Effects of Dioxin on Vascular Endothelial Growth Factor (VEGF) Production in the Retina Associated with Choroidal Neovascularization

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PURPOSE. Cigarette smoking is the most consistent risk factor for age-related macular degeneration (AMD), especially the choroidal neovascularization (CNV)-mediated exudative type. Dioxins and dioxin-like compounds have various effects on living organisms and are also contained in cigarette smoke. However, the effects of dioxins on the eye remain elusive. In this study, the authors examined the association between dioxins and neovascularization in the eye.

METHODS. C57BL/6 mice were injected intraperitoneally with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) every other day for 14 days. Messenger RNA expression of cytochrome P450 (CYP)1A1, CYP1B1, vascular endothelial growth factor (VEGF)-A and VEGF-B, and VEGF production were examined in the eyes of TCDD-treated mice and in human retinal pigment epithelial cell lines (ARPE-19) exposed to TCDD. In addition, CNV was induced by photoagulation in mice injected with TCDD, and the volume of CNV was compared by fluorescence-labeled choroidal flat mount.

RESULTS. TCDD injected intraperitoneally increased CYP1A1 mRNA expression in the iris/ciliary body and retina, indicating that TCDD acts directly on ocular tissues through the aryl hydrocarbon receptor (AhR) to promote the transcription of target genes. TCDD also promoted VEGF-A mRNA expression in the retina and the retinal pigment epithelium. TCDD-induced VEGF production at the molecular level was also observed in vivo by immunohistochemistry and in vitro using ARPE-19. Moreover, the injection of TCDD significantly exacerbated photoagulation-induced CNV in mice.

CONCLUSIONS. The authors demonstrate that dioxins are among the factors inducing abnormal vascularization in the eye through VEGF production mediated by AhR signaling. (Invest Ophthalmol Vis Sci. 2009;50:3410–3416) DOI:10.1167/iovs.08-2299

Age-related macular degeneration (AMD), a disease that affects the central area of the macula, is the most common cause of blindness in the elderly population of developed countries.1–3 The etiology of AMD is poorly understood, although genetic influences and environmental risk factors are implicated. Among the environmental factors, cigarette smoking is the most consistent risk factor for AMD.4–6 Cigarette smoking has negative effects on almost all organs of the body and is related to many pathologic conditions. Smoking has been associated with a twofold to fourfold increase in incidence of exudative type of AMD.4,5,7,8 The growth of abnormal new vessels from the choroids into the space beneath the retinal pigment epithelium (RPE), termed choroidal neovascularization (CNV), is observed in the exudative type of AMD and causes the most severe form of vision loss.9

Gas chromatography-mass spectrometric analyses have revealed that cigarette smoke contains dioxins and dioxin-like compounds, including polychlorinated dibenzop-dioxins, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, coplanar polychlorinated biphenyls, and other polycyclic aromatic hydrocarbons.10,11 Most of the toxic effects of dioxins are mediated by the cytosolic dioxin receptor known as aryl hydrocarbon receptor (AhR).12,13 Once a xenobiotic ligand is recognized by AhR, the AhR-ligand complex translocates to the nucleus and forms a heterodimer with its coactivator, the AhR nuclear translocator (Arnt). The AhR/Arnt heterodimer recognizes and binds to an enhancer DNA sequence, the xenobiotic responsive element. This interaction regulates the expression of dioxin target genes, particularly those encoding several isofoms of cytochrome P450 (CYP) enzymes including CYP1A1, CYP1A2, and CYP1B1, and some phase 2 detoxification enzymes.14,15

With a luciferase-based reporter assay, aged and diluted sidestream cigarette smoke has been shown to induce CYP1A1 expression mediated by activating AhR.10 In addition, AhR signaling has been found to play a major role in mediating cigarette smoke-induced cytogenetic damage.16,17 However, whether AhR-mediated signal is associated with the development of CNV observed in exudative AMD remains unclear because the AhR pathway-mediated toxic effects on the eye have not been studied.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic dioxin that mediates immunotoxicity, hepatotoxicity, teratogenicity, and tumor promotion by activating AhR.12,13,19–21 Here, we report that CYP1A1 expression was observed in the ocular tissues after intraperitoneal injection of TCDD, TCDD promoted vascular endothelial growth factor (VEGF)-A mRNA expression and VEGF production in mouse retina and human retinal pigment epithelial cell lines by way of the AhR pathway.
and injection of TCDD promoted the progression of laser-induced CNV in mice. From these results, we conclude that AhR-mediated signaling is one of the mechanisms by which cigarette smoke increases the risk for exudative AMD.

**MATERIALS AND METHODS**

**Mice and Human RPE Cell Line**

Six- to 8-week-old female C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; all procedures were performed under anesthesia with sodium pentobarbital or a ketamine and xyline mixture.

Human RPE cell line ARPE-19 was obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were passaged and cultured using a slight modification of the technique of Dunn et al.22

**TCDD Treatment**

Mice were injected intraperitoneally with 500 ng TCDD (Cambridge Isotope Laboratories, Inc., Andover, MA) dissolved in 1 mL oil or an equivalent volume of olive oil every other day for 2 weeks.

**Immunofluorescence**

Eyes were enucleated from mice after 2-week treatment with or without TCDD and were embedded in optimum cutting temperature compound (Tissue Tek; Sakura Finetechnical Co., Tokyo, Japan) to prepare frozen sections. Immunofluorescence analysis of VEGF expression was performed as previously described.23 Briefly, 1-μm-thick cryostat tissue sections were fixed in paraformaldehyde and incubated in 5% skim milk diluted in PBS for 20 minutes. The sections were incubated overnight at room temperature with rabbit anti-VEGF antibody, which reacts with VEGF-A (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then were incubated with FITC-conjugated goat anti–rabbit IgG (Molecular Probes, Eugene, OR). The sections were covered in mounting medium (Vector Laboratories, Burlingame, CA) and were analyzed under a confocal microscope equipped with image analysis systems.

**Messenger RNA Preparation from Iris/Ciliary Body, Neural Retina, and RPE**

Eyes were enucleated from mice after 2 weeks of treatment with or without TCDD and were placed in Ca2+/Mg2+-free HBSS on ice for 30 minutes. A circumferential incision was performed below the level of the ciliary body, and the iris and ciliary body tissues were gently separated from the anterior segment. The posterior segment was placed in 0.01 U/mL chondroitinase ABC24 for 30 minutes at 37°C, and the neural retina was gently lifted from the RPE layer by microsurgical forceps. Neural retina-deficient posterior eyecups, consisting of sclera, choroid, and a healthy monolayer of RPE, were incubated in 0.2% trypsin (BioWhittaker, Walkersville, MD) for 1 hour at 37°C in a 5% CO2 atmosphere. Thereafter, the eyeball was transferred to culture medium, and RPE cells were peeled off gently as intact sheets with a pair of fine forceps. The iris/ciliary body, whole retina, and RPE samples of 5 to 10 mice were homogenized (IsoGen; Nippon Gene, Tokyo, Japan), and total RNA was isolated according to the manufacturer’s protocol. RNA purity was detected by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically.

**Messenger RNA Preparation from ARPE-19 Cells**

ARPE-19 cells were plated in 10-cm tissue culture dishes. Monolayers were allowed to remain quiescent for 12 hours. After the quiescence period, the monolayers were incubated in triplicate for 12 hours with the indicated concentrations of TCDD in the presence or absence of α-naphthoflavone (ANF). At the end of culture, the cells were harvested, and total RNA was extracted (IsoGen; Nippon Gene) according to the manufacturer’s protocol.

**Real-Time Polymerase Chain Reaction (PCR) Analysis**

Real-time transcription of 1 μg RNA was performed to synthesize cDNA (Improm II Kit; Promega, Madison, WI). Real-time PCR analysis was performed (Smart Cycler System; Cepheid, Sunnyvale, CA) with dye (SYBR Green I; Cambrex, Washington, DC). The following primers were used: mouse CYP1A1-specific primers, 5′-CACTTGCGGTGCACGATGGAG-3′ and 5′-GTCTAAGCCTGAAGATGC-3′; mouse VEGF-A-specific primers, 5′-CCTTGGGACATCTCCTCAGGACTC-3′ and 5′-GAAAGCCTTCTCCTCATATTGCGGC-3′; mouse VEGF-B-specific primers, 5′-TCTGGGACATCTTATTATCCTCAGG-3′ and 5′-CAGAACCCAAATCCCGTTATTTG-3′; mouse β-actin-specific primers, 5′-ACCTTGCGGTCCAGCATGAGG-3′ and 5′-CYP1B1-specific primers, 5′-TGGCTGCTATTTCTCCATGTG-3′ and 5′-CYP1B1-specific primers, 5′-ACTGTCGGTCACTATG-3′ and 5′-CTTATGACAGTGTCTCCTG-3′ and 5′-CAGTGTGGGATCTTGGAC-3′; and human β-actin-specific primers, 5′-GGGAATTCTGGTTAGCAGAT-3′ and 5′-TTGTTGGCAGCTACAGTTTGG-3′. Reaction mixtures were denatured at 95°C for 30 seconds, then subjected to 40 PCR cycles of either 95°C for 5 seconds, 68°C for 30 seconds, and 85°C for 6 seconds for mouse CYP1A1, VEGF-A, VEGF-B, and β-actin or 95°C for 5 seconds and 68°C for 30 seconds for human CYP1B1, VEGF-A, and β-actin. Mouse CYP1A1, VEGF-A, and VEGF-B signals were normalized to mouse β-actin signal, and human CYP1B1 and VEGF-A signals were normalized to human β-actin signal. mRNA expression experiments were performed in triplicate.

**VEGF Production Assay**

ARPE-19 cells were plated in six-well plates. Monolayers were allowed to remain quiescent for 12 hours. After the quiescence period, the monolayers were incubated in triplicate for 12 hours with the indicated concentrations of TCDD in the presence or absence of 1 μg ANF. At the end of 12 hours, supernatants were collected, divided into aliquots, and stored frozen until assay. VEGF secreted in the supernatants was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

**Induction and Quantification of Laser-Induced CNV**

Laser photoocoagulation (532 nm, 200 mW, 100 ms; Novus Verdi [Coherent Inc.]) was performed on both eyes (four spots per eye) of each animal to induce CNV as described.25,26 One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde. CNV volume was measured by a scanning laser confocal microscope (5 Pascal; Zeiss, Thornwood, NY) using fluorescein-isothiocyanate-conjugated Griffonia simplicifolia isoslect B4 (Vector Laboratories, Burlingame, CA). The vessels were visualized by excitation with blue argon laser at 488 nm, and emission was captured between 515 and 545 nm. Any vessel in the laser-treated area and superficial to this reference plane was considered to be CNV. Horizontal optical sections (1-μm step) were obtained starting from the surface of the RPE/choroidal-sclera complex. The deepest focal plane at which the surrounding choroidal vascular network connecting to the lesion could be identified was judged to be the base of the lesion. Images of all the sections were stored digitally. The area of CNV-related fluorescence on each section was measured by computed image analysis with the microscope software. The sum of the areas of fluorescence measured from all the horizontal sections was used as an index of the CNV volume. CNV areas were measured by NIH Image (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and compared by hierarchical logistic regression using repeated measures. Results are expressed as mean ± SEM.

**Statistical Analyses and Reproducibility**

Experiments were repeated at least twice and usually more than three times. Response patterns were highly reproducible. Statistical analyses...
for parametric data were performed by independent t-test. Nonparametric data were analyzed by Mann-Whitney U test. P < 0.05 was considered significant (significant difference is denoted by an asterisk in the figures).

**RESULTS**

**CYP1A1 mRNA Expression in Iris/Ciliary Body and Retina of Mice Injected with TCDD**

First, to examine whether TCDD absorption by the body directly affects the eye, we injected TCDD (50 μg/mL) dissolved in olive oil into the peritoneal cavities of C57BL/6 mice every other day for 14 days and measured CYP1A1 mRNA expression in the iris/ciliary body and the retina by quantitative real-time PCR. If AhR is expressed on ocular tissues, the AhR activated by ligation with TCDD would promote their mRNA expression of CYP enzyme genes. Representative results are shown in Figure 1A. Although CYP1A1 mRNA expression was sparse in ocular tissues of mice injected with olive oil only, remarkable expression of CYP1A1 mRNA was observed in the iris/ciliary body and was especially marked in the retinas of mice injected with TCDD. These results indicate that TCDD injected into the body circulates to the eye, where it binds to AhR and promotes subsequent transcription of target genes.

**VEGF-A and VEGF-B mRNA Expression in Iris/Ciliary Body and Retina of Mice Injected with TCDD**

VEGF, one of the most potent angiogenic factors identified to date, has been demonstrated to play a significant role in CNV.27–29 Five VEGF isoforms have been characterized that differ in molecular mass and biochemical properties. Of these, VEGF-A has been most extensively studied. VEGF-A is expressed in the subfoveal membranes of patients with AMD,28 whereas VEGF-B is expressed in the retina during retinal vasoobliteration and hypoxia.30 To examine whether VEGF production in the eye is influenced by the absorption of dioxins in the body after exposure such as by ingestion, we analyzed mRNA expression of VEGF-A and VEGF-B in the iris/ciliary body and retina of mice injected intraperitoneally with TCDD (Figs. 1B, C). Quantitative real-time PCR analyses revealed that mRNA expression of VEGF-A did not change in the iris/ciliary body but increased significantly in the retina of TCDD-treated mice compared with control mice treated with olive oil only. On the other hand, mRNA expression of VEGF-B in the TCDD-treated mice increased in the iris/ciliary body and the retina.

**VEGF-A and VEGF-B mRNA Expression in RPE of Mice Injected with TCDD**

The retina consists of 10 layers containing blood vessels and several cell types. Among the retinal layers, the RPE has no blood vessel and has been implicated in the physiological regulation of the choroidal vasculature.28,31,32 Overexpression of VEGF in the RPE is considered an important factor in the pathogenesis of CNV associated with AMD.33 Therefore, we analyzed mRNA levels of VEGF-A and VEGF-B in RPE cells (Fig. 2). In contrast to the results of VEGF mRNA expression in whole retina in which VEGF-A and VEGF-B mRNA increased in mice injected with TCDD, only VEGF-A mRNA was significantly elevated in RPE cells.

**Immunofluorescence for VEGF in the Retinas of Mice Injected with TCDD**

Subsequently, molecular expression of VEGF in retinas isolated from mice injected with TCDD was examined by immunofluorescence using anti-VEGF antibody, which reacts with VEGF-A (Fig. 5). Although mRNA expression of VEGF, especially VEGF-A, was detected in the retinas of control mice.
**Figure 2.** VEGF-A and VEGF-B mRNA expression in RPE cells from mice injected with TCDD. Messenger RNA expression of VEGF-A (A) and VEGF-B (B) in RPE cells obtained from mice injected intraperitoneally with olive oil or TCDD (80 mg/mL) every other day for 14 days. RPE samples of 10 mice were pooled and used in this study. Only VEGF-A mRNA is increased in the RPE of mice injected with TCDD. Data are presented in mean ± SEM of triplicate assays. *Significant difference (P < 0.05). Experiments were repeated three times with similar results.

Injected with olive oil only, remarkable VEGF immunostaining was not shown (Fig 3B). On the other hand, clear VEGF immunostaining was observed in the ganglion cell layer, the photoreceptor cell layer, and the RPE layer of the retinas of TCDD-treated mice (Fig 3C).

**CYP1B1 mRNA Expression in Human RPE Cell Line Cultured with TCDD**

These in vivo studies indicated that TCDD injected intraperitoneally into mice circulated to the eye, acted on the RPE through the AhR, and promoted VEGF-A production at this site. However, it is unclear whether these results are also observed in human cells. ARPE-19 cells are spontaneously immortalized RPE cells with morphologic and functional characteristics similar to those of adult human RPE cells. We cultured ARPE-19 in 1 nM TCDD dissolved in dimethyl sulfoxide (DMSO), then analyzed mRNA expression of CYP1B1 by quantitative real-time PCR. Although minimal CYP1B1 mRNA expression was detected in ARPE-19 cells cultured with DMSO alone, mRNA expression of CYP1B1 increased markedly after TCDD stimulation (Fig. 4A). ANF is an antagonist of TCDD formed by an inactive complex with AhR. When ARPE-19 cells were cultured with TCDD in the presence of ANF, the TCDD-induced increase in CYP1B1 mRNA expression was dramatically abrogated in a dose-dependent manner (Fig. 4B). These results indicate that TCDD stimulates human RPE cells through the AhR, as is observed in mouse retinal tissues.

**VEGF Production in Human RPE Cell Line Cultured with TCDD**

We then analyzed VEGF-A mRNA expression in TCDD-exposed ARPE-19 cells by real-time PCR and measured VEGF secreted in these cultures by ELISA (Fig. 5). Although VEGF-A mRNA was expressed at low levels in ARPE-19 cells not exposed to TCDD, expression increased significantly with exposure to 0.1 or 1 nM TCDD (Fig. 5A). Compatible with the results of VEGF-A mRNA expression, though VEGF was detected in supernatants obtained from ARPE-19 cultures not exposed to TCDD, VEGF secretion was enhanced by exposure to TCDD, reaching a peak at the concentration of 0.1 nM (Fig. 5B). The TCDD-induced increase in VEGF production was significantly abolished by the addition of ANF.

**Laser-Induced CNV in Mice Treated with TCDD**

Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of CNV seen in AMD. Therefore, we evaluated the effect of TCDD on CNV development using a mouse laser-induced CNV model. One week after laser treatment, though CNV was observed at sites of rupture in Bruch’s membrane of mice injected intraperitoneally with olive oil only (Fig. 6A), the extent of CNV was apparently more extensive in mice injected with TCDD (Fig 6B). These results were supported by a volumetric analysis of CNV (Fig. 6C). The total volume of neovascularization in mice given TCDD was $450,683 \pm 36,083 \mu m^3$, which was significantly greater than $341,759 \pm 25,503 \mu m^3$ in mice given olive oil.

**Discussion**

Although AhR serves as a receptor for polycyclic and halogenated aromatic hydrocarbons contained in tobacco smoke, one or more endogenous ligands for AhR have been proposed to affect embryonic development, homeostasis, apoptosis, immunosuppression, and cell proliferation. AhR-null mice are relatively unaffected by TCDD at doses that induce severe toxic and pathologic effects in wild-type mice. However, ocular tissue changes associated with AhR signaling remain unde-
AhR-mediated signaling promotes VEGF production and CNV formation in the eye. This report is the first to demonstrate that AhR-mediated signaling promotes VEGF production and CNV formation in the eye. Experiments were repeated three times with similar results.

In the present study, though both VEGF-A and VEGF-B mRNA expression were upregulated in the retinas of TCDD-injected mice, intraperitoneal injection of TCDD increased mRNA expression of VEGF-B but not VEGF-A in the iris/ciliary body and of mRNA expression of VEGF-A but not of VEGF-B in the RPE. These results are compatible with the differences in the pathogenesis of hypoxia-induced angiogenesis and CNV related to AMD. Hypoxia-induced angiogenesis, such as that observed in neovascular glaucoma mediated by progressive diabetic retinopathy or retinal vein occlusion, occurs in the iris and retina but not in the RPE-choroid, which is probably regulated by VEGF-B. On the other hand, age-related neovascularization occurs in the retina and choroid but not in the iris, in which VEGF-A is involved.

This observation is not consistent with a previous report that mice exposed to cigarette smoke exhibited marked impairment of angiogenesis in response to surgically induced hind limb ischemia. That study suggests that cigarette smoke exposure inhibits VEGF production by downregulating hypoxia inducible factor-1α (HIF-1α) expression. HIF-1α is a member of the basic helix-loop-helix PAS (Per-ARNT-SIM) transcription factors contained in AhR and dimerizes with HIF-1β, which is another basic helix-loop-helix transcription factor identical with Arnt. The hypoxia and dioxin response path-

VEGF production in ARPE-19 cells was increased by TCDD treatment with a peak at the concentration of 0.1 nM and was significantly abolished by the addition of ANF. Data are presented in mean ± SEM of triplicate assays. *Significant difference (P < 0.05).
ways share common cellular factors. Therefore, the DNA-binding activity of the AhR-Arnt complex augmented by cigarette smoke most likely compensates for the cigarette smoke–binding activity of the AhR-Arnt complex augmented by cigarette smoke.

Figure 6. Effects of TCDD on laser-induced CNV. Laser photocoagulation was performed on both eyes of 6- to 8-week-old mice to induce CNV, as described in Materials and Methods. Representative sections of CNV in mice treated with olive oil only (A) and mice treated with TCDD (B) are shown. These results were quantified by measuring the total CNV volume and were analyzed statistically (C). Results are expressed as mean ± SEM (n = 10 in each group). *Significant difference (P < 0.05). Experiments were repeated three times with similar results.

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