Suppression of Retinal Neovascularization by the NF-κB Inhibitor Pyrrolidine Dithiocarbamate in Mice

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PURPOSE. To evaluate the effect of pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor κB (NF-κB), on retinal neovascularization in a murine model of ischemic retinopathy.

METHODS. One-week-old C57BL/6N mice were exposed to 75% ± 2% oxygen for 5 days and then were returned to room air to induce retinal neovascularization. After the return to room air, the left and right eyes were injected intravitreally with PDTC or a vehicle, respectively. Retinal neovascularization was examined by injecting fluorescein dextran and angiography after 5 days in room air and was quantitized histologically with a masked protocol. The effects of PDTC on NF-κB activation were evaluated by immunohistochemistry. To examine the toxicity of PDTC, the histologic change in the retina was examined by light and electron microscopy.

RESULTS. Retinal neovascularization in the eye injected with PDTC by intravitreal methods was reduced in 100% of animals compared with that apparent in the vehicle-treated eye. The inhibitory effect was dose-dependent, with a maximal inhibition of 39% (P < 0.01) at a dose of 1 nmole. The immunostaining intensity for NF-κB in the retina was reduced by PDTC injections. No side effects by PDTC in the retina were observed by light and electron microscopy.

CONCLUSIONS. NF-κB activation appears to be required for retinal angiogenesis, given that the administration of PDTC suppressed retinal neovascularization. PDTC may prove beneficial in the treatment of ischemic neovascular diseases such as diabetic retinopathy and retinal vein occlusion. (Invest Ophthalmol Vis Sci. 1999;40:1624-1629)

Angiogenesis, the formation of new capillary blood vessels, often accompanies wound healing, inflammation, and other pathologic conditions. Retinal neovascularization is a major cause of the blindness associated with such ischemic retinal disorders as diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion. Despite the prevalence of these diseases, an effective treatment for retinal neovascularization remains elusive. Neovascularization is induced by complex interactions among multiple cytokines and adhesion molecules. Several potential inhibitors of retinal neovascularization, including soluble vascular endothelial growth factor (VEGF) receptor and antagonists of both α5-integrin and growth hormone, have been identified with the use of a highly reproducible model of ischemia-induced retinal neovascularization. However, targeting pleiotropic regulators of multiple angiogenic factor and adhesion molecule genes may be required to inhibit neovascularization effectively.

With the animal model of ischemia-induced retinal neovascularization, we have previously shown that nuclear factor κB (NF-κB) activation may be important to induce retinal neovascularization in vivo. In this model, exposure of neonatal animals to hyperoxic conditions results in extensive obliteration of retinal capillaries. When the animals are returned to room air, the inner retina presumably becomes relatively hypoxic, which results in activation of NF-κB, subsequent production of many cytokines and adhesion molecules, and retinal neovascularization.

We have also shown that the expression of interleukin-8 (IL-8), probably mediated by NF-κB activation, may contribute to the pathogenesis of intraocular neovascularization in individuals with diabetic retinopathy or retinal vein occlusion. In addition to the IL-8 gene, NF-κB regulates many other angiogenesis-related genes, including those encoding tumor necrosis factor-α (TNF-α), vascular cell adhesion molecule-1, and intercellular adhesion molecule-1. Pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB activation, and NF-κB antisense oligonucleotides inhibit angiogenesis in an in vitro model. We also have shown that PDTC prevents hypoxia-induced NF-κB activation in retinal glial cells in vitro. These observations suggest that NF-κB may represent a suitable target for therapeutic intervention in retinal neovascularization.

We have now examined the effects of PDTC on retinal neovascularization in the mouse model of proliferative retinop-
Animal Model of Proliferative Retinopathy

The experiments were approved by the Ethics Committee on Animal Experiments of the Faculty of Medicine, Kyushu University, and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The reproducible model of hypoxia-induced neovascularization has been described previously. Briefly, litters of 7-day-old (postnatal day 7 (P7)) C57BL/6N mice, together with their mothers, were exposed to 75% ± 2% oxygen for 5 days and then returned to room air on P12. Intravitreal injections were performed as described below. Mice of the same age that had been kept in room air were used as controls.

Angiography with High-Molecular-Weight Fluorescein-Dextran

Mice (n = 23) were deeply anesthetized by intraperitoneal (IP) injections of sodium pentobarbital, and 80 μl phosphate-buffered saline (PBS) containing 20 mg fluorescein isothiocyanate-dextran was then injected into the left ventricle. The eyes were enucleated, and the retinas were dissected and flat-mounted on microscope slides for examination with a Leica (Wetzlar, Germany) fluorescence microscope.

Intravitreal Injections

Mice were deeply anesthetized by IP injections of sodium pentobarbital. The lid fissure was opened, and the eyes were propidised. Intravitreal injections were performed on P12 and P14 by delivering 0.5 μl (1 pmole to 10 nmole) of PDTC diluted in PBS to the left eye and of PBS alone to the right eye with a 32-gauge Hamilton needle and syringe at 200 μm posterior to the limbus. The eyes were then repositioned, and the lids were approximated over the cornea. Repeated injections were performed through a previously unmanipulated section.

Quantitation of Neovascularization

P17 mice were killed by IP injections of an overdose of sodium pentobarbital. Their eyes were enucleated, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Serial 3-μm paraffin-embedded axial sections of the retina were obtained starting at the optic nerve head. After staining with periodic acid–Schiff reagent and hematoxylin, 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section according to a fully masked protocol. Averaging of all 10 counted sections yielded the mean number of neovascular cell nuclei per 3-μm section per eye. No vascular cell nuclei anterior to the internal limiting membrane were observed in normoxic control animals.

Immunohistochemistry

The eyes were enucleated, fixed with 4% paraformaldehyde in 0.1 M PBS, and embedded in paraffin. Thick sections (3 μm) were cut, and after removal of the paraffin, they were rehydrated and incubated for 1 hour at room temperature with rabbit polyclonal anti-p65 (NF-κB) antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rat monoclonal anti-F4/80 antibody (1:10 dilution; Biosource, Camarillo, CA). Bound antibodies were detected by a conventional avidin-biotin-peroxidase protocol with 3-amin-9-ethylcarbazole as the substrate. For negative controls, rabbit and rat nonimmune IgG was used as the primary antibody.

Assessment of Immunohistochemistry

Four intact sections of equal length, each 30 μm apart, starting from the edge of the optic nerve, were examined for each retina to assess the immunohistochemistry. Because virtually all activated NF-κB is localized in the nucleus, the cells were considered positive for activated NF-κB if any nuclear staining was present. Averaging all four counted sections yielded the mean number of NF-κB-positive cells per section per eye with masked protocol.

Light and Electron Microscopy for Assessment of Side Effects

The possible side effects of PDTC treatment on the retina were assessed by light and electron microscopy 30 days after the PDTC injections (P44). The eyes were enucleated, fixed with 4% paraformaldehyde in 0.1 M PBS or 4% glutaraldehyde in a 0.1 M cacodylate buffer solution for 24 hours. The eyes fixed with paraformaldehyde (n = 24) were embedded in paraffin, cut, and stained by hematoxylin and eosin. The eyes fixed with glutaraldehyde (n = 16) were postfixed for 90 minutes in 1% cacodylate-buffered osmium tetroxide, dehydrated in a series of graded alcohols, and then were embedded in an epoxy resin. Thin sections were cut on an ultramicrotome (Porter-Blum MT II, Sorvall, Newton, CT), stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM-100CX, Nihon Densi, Tokyo, Japan).

We counted the number of cell nuclei per 50 μm length in retinal sections as linear cell densities by light microscopy as previously described. The number of cell nuclei in three layers [the ganglion cell layer, inner nuclear layer, and outer nuclear layer] was counted in a width of 50 μm in retinal sections from both hemispheres at a distance of 0.5 mm from the optic nerve head. The values of retinal cell densities were averaged from 10 measurements of five sections from each eye, and a single mean value for each layer was obtained from each eye. The cell densities were measured under masked conditions.

Statistical Analysis

Data are presented as means ± SD. The significance of differences was evaluated by the Kruskal-Wallis test. A P value < 0.01 was considered statistically significant.

RESULTS

Qualitative Effect of PDTC on Retinal Neovascularization

To evaluate the angiostatic efficacy of PDTC on ischemia-induced retinal neovascularization, retinas were examined by injecting fluorescein-dextran and angiography. Immediately after exposure to hyperoxia (P12), the fine branching capillaries around the optic disc showed almost no perfusion, whereas
The well-developed radial vessels extending from the optic disc were perfused (data not shown). Retinas of the PBS-injected eyes of hypoxic animals at P17 contained prominent neovascular tufts extending into the vitreous body at the junction between the perfused and the nonperfused tissue (Fig. 1B). In contrast, in the P17 retinas from hypoxic animals treated with PDTC (100 pmole to 10 nmole), the amount of neovascular tissue was markedly reduced, despite the presence of comparable pericentral regions of nonperfusion (Fig. 1C). Retinas from normoxic control animals revealed an evenly distributed, stained capillary network (Fig. 1A).

**Quantitative Effect of PDTC on Retinal Neovascularization**

The neovascularization was assessed histologically by counting the endothelial cell nuclei anterior to the inner limiting membrane. This assessment revealed that retinas of PBS-treated eyes from hypoxic mice contained multiple neovascular tufts extending into the vitreous (Fig. 2A), whereas retinas from control eyes of normoxic mice did not contain endothelial cells at this location (data not shown).

Intravitreal injections of PDTC at a dose of 1 nmole in hypoxic mice reduced the histologically evident retinal neovascularization in all 15 animals (Fig. 2B) compared with that evident in the PBS-treated contralateral eyes. Administration of PDTC at doses 1 pmole to 1 nmole resulted in a dose-dependent inhibition of retinal neovascularization, with a maximum inhibitory effect of 39% at the 1 nmole dose (Fig. 3). A further increase in the dose at 10 nmole PDTC did not increase the inhibitory effect.

**Immunohistochemistry**

To determine the inhibition by PDTC of NF-κB activation, immunohistochemistry was performed. NF-κB immunoreactiv-
FIGURE 3. Inhibition of retinal neovascularization by PDTC. Data are means ± SD. *P < 0.01 versus contralateral, PBS-treated eyes; n = 144.

We have now shown that PDTC, an inhibitor of NF-κB activation, inhibited NF-κB activation and reduced retinal neovascularization without discernible toxicity in a mouse model. Our data indicate that NF-κB likely plays a key role in the neovascularization process and may provide a basis for a specific and effective therapeutic regimen for various diseases associated with undesired angiogenesis.

The effect of PDTC on NF-κB activation appears specific for this transcription factor, in that other DNA-binding activities were unaffected by this compound. In our previous study, PDTC also prevented hypoxia-induced NF-κB activation and production of IL-8, a NF-κB-inducible molecule, but not VEGF production in retinal glial cells during exposure to hypoxia. Furthermore, it has also been shown that PDTC could prevent NF-κB activation and production of NF-κB-inducible molecules in several cells, including vascular endothelial cells,

Assessment of Side Effects of PDTC
To examine the toxicity of PDTC, the retinas were examined by light and electron microscopy 30 days after injections (P44). Light microscopy showed that the retina from eyes that received PDTC injections were similar to those from eyes injected with PBS and without injection. The numbers of cells in each layer of the retina did not differ significantly between PDTC-treated, PBS-treated, and untreated eyes (Fig. 5A). Electron microscopic examination of the retina also revealed no evidence of retinal toxicity of 1 nmole and 10 nmole PDTC (Fig. 5B). All retinal layers showed no damage to the organization and cytoarchitecture. The relationship between photoreceptor cells and pigment epithelial cells appeared no different in all groups.

DISCUSSION
We have previously shown that NF-κB activation might play an important role in the induction of retinal neovascularization in vivo. We have also shown that NF-κB activation in human microvascular endothelial cells contributes to angiogenesis. In contrast, a study by Grilli et al. implicates NF-κB activation in neurodegeneration. Inhibition of NF-κB activation might thus help to prevent both excitotoxic neuronal cell death and undesirable angiogenesis.

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Macrophages produce many NF-κB-regulatable, angiogenesis-related factors, such as TNF-α, in response to various stimuli, including hypoxia. We have previously shown that macrophage-derived TNF-α may trigger angiogenesis through the induction of IL-8, VEGF, or basic fibroblast growth factor (bFGF) in an autocrine or paracrine manner. In addition, tumor-associated macrophage infiltration correlates with tumor angiogenesis in individuals with invasive breast cancer, and the accumulation of monocytes-macrophages that produce TNF-α and bFGF is associated with angiogenesis after femoral artery occlusion in the rabbit. Therefore, the inhibitory effect of PDT on retinal neovascularization in vivo is probably due to, at least in part, preventing macrophages from producing angiogenic cytokines.

In this study, administration of 1 nmole PDTC resulted in maximal reduction of 71% of NF-κB-positive cells after exposure to hyperoxia, and a further increase in the amount at 10 nmole PDTC did not increase its inhibitory effect on NF-κB, suggesting PDTC in our experiment could not inhibit NF-κB completely. Improvement of the delivery methods of PDTC, using other NF-κB inhibitors such as antisense oligonucleotides directed against NF-κB or using a combination of these compounds, might be more effective. On the other hand, the maximal inhibitory effect of PDTC on neovascularization was 39%; thus, other angiogenic factors that are not induced by NF-κB likely account for the remaining angiogenic activity. Combination of the NF-κB inhibitor with inhibitors of other pleiotropic factors, such as Sp1 and hypoxia-inducible factor-1, that contribute to angiogenesis may therefore be necessary to achieve a complete suppression of the hypoxia-induced retinal neovascularization.

In a clinical situation, chronic repeated injections might be required. Although we could not observe any toxicity in the

Figure 5. Assessment of the side effects of PDTC in the retina at 30 days after twice injections. (A) Cell densities in P44 retinas from 1 nmole and 10 nmole PDTC-treated, PBS-treated, or untreated eyes of hypoxic mice. Data are means ± SD. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. n = 24. (B) Electron micrograph of the retina treated with 10 nmole PDTC. The left electron micrograph shows no damage to the relationship between photoreceptor cells and pigment epithelial cells; the right electron micrograph shows no damage to the nuclear chromatin pattern in the outer nuclear layer and outer limiting membrane. Bar, 2 μm.
retina by light and electron microscopy 30 days after twice injections of PDTC, chronic repeated injections might induce side effects. Improvement of the drug modification and drug delivery systems such as liposome-, microsphere-, scleral plug-, or pellet-mediated delivery that can maintain sustained and effective concentration for a specific period and reduce the damage to the retina by drugs should be required.

The findings in this study suggest that PDTC can be used to prevent intraocular neovascularization without retinal destruction and that a therapeutic strategy involving the inhibition of NF-κB activation may prove effective in retinal neovascularization.

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References


