAs may prevent CNV and lower the risk of blindness due to age-related macular degeneration. (*Invest Ophthalmol Vis Sci*. 2007;48:4328–4334) DOI:10.1167/iov.06-1148

Age-related macular degeneration (AMD) is the most common cause of adult blindness in developed countries.<sup>1</sup> It is complicated by choroidal neovascularization (CNV) and subsequent bleeding and exudation beneath the macula, leading to severe vision loss and blindness. CNV associated with AMD, with relation to both genetic and environmental factors, develops after chronic inflammation adjacent to the retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris. In previous studies of human surgical samples<sup>2</sup> and animal models,<sup>3,4</sup> vascular endothelial growth factor (VEGF) was shown to be a key molecule in the development of CNV. Recent clinical trials, in accordance with the experimental results, revealed that the intravitreal administration of VEGF antagonists ameliorates the visual outcome compared with sham injections.<sup>5,6</sup> In addition to its angiogenic property, VEGF has recently been recognized as a proinflammatory cytokine in the eye.<sup>7,8</sup> Inflammatory processes, including macrophage infiltration<sup>9–14</sup> and cytokine network,<sup>15</sup> play crucial roles in CNV, as well as in the pathologic neovascularization seen in solid tumors and rheumatoid arthritis.

Polyunsaturated fatty acids (PUFAs) are classified into two groups,  $\omega$ -3 and  $\omega$ -6, based on their chemical structures. The nomenclature of  $\omega$ -3 (or n-3) refers to the first double bond as being three carbons from the methyl end of the PUFA molecule. It is well known that supplemental fish oil, a rich source of  $\omega$ -3 PUFAs, reduces elevated triglyceride levels.<sup>16</sup> The major  $\omega$ -3 PUFA eicosapentaenoic acid (EPA) is clinically used as an anti-hyperlipidemic agent. A recent interventional study on the effect of EPA treatment of hypercholesterolemia has revealed a significant decline of major coronary events in Japanese patients.<sup>17</sup> Reportedly, risk factors for AMD include hyperlipidemia and atherosclerosis.<sup>18–20</sup> Inflammation mediates the pathogenesis of atherosclerosis, which develops macrophage infiltration and lipid uptake, resulting in the formation of atheromatous plaque with foam cells (i.e., lipid-laden macrophages). In vitro data in several studies have demonstrated that EPA application inhibits endothelial and macrophage expression of inflammation-related adhesion molecules.<sup>21–24</sup> Recent reports have revealed that the anti-inflammatory property of EPA is attributable in part to peroxisome proliferator-activated receptor (PPAR)- $\gamma$ -mediated suppression of nuclear factor (NF)- $\kappa$ B activation,<sup>25,26</sup> in addition to the less potent effect of EPA-derived eicosanoids than the arachidonic acid (AA) cascade metabolites. Reasonably, several clinical studies have shown the efficacy of fish oil in various inflammatory diseases, including rheumatoid arthritis<sup>27</sup> and ulcerative colitis.<sup>28</sup>

Excessive dietary intake of saturated fatty acids and elevated cholesterol levels were shown to increase the risk of AMD development.<sup>29</sup> In contrast, supplemental  $\omega$ -3 PUFAs or frequent consumption of fatty fish decreased the risk of

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AMD,<sup>30-33</sup> suggesting the protective role of  $\omega$ -3 PUFAs in the aging eye.<sup>34</sup> No data have been reported, however, that show the direct evidence of the in vivo effect of  $\omega$ -3 PUFA intake on CNV generation. In this article, we report the first evidence of dietary EPA as a negative regulator of CNV together with underlying molecular mechanisms related to inflammation.

## METHODS

### Animals and Laboratory Chow

Six-week-old C57BL/6 mice (CLEA, Tokyo, Japan) were used. Mice were fed with laboratory powder chow that did not contain fish products (F1; Funabashi Farm, Chiba, Japan) or chow supplemented with 5% EPA ethyl ester or 5% linoleic acid (LA; Sigma-Aldrich, St. Louis, MO). This ethyl-ester derivative of 5,8,11,14,17-*cis*-EPA (20:5, n-3) was the kind gift of Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). EPA and LA are the precursors of docosahexaenoic acid (DHA) and AA, the major  $\omega$ -3 and  $\omega$ -6 PUFAs in the eye, respectively. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ethics committee of our institution approved all surgical interventions and animal care procedures, which were in accordance with the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals of Keio University.

### Induction of CNV

Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of inflammation-related CNV in AMD. In this animal model, new vessels from the choroid invade the subretinal space through Bruch's membrane after photocoagulation. Animals were fed with laboratory chow with 5% EPA or 5% LA for 4 weeks before laser treatment. Laser photocoagulation was performed with the wavelength of 532 nm, the power of 200 mW, the duration of 100 ms and the spot size of 75  $\mu$ m by a single individual, as described previously.<sup>10</sup> Laser spots were applied in a standardized fashion around the optic nerve, using a slit lamp delivery system (Novus Spectra; Lumenis, Tokyo, Japan). The morphologic end point of the laser injury was the appearance of a cavitation bubble, a sign thought to correlate with the disruption of Bruch's membrane.

### Quantification of Laser-Induced CNV

One week after laser injury, the eyes were enucleated and fixed with 4% paraformaldehyde (PFA) for 30 minutes. Eyecups obtained by removing anterior segments were pretreated with buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA; Sigma], and 0.5% Triton X-100 [Sigma]) for 30 minutes at room temperature, and incubated overnight at 4°C with 0.5% fluorescein isothiocyanate (FITC)-isolectin B4 (Vector, Burlingame, CA). After two washes with PBS containing 0.1% Triton X-100, the neurosensory retina was detached and the choroid-sclera complex was flatmounted. Flatmounts were evaluated with a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan), and CNV was visualized with a blue argon laser (wavelength, 488 nm). Horizontal optical sections of CNV were obtained in 1- $\mu$ m intervals from the surface to the deepest focal plane in which the surrounding choroidal vascular network connecting to the lesion could be identified. The area of CNV-related fluorescence was measured by NIH Image (available by ftp at [zippy.nimh.nih.gov/](http://zippy.nimh.nih.gov/) or at <http://rsb.info.nih.gov/nih-image/>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The summation of the whole fluorescent area was used as the volume of CNV, as described previously.<sup>10</sup>

### RT-PCR Analyses

Total RNA was isolated from the RPE-choroid complex 3 days after photocoagulation by RNA extraction (TRIzol; Invitrogen, Carlsbad, CA)

and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. PCR was performed with *Taq* DNA polymerase (Toyobo, Tokyo, Japan) in a thermal controller (Gene Amp PCR system; Applied Biosystems, Foster City, CA). The primer sequences and the expected size of amplified cDNA fragments were as follows: 5'-TTC CTC TCT GCA AGA GAC T-3' (sense) and 5'-TGT ATC TCT CTG AAG GAC T-3' (antisense) (430 bp) for interleukin (IL)-6; 5'-GAA GTC CCA TGA AGT GAT CCA G-3' (sense) and 5'-TCA CCG CCT TGG CTT GTC A-3' (antisense) (319 bp) for VEGF120, 451 bp for VEGF164 for VEGF; and 5'-ATG TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' (sense) and 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3' (antisense) (837 bp) for  $\beta$ -actin. Commercial primer pairs (Mouse/rat ICAM-1 PCR Primer Pair, 500 bp; and mouse/rat JE/MCP-1/CCL2 PCR primer pair, 246 bp; R&D Systems, Minneapolis, MN) were used for intercellular adhesion molecule (ICAM)-1 and monocyte chemoattractant protein (MCP)-1, respectively.

### Enzyme-Linked Immunosorbent Assay

The RPE-choroid complex was carefully isolated from the eyes 1 week after photocoagulation and placed into 200  $\mu$ L of lysis buffer (0.02 M HEPES, 10% glycerol, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1% Triton, 100 mM NaF, and 4 mM EDTA [pH 8.0]) supplemented with protease inhibitors, and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the levels of ICAM-1, MCP-1, VEGF, and IL-6 were determined with the mouse ICAM-1, MCP-1, VEGF, and IL-6 ELISA kits (R&D Systems), according to the manufacturer's protocols. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

### In Vitro Assays

Murine brain-derived capillary endothelial cells (b-End3) and murine macrophages (RAW264.7) were cultured with DMEM (Dulbecco's modified Eagle's medium; Sigma-Aldrich) containing 10% fetal bovine serum (FBS) at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere. EPA as a sodium salt (Sigma-Aldrich) was dissolved in DMEM with 10% FBS to make a 100-mM stock solution. After it was held at 37°C for 30 minutes, the solution was aliquoted and frozen. Twelve hours before the experiments with b-End3 cells, the culture medium was changed to serum-free DMEM. After a 6-hour incubation with tumor necrosis factor (TNF)- $\alpha$  (1 ng/mL; Sigma-Aldrich) alone, TNF- $\alpha$  plus EPA (10, 50, or 100  $\mu$ M; Sigma-Aldrich), or TNF- $\alpha$  plus EPA and a selective PPAR- $\gamma$  antagonist, GW9662 (10  $\mu$ M; Alexis Biochemicals, San Diego, CA), total cellular RNA was isolated and processed for RT-PCR analyses for ICAM-1 and MCP-1. For protein analyses, supernatant, and cell lysates were collected after a 6- or 24-hour incubation and processed for ELISA for MCP-1 and ICAM-1, respectively. RAW264.7 cells were treated with serum-free DMEM containing lipopolysaccharide (100 ng/mL; LPS) alone, LPS plus EPA (10, 50, or 100  $\mu$ M), or LPS plus EPA and GW9662 (10  $\mu$ M). After a 6-hour incubation, total cellular RNA was processed for RT-PCR analyses for VEGF and IL-6. For protein analyses, supernatant was collected after 6- or 24-hour incubation and processed for ELISA for VEGF and IL-6. RT-PCR analyses and ELISA were performed with the same procedure as was used for the in vivo assays.

### In Vivo Blockade of PPAR- $\gamma$ Signaling

To evaluate whether the EPA diet inhibits CNV via the PPAR- $\gamma$  pathway, animals were treated with GW9662, as described previously.<sup>35</sup> GW9662 was administered to EPA-fed mice at a dose of 0.1 or 1 mg/kg body weight daily for 6 days before photocoagulation, and the treatment was continued daily until the end of the study. The volume of CNV was evaluated as described earlier.

### Gas Chromatography and ELISA for Serum Analyses

One day before and a week after photocoagulation, blood was obtained from the tail vein. Fatty acid composition in the serum lipids was

determined by gas chromatography at the SRL Clinical Laboratory (Tokyo, Japan). Briefly, total lipids in the serum were extracted according to the procedure of Folch et al.,<sup>36</sup> followed by hydrolysis, to free the fatty acids. The free fatty acids were esterified with potassium methoxide-methanol and boron trifluoride-methanol. The methylated fatty acids were analyzed by gas chromatography (GC-17A gas chromatograph; Shimadzu, Kyoto, Japan) with a capillary column (Omega-wax 250 column; Sigma-Aldrich, Tokyo, Japan). ELISA kits were used for the measurement of serum levels of C-reactive protein (CRP; Life Diagnostics, West Chester, PA) and ICAM-1, MCP-1, VEGF, and IL-6 (R&D Systems).

### HPLC for Fatty Acid Composition in the RPE-Choroid

Total lipids in the RPE-choroid complex were extracted according to the method of Folch et al.,<sup>36</sup> with minor modifications. The residue of total lipids was hydrolyzed with 0.5 M hydrochloric acid in acetonitrile and water for 60 minutes at 80°C. After extraction with chloroform and evaporation to dryness, the residue was derivatized with DMEQ (6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionyl-carboxylic acid)-hydrazide, as described before,<sup>37</sup> with minor modifications. The DMEQ derivatives were extracted from the reaction mixture with diethylether and evaporated to dryness. The residue was dissolved in ethanol-dimethylformamide-water and analyzed with an HPLC system equipped with a C8 column (Capcell Pak C8 DD; Shiseido, Tokyo, Japan) and a fluorescence detector (367 nm excitation; 445 nm emission). Peaks of the DMEQ derivatives were identified as corresponding fatty acids by their retention times.

### Statistical Analyses

All results are expressed as the mean  $\pm$  SD. The values were processed for statistical analyses by the Mann-Whitney test. Differences were considered statistically significant at  $P < 0.05$ .

## RESULTS

### Suppression of CNV by EPA, but Not LA, Supplementation

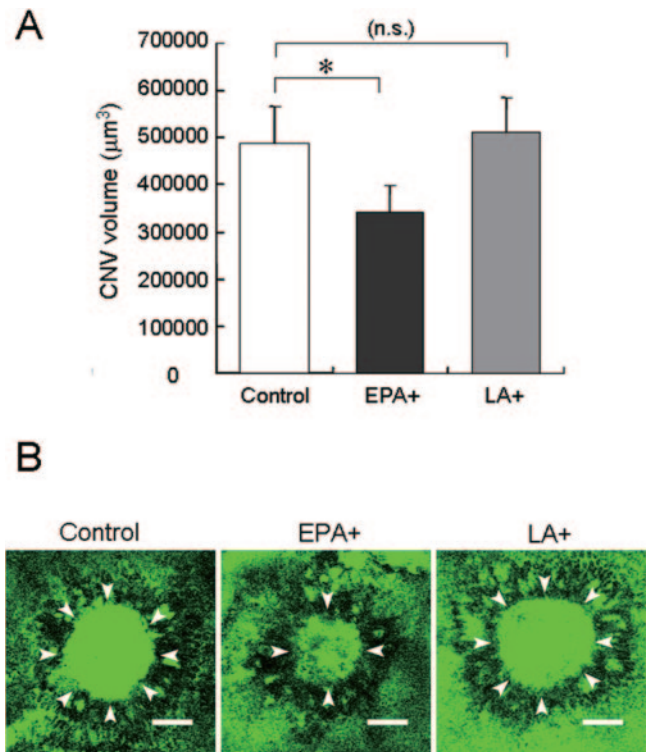
The volume of CNV was measured to evaluate and compare the effects of dietary intake of  $\omega$ -3 or  $\omega$ -6 PUFA on the development of CNV. Compared with control animals ( $488,588 \pm 77,324 \mu\text{m}^3$ ), the  $\omega$ -3 PUFA EPA-fed mice showed a significant ( $P < 0.01$ ) decrease in the volume of CNV ( $342,379 \pm 56,510 \mu\text{m}^3$ ), whereas the  $\omega$ -6 PUFA LA-fed mice ( $510,689 \pm 75,548 \mu\text{m}^3$ ) did not show a significant difference (Fig. 1).

### Inhibitory Effects of EPA on the In Vivo Expression of Inflammatory and Angiogenic Molecules

To determine whether supplemental EPA affects inflammatory and angiogenic molecules related to the pathogenesis of CNV, mRNA expression and protein production of ICAM-1, MCP-1, VEGF, and IL-6 in the RPE-choroid complex were analyzed by semiquantitative RT-PCR and ELISA. Both mRNA (Fig. 2A) and protein levels (Figs. 2B–E) of these inflammatory and angiogenic molecules were substantially suppressed in EPA-supplemented mice. Differences in protein levels were statistically significant ( $P < 0.01$  for ICAM-1, MCP-1, and VEGF;  $P < 0.05$  for IL-6).

### PPAR- $\gamma$ -Mediated Inhibitory Effects of EPA on the In Vitro Expression of Inflammatory and Angiogenic Molecules

To confirm the in vivo inhibitory effects of EPA on the expression of various inflammatory and angiogenic molecules (Fig. 2),



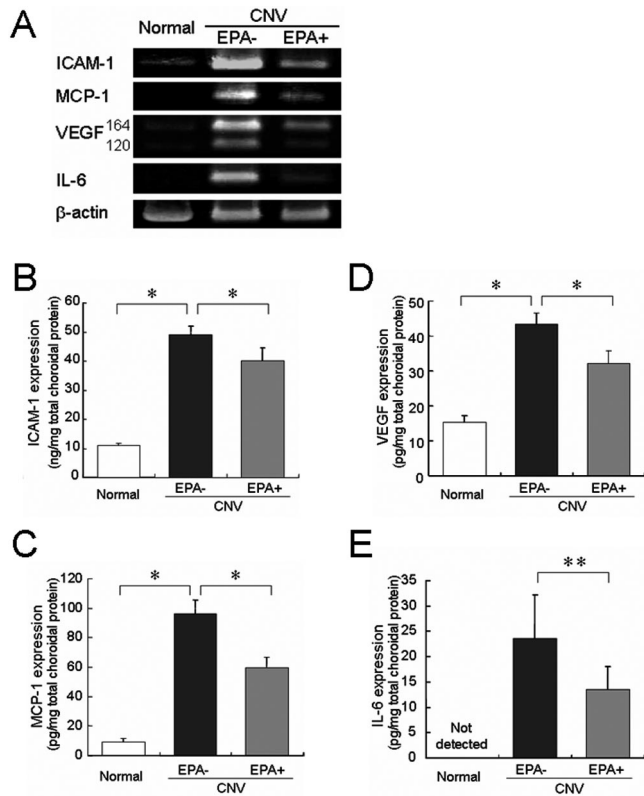
**FIGURE 1.** Inhibitory effect of EPA, but not LA, intake on CNV. (A) Volume of CNV. (B) Flatmounted choroids from control, EPA-fed (EPA+), and LA-fed (LA+) mice. Arrowheads: lectin-stained CNV. The volume of CNV was significantly reduced by EPA, but not by LA, intake. The results are expressed as the mean  $\pm$  SD ( $n = 32$ ). \* $P < 0.01$  by Mann-Whitney test. Scale bar, 100  $\mu\text{m}$ .

we further performed in vitro analyses, using murine cell lines including b-End3 for vascular endothelial cells (Figs. 3A–C) and RAW264.7 for macrophages (Figs. 3D–F). We analyzed mRNA expression (Fig. 3A, D) and protein levels (Figs. 3B, 3C, 3E, 3F) of ICAM-1 and MCP-1 in endothelial cells and VEGF and IL-6 in macrophages. These angiogenic and inflammatory molecules, upregulated in the RPE-choroid complex from mice with CNV, were suppressed by EPA supplementation in vivo (Fig. 2). As for the protein analyses, the maximum effect of EPA was obtained at a dose of 100  $\mu\text{M}$  after 24-hour incubation (Figs. 3B, 3C, 3E, 3F), whereas 6-hour incubation with EPA did not show any significant effect (data not shown). The mRNA expression of ICAM-1 and MCP-1, strongly induced by the 6-hour exposure to TNF- $\alpha$ , was substantially suppressed by EPA application (Fig. 3A). Simultaneous administration of a PPAR- $\gamma$  antagonist, GW9662, reversed the inhibitory effect of EPA (Fig. 3A). Administration of EPA significantly reduced protein levels of ICAM-1 (Fig. 3B) and MCP-1 (Fig. 3C). The EPA-induced reduction of ICAM-1 and MCP-1 levels was significantly reversed by the simultaneous administration of GW9662 ( $P < 0.01$ ). The mRNA (Fig. 3D) and protein (Figs. 3E, 3F) levels of VEGF and IL-6, strongly induced by LPS stimulation, were significantly suppressed by treatment with EPA ( $P < 0.01$  for VEGF and  $P < 0.05$  for IL-6). Administration of GW9662 reversed the inhibitory effect of EPA on VEGF and IL-6 production ( $P < 0.01$  for VEGF and  $P < 0.05$  for IL-6).

### Negligible Role of PPAR- $\gamma$ in the In Vivo Suppressive Effect of EPA Supplementation on CNV

To examine whether the PPAR- $\gamma$ -activating action of EPA observed in vitro functions in vivo as well, GW9662 was admin-





**FIGURE 2.** In vivo effects of EPA on the mRNA expression (A) and protein levels (B–E) of angiogenic and inflammatory molecules. (A) EPA substantially reduced mRNA expression of ICAM-1, MCP-1, VEGF, and IL-6. (B–E) Dietary intake of EPA significantly suppressed protein levels of ICAM-1 (B), MCP-1 (C), VEGF (D), and IL-6 (E). The results are expressed as the mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.01$ , \*\* $P < 0.05$  by Mann-Whitney test.

istered to EPA-fed mice. Of interest, the suppressive effect of EPA supplementation on CNV ( $342,379 \pm 56,510 \mu\text{m}^3$ ) was not reversed by PPAR- $\gamma$  blockade with GW9662 at the low ( $355,681 \pm 60,429 \mu\text{m}^3$ ) or high ( $329,946 \pm 80,036 \mu\text{m}^3$ ) dose (Fig. 4).

### Effects of EPA Supplementation on Fatty Acid Composition in the Serum and the RPE-Choroid

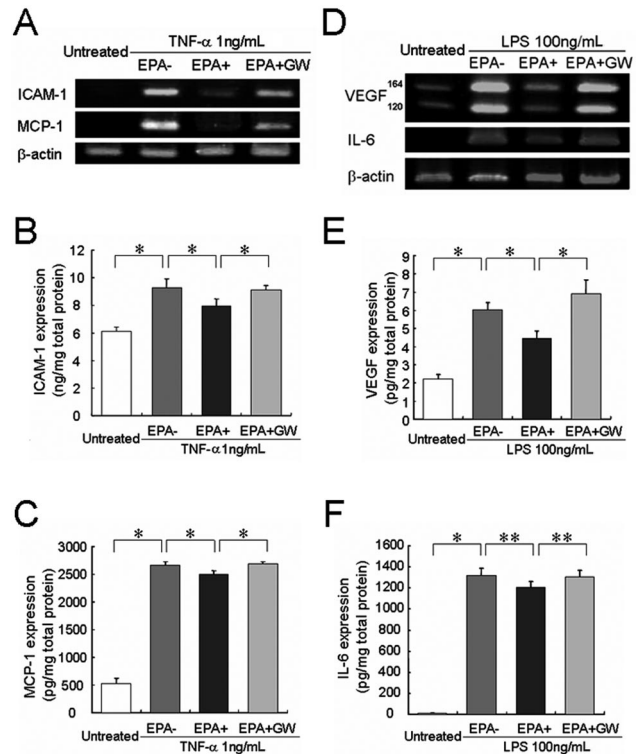
Fatty acid composition in the serum (Figs. 5A, 5B) and the RPE-choroid (Figs. 5C, 5D) was examined by gas chromatography and HPLC, respectively. The serum and RPE-choroid composition levels of total  $\omega$ -3 PUFAs, including EPA and its downstream metabolite DHA, were significantly ( $P < 0.01$ ) higher in EPA-fed mice than in control mice (Figs. 5A, 5C). In contrast, the serum and RPE-choroid composition levels of total  $\omega$ -6 PUFAs including AA and LA were significantly ( $P < 0.01$ ) lower in EPA-supplemented mice than in control animals (Figs. 5A, 5C). Particularly, the major fatty acids responsible for the significant changes in the PUFA levels were EPA (75.4- and 33.3-fold increases in the serum and the RPE-choroid, respectively) and AA (81.6% and 78.4% decreases in the serum and the RPE-choroid, respectively; Figs. 5B, 5D). In contrast, the serum and RPE-choroid composition levels of total saturated fatty acids (SFAs) including palmitic acid and stearic acid showed little or no difference between the two groups (Figs. 5A, 5C).

### Effects of EPA Supplementation on the Serum Levels of Inflammatory and Angiogenic Molecules

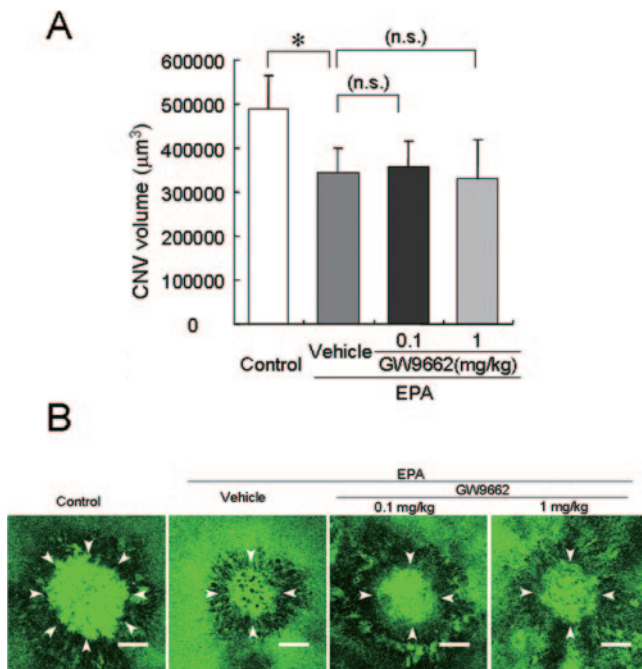
To determine the systemic effect of EPA intake, serum levels of CRP, soluble ICAM-1, MCP-1, VEGF, and IL-6 were measured by ELISA. EPA-fed mice exhibited significantly lower levels of CRP after laser injury (Fig. 6A) and IL-6 (Fig. 6B) than did the control animals. No significant difference was detected in any of the levels of soluble ICAM-1, MCP-1, and VEGF (data not shown).

### DISCUSSION

The present study reveals, for the first time to our knowledge, several important findings. First, dietary  $\omega$ -3 PUFA EPA, but not  $\omega$ -6 PUFA LA, consumption resulted in significant suppression of CNV formation in mice (Fig. 1). Second, EPA-fed mice exhibited significant decreases in the expression and production of ICAM-1, MCP-1, VEGF, and IL-6 in the RPE-choroid (Fig. 2). Third, in vitro application of EPA led to significant suppression of inflammation-related induction of ICAM-1 and MCP-1 in endothelial cells and VEGF and IL-6 in macrophages (Fig. 3). Although the anti-inflammatory effect of EPA application in vitro was mediated by PPAR- $\gamma$  activation (Fig. 3), the PPAR- $\gamma$ -mediated effect of EPA consumption in vivo was negligible (Fig. 4). Furthermore, EPA-fed mice exhibited significantly higher  $\omega$ -3 PUFA and lower  $\omega$ -6 PUFA levels in the serum and the RPE-choroid than control animals (Fig. 5). EPA supplementation also led to significant reduction of serum levels of IL-6 and CRP after photocoagulation (Fig. 6).



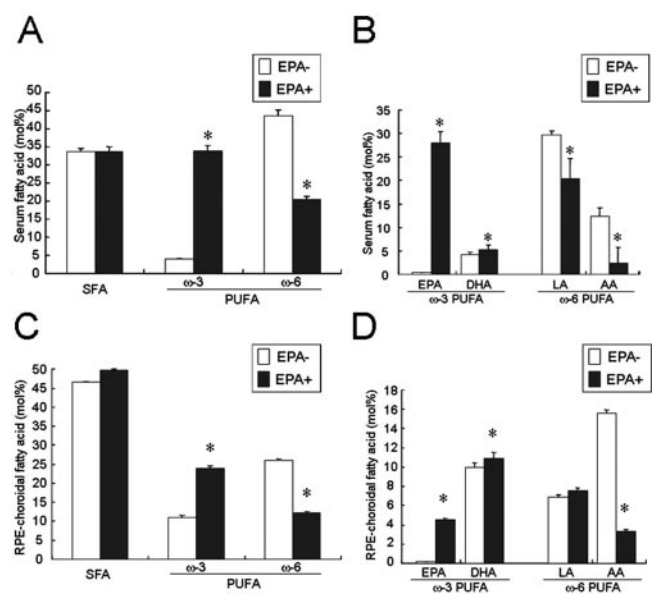
**FIGURE 3.** In vitro effects of EPA on mRNA expression (A, D) and protein levels (B, C, E, F) of angiogenic and inflammatory molecules in b-End3 endothelial cells (A–C) and RAW264.7 macrophages (D–F). EPA application led to significant inhibition of mRNA and protein levels of ICAM-1 and MCP-1 in endothelial cells (A–C) and VEGF and IL-6 in macrophages (D–F), each of which was significantly reversed by a PPAR- $\gamma$  antagonist GW9662. The results are expressed as the mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.01$ , \*\* $P < 0.05$  by Mann-Whitney test.



**FIGURE 4.** Negligible role of PPAR- $\gamma$  in the suppressive effect of EPA supplementation on CNV. (A) Volume of CNV. (B) Flatmounted choroids from control mice and EPA-fed mice receiving vehicle or GW9662. Arrowheads: lectin-stained CNV. The suppressive effect of EPA on CNV was not reversed by PPAR- $\gamma$  blockade with GW9662 at the low or high dose. The results are expressed as the mean  $\pm$  SD ( $n = 24$ ). \* $P < 0.01$  by Mann-Whitney test. Scale bar, 100  $\mu$ m.

Our data on the in vivo effect of high  $\omega$ -3 PUFA diet on experimental CNV revealed a significant reduction of CNV volume in EPA-fed mice compared with control animals taking in the EPA-subtracted laboratory chow (Fig. 1). In an important finding,  $\omega$ -6 PUFA supplementation with LA did not alter the volume of CNV. As for molecular mechanisms in the EPA-mediated prevention of CNV, the present in vivo data showed that CNV-associated induction of ICAM-1, MCP-1, VEGF, and IL-6 was significantly suppressed by dietary EPA intake (Fig. 2). The expression of these angiogenic and inflammatory molecules are induced by  $\omega$ -6 PUFA (AA and its precursor LA)-derived eicosanoids, including prostaglandin (PG) E<sub>2</sub> and leukotriene (LT) B<sub>4</sub>.<sup>38-41</sup> In contrast,  $\omega$ -3 PUFA (DHA and its precursor EPA)-derived eicosanoids including PGE<sub>3</sub> and LTB<sub>5</sub> are slightly different in structure, but substantially less pathogenic than these AA cascade metabolites.<sup>38</sup> The anti-inflammatory effect of EPA consumption is attributable to the  $\omega$ -3 PUFA-derived less-inflammatory mediators, which are processed from the cell membrane where  $\omega$ -6 PUFAs are replaced with  $\omega$ -3 PUFAs.<sup>42-47</sup> Reasonably, the present data showing that supplementation with EPA, but not LA, prevented CNV indicate the specificity of a  $\omega$ -3 PUFA-induced anti-inflammatory effect.

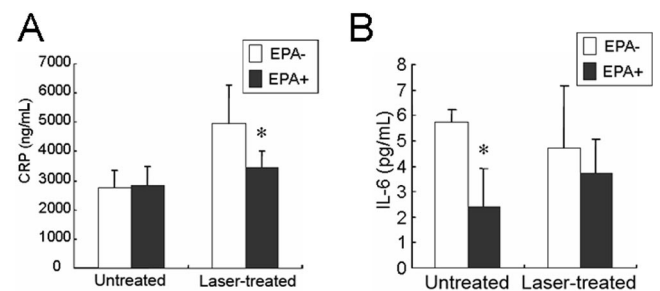
Previous reports concerning the molecular mechanisms underlying CNV generation showed VEGF to be a critical angiogenic factor. VEGF was detected in both the experimental model of laser-induced CNV<sup>3</sup> and surgically excised CNV tissues from patients with AMD.<sup>2</sup> Antibody-based neutralization of VEGF led to significant suppression of experimental CNV.<sup>4</sup> More recently, several in vivo experiments in genetically altered mice demonstrated critical roles of adhesion molecules and chemotactic factors including ICAM-1 and MCP-1, indicating the substantial involvement of inflammatory processes in CNV generation.<sup>12,48</sup> Moreover, our new in vivo data also show



**FIGURE 5.** Fatty acid composition in the serum (A, B) and the RPE-choroid (C, D). The serum and RPE-choroid composition levels of total  $\omega$ -3 PUFAs were significantly ( $P < 0.01$ ) higher in EPA-fed mice than in control mice, whereas those of total  $\omega$ -6 PUFAs were significantly ( $P < 0.01$ ) lower in EPA-supplemented mice than in control animals (A, C). The serum and RPE-choroid composition levels of total SFAs showed little or no difference between the two groups (A, C). Both EPA and DHA levels were increased by EPA intake, whereas AA levels declined (B, D). The results are expressed as the mean  $\pm$  SD ( $n = 9$  in serum and  $n = 15$  in RPE-choroid). \* $P < 0.01$  by Mann-Whitney test.

that IL-6 receptor-dependent activation of inflammatory signaling promotes CNV.<sup>49</sup>

Parallel in vitro experiments also showed the inhibitory effect of EPA on ICAM-1 and MCP-1 expression in the stimulated endothelial cells (Fig. 3A), in accordance with the previous in vitro data showing that EPA application reduced the expression of ICAM-1 induced by TNF- $\alpha$  or IL-1 $\beta$  in human umbilical vascular endothelial cells<sup>21,23</sup> and MCP-1 induced by LPS in HK (human kidney proximal tubule)-2 cells.<sup>26</sup> MCP-1-recruited macrophages, producing VEGF, facilitate the development of CNV.<sup>2,3</sup> Reasonably, our present data on the inhibitory effect of EPA on VEGF and IL-6 expression in macrophages (Fig. 3B) provide a possible explanation for the EPA-mediated antiangiogenic and anti-inflammatory mechanisms in vivo. Collectively, the EPA-mediated suppression of CNV formation observed in the present study is probably



**FIGURE 6.** Serum levels of CRP and IL-6. EPA-fed mice exhibited significantly lower levels of post-laser CRP (A) and pre-laser IL-6 (B) than did control animals. IL-6 levels after laser injury tended to be reduced by EPA intake (B), although the difference was not statistically significant. The results are expressed as the mean  $\pm$  SD ( $n = 8$  for untreated and 11 for laser-treated). \* $P < 0.01$  by Mann-Whitney test.

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 and IL-6 secretion.

In addition to its essential property as the substrates of less inflammatory eicosanoids, recent *in vitro* studies have shown that the anti-inflammatory effect of EPA depends on PPAR- $\gamma$ -mediated suppression of NF- $\kappa$ B activation.<sup>25,26</sup> PPAR- $\gamma$ , a ligand-responsive, intranuclear receptor-type transcription factor, is known to function as a major regulator of lipid and glucose metabolism. In addition, the anti-pathogenic role of PPAR- $\gamma$  has recently been elucidated. It has been shown to antagonize NF- $\kappa$ B-mediated transcription of various inflammation-related genes.<sup>50</sup> In consistence with these recent findings, the present study showed that a PPAR- $\gamma$  antagonist, GW9662, reversed the *in vitro* EPA-induced inhibition of ICAM-1 and MCP-1 expression in endothelial cells and VEGF and IL-6 expression in macrophages (Fig. 3). However, the PPAR- $\gamma$ -mediated anti-inflammatory phenotype by dietary EPA has not been clearly demonstrated in the literature, leading us to perform the *in vivo* blockade of PPAR- $\gamma$  pathway. In fact, the PPAR- $\gamma$  antagonist did not reverse the suppressive effect of EPA on CNV development (Fig. 4). Actually, the *in vitro* mechanism underlying EPA-induced activation of PPAR- $\gamma$  pathway remains unclear, whereas the *in vivo* incorporation of dietary  $\omega$ -3 PUFAs into the cell membrane was reported to require several weeks to reach the maximum plateau of fatty acid composition.<sup>45,51</sup> The present data suggest that the PPAR- $\gamma$ -mediated effect was limited or masked, at least *in vivo*, by the less inflammatory property of  $\omega$ -3 PUFA-derived eicosanoids.

In the present study, dietary EPA consumption led to significant reduction of the serum and RPE-choroid composition levels of the major  $\omega$ -6 PUFA AA, together with increased levels of the major  $\omega$ -3 PUFAs, EPA, and its downstream metabolite DHA (Fig. 5). This reduction is compatible with the previous report showing that dietary supplementation with EPA or DHA reduces plasma levels of AA in rats.<sup>52</sup> Increased levels of  $\omega$ -3 PUFAs are thought to inhibit the synthesis of AA because of the higher affinity of  $\Delta$ 6-desaturase, a common rate-limiting enzyme in the PUFA metabolism, with  $\omega$ -3 PUFAs than with the  $\omega$ -6 PUFA LA, the precursor of AA.<sup>53</sup> The present data showing that EPA supplementation led to decreased serum levels of IL-6 and CRP after laser injury (Fig. 6) may be explained by the finding that EPA-supplemented mice are less prone to inflammation. This is supported by the previous clinical studies showing that  $\omega$ -3 PUFA consumption reduced several inflammation-related biomarkers including CRP and IL-6.<sup>54-56</sup> Epidemiologic risk factors for AMD include several components of the metabolic syndrome,<sup>18,19,57-59</sup> the pathogenesis of which has been shown to be mediated by silent, chronic inflammation.<sup>60,61</sup> Of note, a recent prospective cohort study concerning the relationship of AMD with cardiovascular biomarkers has revealed that increased levels of CRP and IL-6 are independently associated with progression of AMD.<sup>62</sup> Accordingly, dietary EPA intake is suggested to improve systemic inflammation as a predisposing background of AMD.

In the present study, supplemental EPA was shown to exert anti-inflammatory action locally in the eye, as well as systemically in the circulation. The anti-inflammatory property of EPA may lead not only to prevention of CNV, but also to prophylactic improvement of background conditions related to AMD. Clinically, EPA is safely and widely used in patients with hyperlipidemia and/or atherosclerosis. Consequently, our present data warrant future clinical trials to verify the beneficial effect of EPA on human CNV.

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