Inflammation induced by photocoagulation laser is minimized by copper chelators

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Abstract

The effect of trientine hydrochloride (TRIENT), a copper-selective chelating agent, on retinal inflammation induced by photocoagulation laser treatment was studied. Nine Long-Evans rats were treated with TRIENT (0.5 mmol/kg per day, intraperitoneal injection) for 9 days. On day 8, each animal underwent unilateral photocoagulation laser treatment with an argon dye laser. On day 9, animals were killed and the eyes processed for immunohistochemistry and light microscopy. In the TRIENT-treated group, retinal thickness and number of macrophages (ED-1) were both significantly lower than in the saline-treated, control group exposed to laser photocoagulation. The results support the hypothesis that selective copper chelation prior to laser treatment may inhibit ocular inflammation. The results suggest that pretreatment with a selective copper-chelating compound can minimize retinal inflammation secondary to laser photocoagulation treatment, which may improve overall outcome of photocoagulation treatment for diabetic retinopathy.

Keywords

Inflammation; Trientine; Immunohistochemistry; Laser therapy; Retinal edema; Diabetic retinopathy

Introduction

Abnormal cellular proliferation and angiogenesis are hallmarks of several sight-threatening eye diseases such as proliferative diabetic retinopathy (PDR). PDR is commonly treated by laser photocoagulation in which a thermal laser is used to seal leaky retinal capillaries (focal

photocoagulation) or to destroy tissue to slow the growth of new abnormal blood vessels (scatter photocoagulation). Both procedures result in nonselective retinal damage that may result in permanent loss of vision [1]. Furthermore, studies have shown that laser photocoagulation also results in the excessive formation of reactive oxygen species (ROS), or free radicals, in the surrounding tissue [2, 18]. Free radicals are known to promote inflammation in tissues after laser treatment. The inflammation may then trigger neovascular events by stimulating cell proliferation in fibroblasts and retinal pigment epithelial cells, two cell types implicated in the recurrence of neovascularization in the eye [3–7]. Thus, the formation of free radicals in ocular tissues secondary to laser treatment may initiate an inflammatory response that could substantially compromise therapeutic outcome. Obviously, a need exists for a method of inhibiting inflammation and preventing recurrence of neovascularization [8]. So far, there is no antiinflammatory treatment available that is used in combination with photocoagulation laser therapy for diabetic retinopathy.

Our earlier study showed that the transition metal, copper, plays a critical role in the generation of intermediate ROS including the hydroxyl radical [9]. Free copper ions are highly redox-active and may contribute to tissue damage via catalyzing the generation of ROS. Copper acts as an electron carrier to catalyze electron transfer from many reducing agents, such as cysteine and glutathione, to molecular oxygen (O_2). During this process, intermediate reactive oxygen species (ROS), including superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), are produced. The hydroxyl radical causes the breakdown of cellular DNA, membranes and proteins, and is an important mediator of ocular inflammation. Based on the significance of free radicals in cell proliferation and of copper in the generation of free radicals, we explored the potential therapeutic application of a copper-selective transition metal chelator, TRIEN, in inhibiting ocular inflammation secondary to photocoagulation laser treatment.

Methods and materials

Inhibition of copper-catalyzed generation of hydroxyl radicals by TRIEN

Production of $\cdot OH$ was estimated using coumarin-3-carboxylic acid (CCA). Nonfluorescent CCA is converted by $\cdot OH$ to highly fluorescent 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) [10]. For measuring $\cdot OH$ produced during autoxidation of $100\ \mu M$ cysteine in the presence of $0.2\ \mu M Cu^{2+}$, the reaction mixture was prepared as follows in sequence: $0.885\ ml$ of PBS, $0.1\ ml$ of $10\ mM$ CCA (final concentration $1\ mM$), $10\ \mu l$ of $10\ mM$ cysteine, and $5\ \mu l$ of $40\ \mu M Cu^{2+}$. The solution was left at $37^\circ C$ in 100% air to finish the reaction completely. After 4 h incubation, the samples were measured using a fluorescence spectrometer, with an excitation wavelength of $400\ nm$ and emission wavelength of $450\ nm$. CCA ($1\ mM$) in PBS was used as the reference. A standard curve was calculated by measuring the fluorescence intensities of a series of concentrations of 7-OH-CCA. The $\cdot OH$ produced from cysteine autoxidation was represented by the corresponding 7-OH-CCA concentrations.

Animal preparation for laser photocoagulation

All procedures on animals in this study were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nine Long-Evans rats weighing between 400 and 450 g were used for the photocoagulation study. They were divided into a normal (no injection, no laser) group (N), a laser + saline-injected group (LS), and a laser + TRIEN-treated group (LT). Each group contained three animals. Animals in the LT group were treated with TRIEN for a total of 9 days. Approximately 110 mg/kg per day was injected intraperitoneally once a day for 7 days before laser treatment, on the day of laser treatment and 1 day after laser treatment. Animals in the LS group received intraperitoneal injections of sterile saline (1 ml) for 7 days before laser treatment, on the day of laser treatment and 1 day after laser treatment.

On the day of laser photocoagulation treatment, all nine animals were sedated with an intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg). A glass microscope cover slip was applied to the cornea using gonioscopic solution (gonioscopic prism solution, Alcon, Fort Worth, TX) and the anesthetized animal placed on the chin rest of an argon dye laser (Lumenis, Santa Clara, CA). Dye laser irradiation at 514 nm wavelength was delivered through a slit lamp. A total of six laser spots were made separately using a setting of 50 μ m diameter, 0.1 s duration and 150 mW intensity. The laser spots were positioned between major retinal veins in the right eye only, avoiding major retinal vasculature. The untreated fellow eyes served as controls.

Histological preparation of experimental eyes

All rats were killed 24 h after laser treatment by intracardial perfusion of buffered 4% paraformaldehyde under deep anesthesia with isoflurane. From the nine rats, 18 eyes were immediately enucleated, placed in 2% paraformaldehyde for 48 h at 4°C and embedded in individual molds in OCT compound (Optimal Cutting Temperature compound; Tissue-Tek, Torrance, CA, USA) and frozen in dry ice. Cryostat sections were obtained at 6 μ m thickness in the sagittal plane from the central portions of the retina.

Immunohistochemistry

Sections of tissue were processed on glass slides. Endogenous peroxidases were removed by incubation with 0.3% hydrogen peroxide for 15 min. The sections were then blocked for nonspecific binding in a solution of 5% normal swine serum (NSS) and 0.3% TX-100 in PBS for another 20 min. The retinas were transferred to primary antibody diluted in 5% NSS and incubated for 1 h at room temperature and overnight at 4°C in ED-1 antibody for total macrophages. The dilution of the mouse anti-rat ED-1 antibody (Serotec) was 1:100. After primary antibody incubation, sections were rinsed and then placed in a solution containing the appropriate secondary antibody for 30 min at room temperature and developed with amino-ethyl-carbazole (AEC, Vector Laboratories, Burlingame, CA) as a red chromogen.

Results

Reduction of copper-catalyzed generation of hydroxyl radicals by TRIEN

The effect of chelating copper using TRIEN on the generation of $\cdot\text{OH}$ from cysteine autoxidation was tested. Production of $\cdot\text{OH}$ was measured with CCA fluorescence assay. The control reaction mixture contained all components except TRIEN, as shown in Fig. 1. Addition of TRIEN significantly reduced the $\cdot\text{OH}$ generation from copper-catalyzed cysteine autoxidation by 43%.

Retinal thickness after photocoagulation treatment

We compared the retinal thickness of eyes from normal animals, animals in the LS group, and animals in the LT group. The full thickness of the retina was measured from the nerve fiber layer to retinal pigment epithelium layer (Fig. 2a–c). At 24 h after photocoagulation treatment, the retinal thickness in the eyes of the LS animals was 50% greater than in the eyes of normal animals. Treatment with TRIEN before laser photocoagulation resulted in a 30% reduction in retinal thickness in the LT group compared to the LS group. However, retinal thickness in LT animals was still greater than in normal animals (Fig. 2d). Taking the retinal thickness in normal animals as $100 \pm 4\%$ (mean $\pm$ SD), the retinal thicknesses in animals in the LS and LT groups were $150 \pm 22\%$ and $120 \pm 5\%$, respectively. These values were significantly different ($p < 0.05$, Student's *t*-test).

ED-1 immunohistochemistry after photocoagulation treatment

The immunoresponse to photocoagulation treatment was also assessed with immunohistochemistry using an antibody against total macrophages and microglia (ED-1). Macrophages and retinal microglia are important cellular mediators of the immune response and their numbers increase in response to tissue damage caused by many factors, including oxidative stress. ED-1 immunohistochemistry demonstrated more macrophages in retinal sections from animals in the LS group compared to animals in the LT group (Fig. 3a,b). At 24 h after laser treatment, the numbers of ED-1-immunoreactive cells within the laser lesion site were 17 ± 4 (mean $\pm$ SD) and 9.7 ± 4 in the LS and LT groups, respectively (Fig. 3c). The number of ED-1-immunoreactive cells in the LT group was significantly lower than in the LS group ($p < 0.05$, Student's *t*-test).

Discussion

Evidence for the significant role of ROS in the inflammation process is accumulating [11]. The inflammatory process is characterized by the migration of neutrophils and macrophages into the laser injury site, where they eliminate cellular debris by phagocytosis. During phagocytosis, there is a rapid increase in oxygen consumption by neutrophils and macrophages, known as the respiratory burst. The oxidase complex in the plasma membrane of inflammatory cells will make use of NADPH to reduce O_2 , generating superoxide radicals ($\cdot\text{O}_2^-$): $\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + 2\cdot\text{O}_2^-$. The superoxide species, $\cdot\text{O}_2^-$, is the ultimate precursor of other prooxidants, including hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$). These ROS cause tissue damage indiscriminately, cause affected cells to further release other inflammatory factors, and may even stimulate recurrent neovascular

events in the retina [2, 12, 13] (Fig. 4). Given that laser photocoagulation treatment is used to minimize retinal neovascular events in diabetic retinopathy, iatrogenic causes of recurrent neovascularization, such as inflammation, should be minimized.

Inflammation of retinal tissues may also occur after intense light exposure, such as during endoillumination during vitreoretinal surgery, and during cataract surgery when the removal of the lens causes a temporary, unobstructed light axis to the retina [17]. As intense light exposure, even for brief periods, will result in an increased chemical generation of ROS, it is possible that secondary inflammation after vitreoretinal or cataract surgery may result in a compromised therapeutic outcome due to the proliferative cellular events triggered by excess ROS or free radicals in the surrounding tissues.

In an earlier cell culture study, we found that of several transition metals (manganese, chromium, zinc, iron and copper), free copper was the most potent transition metal to catalyze the generation of ROS, particularly hydroxyl radical [9]. In this study we found that copper substantially accelerates the autooxidation rate of cysteine, and that this process results in the build-up of free radicals formed during the process. A copper-selective chelator, such as TRIEN, can inhibit the generation of hydroxyl radicals (Fig. 1). While large quantities of ROS can kill cells, exposure to low levels of free radicals has been reported to stimulate cell proliferation [13, 14]. If the inflammation-induced cell proliferation depends on free radicals generated during the inflammation process, inhibition of free radical generation should inhibit cell proliferation, thus reducing unwanted side effects, such as secondary damage of normal tissue, subsequent upregulation of growth and angiogenic factors, and the recurrence of neovascularization.

In conclusion, this study supports the hypothesis that treatment with the selective copper chelator, TRIEN, can attenuate inflammation after laser treatment. Photocoagulation treatment caused a significant increase in retinal thickness (edema) that was attenuated in animals treated with TRIEN. The number of total macrophages and microglial cells after laser photocoagulation in the retinal tissues was also reduced in animals treated with TRIEN. By reducing the number of activated macrophages and microglia, the inflammatory response associated with photocoagulation laser treatment may be minimized. It is proposed that attenuation of the inflammatory events after photocoagulation laser treatment may, in turn, attenuate the release of growth factors and cytokines and thus minimize recurrent neovascular events associated with photocoagulation laser treatment in the eye [15, 16].

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Fig. 1.

Effect of TRIEN on the generation of $\cdot\text{OH}$ from Cu^{2+} -catalyzed cysteine autoxidation. The reaction mixture included 1 mM TRIEN, $0.2\ \mu\text{M}\ \text{Cu}^{2+}$, $100\ \mu\text{M}$ cysteine (CSH), and 1 mM CCA in PBS. The control mixture contained all components except TRIEN. The reaction conditions were pH 7.4 and 37°C in a humidified atmosphere of 100% air. Fluorescence was measured 4 h after the reaction began (* $p < 0.01$, Student's t -test)

Fig. 2.

a–c. Histology of retinas at 24 h after photocoagulation treatment in rats. Argon dye laser irradiation at 514 nm wavelength was delivered through a slit lamp using a setting of 50 μ m diameter, 0.1 s duration and 150 mW intensity. **a** Cross section of a normal rat eye (*N*). **b** Cross section of a laser lesion from an eye of a saline-injected animal (*LS*). **c** Cross section of a laser lesion from an eye of a TRIEN-treated animal (*LT*) ($\times 10$, scale bar 100 μ m). **d** Retinal thickness 24 h after photocoagulation in animals treated with TRIEN (*LT*) was compared with the retinal thickness in saline-injected animals (*LS*) and with the thickness of normal retina (*N*). Argon dye laser irradiation at 514 nm wavelength was delivered through a slit lamp. A total of six laser spots were placed separately using a setting of 50 μ m diameter, 0.1 s duration and 150 mW intensity. The retinal thickness of eyes from saline-injected animals (*LS*) is greater than that of normal (*N*) retina. Retinal thickness of eyes from TRIEN-treated animals (*LT*) is greater than that of normal retina (*N*), and significantly less than that of saline-treated animals (*LS*) ($p < 0.05$, Student's *t*-test)

Fig. 3.

a, b Light microscopic photographs of cross sections of retinas at the laser site 24 h after photocoagulation treatment showing ED-1 immunostaining. Labeling is red due to the AEC chromogen. **a** Retina of a saline-injected animal (*LS*) shows numerous ED-1-positive cells (*arrows*). **b** Retina of a TRIEN-treated animal shows significantly fewer macrophages and microglial cells (*arrows*). Sections were counterstained with hematoxylin (purple) ($\times 40$, scale bar 20 μm). **c** Number of ED-1 immunoreactive cells in the retinas of animals in the LS and LT groups 24 h after photocoagulation treatment. Argon dye laser irradiation at 514 nm wavelength was delivered through a slit lamp using a setting of 50 μm diameter, 0.1 s duration and 150 mW intensity. The inflammatory response within the lesions was measured by taking the mean number of ED-1-positive cells in four $\times 40$ fields-of-view. The mean number of ED-1-positive profiles was less in the retinas of TRIEN-treated animals (*LT*) than in those of saline-injected (*LS*) animals ($*p < 0.05$, Student's *t*-test)

Fig. 4. Copper-chelators inhibit free radical formation in the extracellular matrix

Proposed mechanism showing the central role of copper in inflammation-induced tissue damage. Many factors, such as trauma, chemicals, infection, UV radiation, cause inflammatory reactions. Neutrophils and phagocytes can generate •O₂⁻ by oxidase during a process known as the respiratory burst. Further converting of •O₂⁻ to H₂O₂ and •OH needs electron transfer from reducing agents, such as cysteine, glutathione. Copper ion is the major catalyst mediating the electron transfer. •OH generated during inflammation is the major radical that causes bactericidal effect and tissue damage. •OH can also induce cell proliferation. Copper chelator can inhibit the catalyzing effect of copper and exerts its antiinflammation effect by preventing the further formation of •OH from •O₂⁻