

# Effects of Tetrathiomolybdate in a Mouse Model of Retinal Neovascularization

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**PURPOSE.** To determine the effects of tetrathiomolybdate (TM), a copper-chelating agent, on retinal angiogenesis and vascular endothelial growth factor (VEGF) in a mouse model of retinal neovascularization.

**METHODS.** Postnatal day (P)7 C57BL/6N mice were exposed to 75% ± 2% oxygen for 5 days (P7–P11) and then returned to room air for 5 days (P12–P17) to induce retinal neovascularization. Beginning on P10 or P12, mice received daily intraperitoneal injections of TM or phosphate-buffered saline (PBS; control) through P17. Retinal neovascularization was examined by fluorescein dextran angiography after 5 days in room air and was quantitated histologically by counting the neovascular endothelial cell nuclei anterior to the inner limiting membrane. TM's effects on VEGF expression were measured by ELISA.

**RESULTS.** TM-treated and control animals demonstrated comparable regions of retinal nonperfusion. Retinas from control mice at P17 contained neovascular tufts at the junction between perfused and nonperfused retina. The tufts contained numerous neovascular nuclei. Retinas from mice treated with TM beginning on P10 (2 days before returning to room air), but not P12, demonstrated a 41% reduction in neovascular cell nuclei compared with control mice ( $P < 0.01$ ). The P10-treated mice also demonstrated a 24% reduction of VEGF compared with control animals ( $P = 0.01$ ).

**CONCLUSIONS.** TM significantly inhibits retinal neovascularization and VEGF production in a mouse model of retinal neovascularization. (*Invest Ophthalmol Vis Sci.* 2005;46:299–303) DOI:10.1167/iovs.04-0180

Retinal neovascularization contributes to vision loss in many ischemic retinal diseases, including retinopathy of prematurity, proliferative diabetic retinopathy, retinal vein occlusion, and sickle cell retinopathy. Retinal angiogenesis results from the production of ocular-derived growth factors, cytokines, and cell adhesion molecules that promote angiogenesis<sup>1–7</sup> through complex interactions that are not fully understood.

Originally described as a strong inducer of angiogenesis in a corneal pocket model,<sup>8</sup> copper is now known to play multi-

ple important roles in angiogenesis.<sup>9–12</sup> It activates many proangiogenic molecules, including ceruloplasmin<sup>9</sup> and prostaglandin E,<sup>10</sup> resulting in increased angiogenic activity. Copper also has differential effects on many of the major proangiogenic cytokines that have been identified in human retinal or choroidal neovascularization.<sup>1,2,13–16</sup> These effects include the induction of vascular endothelial growth factor (VEGF) and interleukin (IL)-8 and the inhibition of basic fibroblast growth factor (bFGF) activity.<sup>17–22</sup>

In vitro, copper has been shown to have direct effects on vascular endothelial cells by increasing their adhesion and migration,<sup>23</sup> as well as by stimulating endothelial cell proliferation.<sup>24</sup> It also affects the composition of the extracellular matrix in vitro, by inducing fibronectin production<sup>25</sup> and by modulating matrix metalloproteinase (MMP)-2.<sup>26</sup> Copper complexed with the matrix-derived tripeptide glycyl-histidyl-lysine stimulates MMP-2 production and the secretion of tissue inhibitor of metalloproteinase (TIMP)-1 and -2, thereby promoting extracellular matrix remodeling during angiogenesis. With reduced copper levels, the function of many proangiogenic molecules may be reduced, preventing or abating neovascularization.

Tetrathiomolybdate (TM) is a copper-complexing drug that has two mechanisms of action to reduce copper stores and availability. The first involves the formation of a tripartite complex together with food proteins and dietary copper in the gastrointestinal tract, thus blocking copper absorption.<sup>27,28</sup> The second involves the ability of absorbed TM to form an inactive tripartite complex with copper and albumin in blood, rendering the copper unavailable for cellular uptake and therefore preventing it from participating in angiogenesis. TM is a powerful copper complexing agent that is well tolerated when taken orally. It was developed for the treatment of Wilson's disease, an autosomal recessive disease of copper transport that results in abnormal copper accumulation and toxicity.<sup>27,28</sup>

In this study, we evaluated whether this unique copper chelator, TM, could suppress the development of retinal neovascularization in a murine model of ischemic retinopathy. The effect of TM on the production of VEGF, a key growth factor in retinal neovascularization, was also evaluated.

## MATERIALS AND METHODS

Ammonium tetrathiomolybdate (TM) was kindly prepared and provided by Dimitri Coucouvanis (Department of Chemistry, University of Michigan) and stored in argon at 4°C until use.

## Animal Model of Proliferative Retinopathy

All experimental procedures involving animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A reproducible model of ischemia-induced retinal neovascularization has been described in detail.<sup>5</sup> Briefly, on postnatal day (P)7, litters of C57BL/6N mice pups with their mothers were exposed to 75% ± 2% oxygen (hyperoxia) for 5 days and then returned to room air for 5 days, producing retinal ischemia and neovascularization by P17. Mice of the

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same strain and of the same age were kept in room air and used as control subjects (normoxia).

### TM Treatment

Mice were injected intraperitoneally with 0.2 mg TM in 0.25 mL phosphate-buffered saline (PBS) per day. In some experiments, mice received TM on P12 through P17. In other experiments, animals received TM through daily intraperitoneal injections on P10 through P17. These animals are called "pretreatment TM," because they were pretreated with TM 2 days (P10–P12) before exposure to room air. Control animals of the same age and same hyperoxia exposure were injected with 0.25 mL PBS alone instead of TM.

### Angiography with High-Molecular-Weight Fluorescein-Dextran

Control and TM-treated P17 mice were deeply anesthetized by intraperitoneal (IP) injections into the cardiac left ventricle of pentobarbital sodium and 80  $\mu$ L PBS containing 20 mg fluorescein isothiocyanate-dextran. The eyes were enucleated, and the retinas were dissected and flatmounted on microscope slides with glycerol-gelatin, for examination with a fluorescence photomicroscope.

### Quantitation of Neovascularization

Control and TM-treated P17 mice were killed by IP injections with an overdose of pentobarbital sodium. Their eyes were enucleated, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Six-micrometer axial step sections of each retina were obtained 30  $\mu$ m apart, starting at the optic nerve head. The sections were stained with hematoxylin and eosin. To determine the extent of retinal neovascularization we counted all retinal neovascular cell nuclei anterior to the internal limiting membrane (ILM), along equal lengths of each step section, with a masked protocol. Averaging of the 10 counted sections yielded a mean number of neovascular cell nuclei per section per eye.<sup>5</sup> No neovascular cell nuclei anterior to the ILM were observed in normoxic control animals.

### ELISA for VEGF

VEGF levels were measured with an ELISA kit for VEGF (R&D Systems, Minneapolis, MN), as previously described.<sup>29</sup> Retinas were removed from mice on P17. Each retina was immersed in 500  $\mu$ L lysis buffer containing 20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM sodium molybdate, 1 mM EDTA (pH 6.8), and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). They were then stored at  $-80^{\circ}\text{C}$  until use. When used, the samples were thawed, homogenized (Polytron homogenizer; Kinematica AG, Littau-Lucerne, Switzerland), sonicated for 30 seconds, and clarified by centrifuging at 150 g for 10 minutes. The clarified retinal lysates were then evaluated with ELISA.

Total protein was determined with a Coomassie-plus protein assay reagent kit (Endogen; Pierce Biotechnology, Rockford, IL). The sensitivity of this assay was 3 pg/mL.

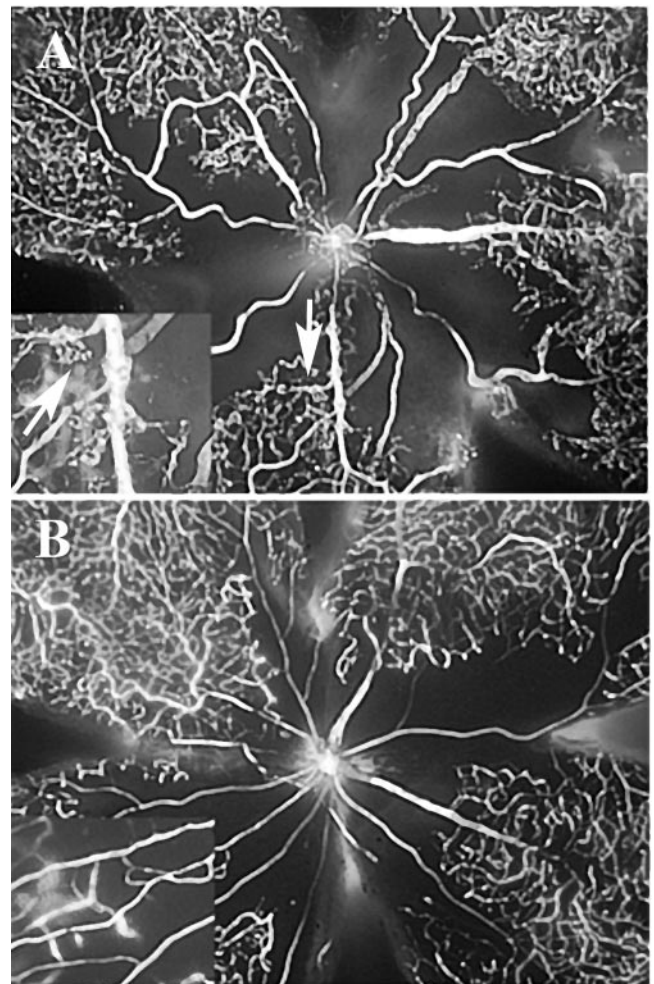
### Statistical Analysis

Data are presented as the mean  $\pm$  SD. The significance of differences was evaluated by the Kruskal-Wallis test. All  $P \leq 0.01$  were considered statistically significant.

## RESULTS

### Fluorescein Angiographic Assessment of the Effect of TM on Retinal Neovascularization

Flatmount retinal preparations were examined after injection of fluorescein-dextran, to study the effect of TM on ischemia-induced retinal neovascularization. P17 TM-treated and control mice subjected to hyperoxia demonstrated no perfusion of

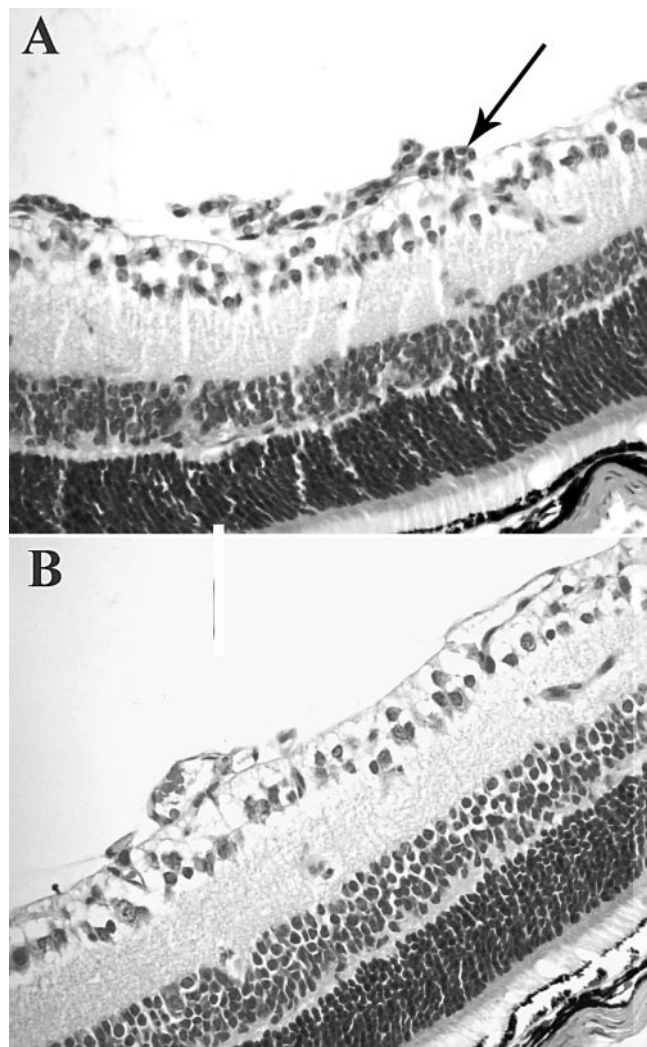


**FIGURE 1.** Angiographic analysis of the effect of TM on retinal neovascularization. Retinal flatmounts were examined by fluorescein dextran angiography. (A) Retina from the PBS-treated, hyperoxia-exposed P17 control animal. A neovascular response was visible at the junction of the perfused and the nonperfused junction of the retina (arrows and inset). (B) P17 retina from the eye of a hyperoxia-exposed animal treated with 0.2 mg TM from P10 to P17 (pretreatment TM). Few neovascular tufts were apparent.

normal, fine peripapillary retinal capillaries, whereas the larger, well-developed radial retinal vessels extending from the optic disc remained perfused (data not shown). Retinas from P17 control mice exposed to hyperoxia contained neovascular tufts extending from the surface of the retina at the junction between the perfused and nonperfused retina (Fig. 1A). In contrast, retinas from P17 mice treated with TM from P10 to P17 demonstrated markedly reduced neovascular tissue, despite the presence of comparable peripapillary regions of non-perfusion (Fig. 1B).

### Assessment of Effects of TM by Histologic Quantitation of Retinal Neovascularization

Retinal neovascularization was also assessed histologically by counting the endothelial cell nuclei (neovascular nuclei) anterior to the ILM. Control retinas of P17 normoxic mice did not contain nuclei anterior to the ILM (data not shown). Retinas from P17 PBS-treated control mice subjected to hyperoxia contained multiple neovascular tufts on the surface (Fig. 2A), with some extending into the vitreous. These tufts contained a



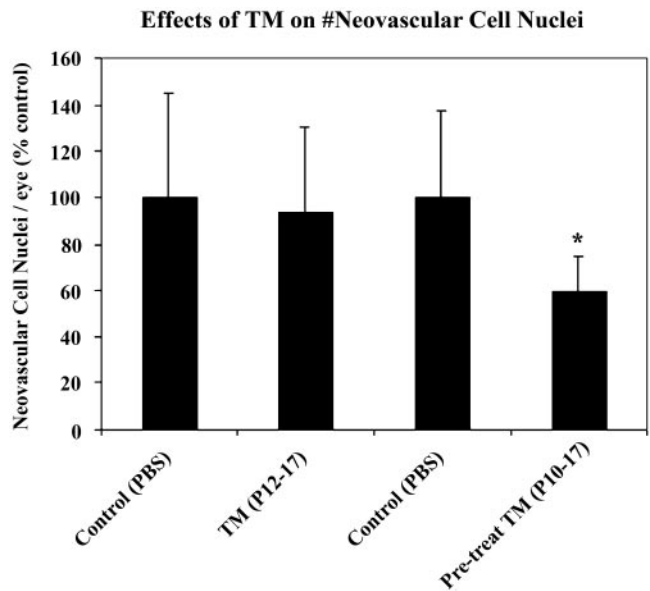
**FIGURE 2.** Histologic demonstration of TM inhibition of ischemia-induced retinal neovascularization. (A) Histologic section of P17 retina from PBS-treated control eye of mouse exposed to hyperoxia. Extensive preretinal neovascular tufts were apparent (arrow). (B) Histologic section of P17 retina from pretreatment TM eye. A marked reduction in preretinal neovascularization was apparent compared with that in the PBS-treated control eye.

significant number of neovascular nuclei anterior to the ILM (Figs. 2A, 3).

Retinas from hyperoxia-exposed, P17 mice treated with TM beginning on P12 demonstrated no significant differences in the number of neovascular nuclei anterior to the ILM compared to control mice from the same litters ( $P = 0.84$ ; Fig. 3, left hand columns). However, retinas from hyperoxia-exposed P17 mice treated with TM beginning on P10, 2 days before returning to room air (pretreatment TM), demonstrated fewer preretinal neovascular tufts (Fig. 2B) and 41% fewer neovascular cell nuclei anterior to the ILM, compared with control mice from the same litters ( $P < 0.01$ ; Fig. 3, the two right histograms).

**Effect of TM on VEGF in Mouse Retinas with Oxygen-Induced Ischemic Retinopathy**

VEGF plays a critical role in the pathogenesis of retinal neovascularization in several ischemic injury models.<sup>2,30</sup> ELISA was performed to determine the protein levels of VEGF in

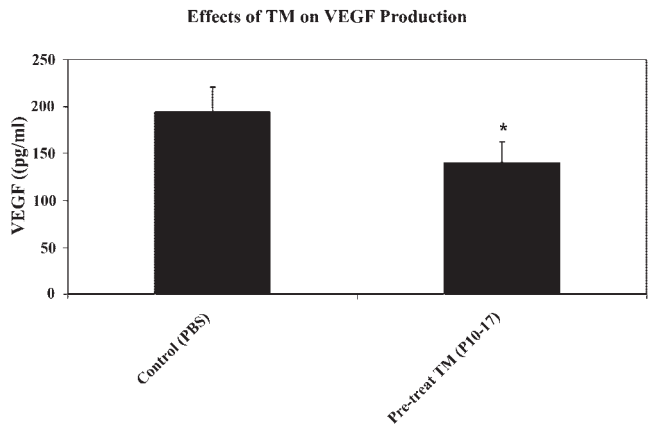


**FIGURE 3.** The number of neovascular nuclei anterior to the ILM in 6- $\mu$ m retinal sections. *Two left histograms:* data from animals treated with TM on P12 to P17 and the data from corresponding PBS control animals. No significant difference in the number of nuclei anterior to the ILM was noted between the TM-treated and control animals ( $P = 0.84$ ). *Two right histograms:* data from animals treated with TM on P10 to P17 (pretreatment TM) and the corresponding data from PBS-treated control mice. A significant reduction in the number of neovascular nuclei anterior to the ILM ( $*P < 0.01$ ) was found in animals pretreated with TM. The number of neovascular nuclei in control mice for P12 to P17 and P10 to P17 TM treatments were  $49 \pm 22$  and  $56 \pm 21$  nuclei/section, respectively ( $P = 0.58$ ). Data shown are % PBS-treated control (mean %  $\pm$  SD).

retinas removed from P17 mice treated with PBS (control) or pretreated with TM on P10 to P17. As shown in Figure 4, TM-pretreated mice had 24% lower levels of VEGF than did littermate control animals ( $140 \pm 22$  vs.  $194 \pm 26$  pg/mL;  $P = 0.01$ ).

**DISCUSSION**

TM was initially developed for the treatment of Wilson’s disease, an inherited disorder in which abnormally high levels of



**FIGURE 4.** Effect of TM on VEGF levels in retinal lysates in a mouse retinal neovascularization model. ELISA demonstrated  $194 \pm 26$  pg/mL VEGF in retinas of PBS-treated P17 mice, whereas mice receiving TM on P10 and P17 (pretreatment TM) had significantly less VEGF ( $140 \pm 22$  pg/mL).  $*P = 0.01$ . Data are expressed as the mean  $\pm$  SD.

copper accumulate in the body leading to liver and neurologic dysfunction.<sup>31,32</sup> TM has since been found to be a promising antiangiogenic agent for use in cancer therapy. We have tested the antiangiogenic anticancer effect of TM in rodent tumor models and have demonstrated that the antiangiogenic activity is responsible for the antitumor effects.<sup>33-36</sup> TM has been shown to inhibit blood vessel formation in animal models of breast cancer.<sup>34</sup> TM-treated animals were found to have smaller tumors that were more sparsely vascularized than those in nontreated controls.<sup>34</sup> A human clinical trial of TM as an anti-tumor agent has shown encouraging results.<sup>37</sup> In one patient, ultrasound imaging indicated reduced blood flow in a tumor in a TM-treated patient.<sup>37</sup>

A likely mechanism of the inhibition of angiogenesis is the effect of TM on angiogenic mediators,<sup>11,12,33,34,37</sup> particularly VEGF.<sup>34,38</sup> VEGF expression is controlled by several different transcription and hypoxia-inducible factors (HIFs).<sup>39</sup> One mechanism by which TM may have suppressed VEGF by 24% in this study is by its known property of inhibiting the activation of NF- $\kappa$ B,<sup>34,38</sup> a necessary transcription factor for VEGF.<sup>40</sup> TM may also have reduced VEGF by inhibiting NF- $\kappa$ B-dependent expression of HIF-1, a potent VEGF inducer that binds to the promoter region of the VEGF gene.<sup>41,42</sup> Nevertheless, the greater inhibition that we observed in actual retinal neovascularization (41%) may have occurred because NF- $\kappa$ B activation is also essential for the expression of other angiogenic agents, including IL-6, chemokines, MMPs, and cell adhesion molecules.<sup>34</sup>

Retinal neovascularization is a frequent complication of blinding retinal diseases in which retinal ischemia develops, most notably diabetic retinopathy and retinopathy of prematurity. VEGF, produced by retinal glial cells, has been strongly implicated to be a major angiogenic factor in the development of retinal neovascularization in hypoxia-induced retinal neovascularization.<sup>1,2,30</sup> Other factors also have been suggested to contribute to the neovascular response in retinal ischemia.<sup>13,16</sup> Studies of vitreous fluid of patients with diabetic retinopathy have demonstrated significantly increased levels of the chemokine IL-8 in the vitreous fluid of patients with neovascular disease compared with that in patients without the disease.<sup>13,16</sup> Models of retinal neovascularization similar to that used in this study have demonstrated the roles of NF- $\kappa$ B,<sup>5,13</sup> cytokine-induced neutrophil chemoattractant (CINC; functionally equivalent to IL-8 in the rat),<sup>13</sup> and other chemokines<sup>7</sup> in the glial cells of nonperfused retina and neovascular endothelial cells. The levels of these factors correlate with the development and regression of the retinal neovascularization in these models.<sup>13</sup>

Antiangiogenic drugs, particularly inhibitors of VEGF, are currently being investigated as potential therapies for ocular neovascularization.<sup>43,44</sup> In a previous human study, TM failed to inhibit already-developed choroidal neovascularization in age-related macular degeneration.<sup>45</sup> In the present study, however, TM significantly reduced neovascularization only if used before its onset, potentially explaining its failure in the human study of existing choroidal neovascularization.<sup>45</sup> It is possible that TM treatment of patients with incipient neovascularization will be effective in preventing it. In this study, we also found that the reduced retinal neovascularization, at least in part, may be the result of inhibition of VEGF. Elucidation of other mechanisms through which TM acts to inhibit retinal neovascularization, including its effects on other proangiogenic cytokines, deserves further study.

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