Tissue Plasminogen Activator as an Antiangiogenic Agent in Experimental Laser-Induced Choroidal Neovascularization in Mice

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Submitted: March 23, 2016
Accepted: August 27, 2016


PURPOSE. We investigate the antiangiogenic efficacy of tissue plasminogen activator (tPA) on experimental laser-induced choroidal neovascularization (CNV) in mice.

METHODS. After CNV was induced by laser photocoagulation in 92 C57BL/6J wild-type mice, tPA (4 or 40 international units [IU]/µl) or PBS was injected intravitreally immediately after laser injury. Fluorescein angiography was performed on day 7 to grade CNV leakage. The CNV volume was measured by confocal microscopy in eyes enucleated 7 days after laser injury. Immunohistochemical studies were performed 3 days after laser injury to evaluate fibrin/fibrinogen and CD31 expression. The possible adverse effects of tPA were assessed by electroretinography (ERG) and histology on day 7.

RESULTS. Intravitreal administration of tPA significantly suppressed CNV leakage and CNV volume in a dose-dependent manner (P < 0.01). Intravitreal injection of tPA suppressed fibrin/fibrinogen and CD31 expression in laser-induced lesions. Histologic examination and ERG showed no evidence of retinal toxicity in eyes injected with tPA.

CONCLUSIONS. Intravitreal injection of tPA suppressed fibrin/fibrinogen expression and laser-induced CNV. The current results suggested that tPA may be a potential therapeutic adjuvant for treating CNV.

Keywords: choroidal neovascularization, fibrin, tissue plasminogen activator, mice, experimental model

A ge-related macular degeneration (AMD) is a leading cause of blindness in industrialized nations. In exudative AMD, choroidal neovascularization (CNV) develops in the subretinal space, accompanies hemorrhage and/or serous exudation, and often impairs central vision due to irreversible damage in the outer retina. The current standard for treating CNV is anti-VEGF therapy, which dramatically suppresses exudation from CNV and consequently reduces the risk of blindness. However, anti-VEGF therapy requires repeated intravitreal injections of drugs in cases with recurrent or persistent CNV. Moreover, severe visual loss occurs due to subfoveal fibrovascular scar formation or atrophy of the outer retina despite adequate treatments.

Tissue plasminogen activator (tPA) is a fibrinolytic compound used to treat embolic or thrombotic stroke; in Ophthalmology, tPA is used as an adjuvant to displace submacular hemorrhages. The displacement of subfoveal hemmorhages minimizes irreversible damage to photoreceptor cells and RPE cells. In addition, marked regression of subretinal fibrovascular masses occurred immediately after therapy in some eyes with type 2 CNV, possibly associated with the specific efficacy of tPA. Based on these experiences, we recently conducted a pilot study to assess the feasibility of combination therapy with intravitreal ranibizumab (Lucentis; Genentech Inc., South San Francisco, CA, USA) and tPA (Eisai, Inc., Tokyo, Japan) in eyes with subfoveal type 2 CNV secondary to exudative AMD and confirmed prompt, marked regression of fibrinous tissue and contraction of the fibrovascular tissue complex with additional tPA injections. However, it is unknown if tPA is effective for newly developed CNV or established fibrovascular tissue complexes.

In the current study, we evaluated the impact of intravitreal tPA monotherapy on experimental laser-induced CNV in mice to investigate mechanisms affecting the specific efficacy of tPA.

MATERIALS AND METHODS

Animals and Anesthesia

Male wild-type C57BL/6J mice (Japan SLC, Shizuoka, Japan) between 6 and 8 weeks of age were used to minimize variability. The Nagoya City University Animal Care and Use Committee approved the study protocol. All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We anesthetized 92 mice with an intraperitoneal injection of 10 µl/g of 2.5% 2,2,2-tribromoethyl alcohol and tertiary amyl alcohol (Avertin; Sigma-Aldrich Corp., St. Louis, MO, USA) and the pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydriar-P; Santen Pharmaceutical, Osaka, Japan). The mice were killed with an overdose of intravenous pentobarbital sodium.
Murine Laser-Induced CNV Model

Laser photocoagulation spots (Elite Ultra 532 nm Laser System; Lumenis, Salt Lake City, UT, USA) were applied using the following settings: 100-μm diameter, 100-ms duration, and 200-mW intensity, consistently inducing bubble formation, which suggested a break in Bruch’s membrane. On day 0, four to six laser spots were irradiated between the major retinal vessels in the right eye as described previously. To enhance reproducibility, one investigator masked to the drug group assignment of the animals performed laser photocoagulation. Laser burns without bubble formation or with bleeding were excluded. The following experiments were designed to be completed within 1 week, because spontaneous regression of CNV might be observed 1 to 2 weeks after laser photocoagulation.

Intravitreal Injection of tPA

Tissue plasminogen activator was dissolved in PBS (Sigma-Aldrich Corp.). Either tPA (4 or 0 international units [IU]/μl) in a dose equivalent to that used clinically in human eyes or the same volume of PBS was injected intravitreally into the vitreous cavity using a 33-gauge needle (Ito Corporation, Tokyo, Japan) immediately after laser photocoagulation.

Fluorescein Angiography

Fluorescein angiography was performed using a fundus camera with a 20-diopter lens (TRC-50AX; Topcon, Tokyo, Japan) or the Optos 200Ttx imaging system (Optos, Dunfermline, Scotland) 7 days after laser photocoagulation. Fluorescein angiograms were captured after intraperitoneal injection of 0.1 mL of 1% fluorescein sodium (Alcon, Tokyo, Japan). Three of 42 eyes (14 eyes in each group) were excluded because of cataract formation. As a result, 144 of 176 spots (81.8%) were available for analysis.

An operator masked to the treatment group assignments graded the lesions as described previously using an ordinal scale based on the spatial and temporal evolution of fluorescein leakage in which 0 (no leakage) indicated no leakage or faint or mottled fluorescence without leakage, 1 (questionable leakage) indicated a hyperfluorescent lesion with no progressive increase in size or intensity, 2 (leakage) indicated hyperfluorescence increasing in intensity but not size with no definite leakage, and 3 (pathologically significant leakage) indicated hyperfluorescence increasing in intensity and size with definite leakage.

Measurement of CNV Volume

One week after laser photocoagulation, 24 eyes (8 eyes in each group) were enucleated and fixed with 4% paraformaldehyde. The eyecups obtained by removing the anterior segments were incubated with 0.5% fluorescein-isothiocyanate-isolectin B4 (Vector Laboratories, Burlingame, CA, USA) for 3 hours at room temperature. Nuclei were counterstained in 4,6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories). Sections were incubated with appropriate secondary antibodies (Alexa Fluor488 for CD31 and Alexa Fluor546 for fibrinogen; Invitrogen, Carlsbad, CA, USA) for 3 hours at room temperature. The sections were visualized using a confocal microscope (A1-Nikon; Nikon, Melville, NY, USA). Primary antibody omission or substitution with an irrelevant antibody of the same species and staining with chromogen alone served as negative controls.

Electroretinography (ERG) and Histology

To evaluate the possible functional and morphologic adverse effects of tPA, ERG and histologic analysis were performed 7 days after application of laser photocoagulation in eyes treated with intravitreal tPA (40 IU; n = 8) or PBS (n = 8). The mice were adapted to darkness overnight. Under anesthesia, a contact lens electrode embedded with gold wire was placed on the cornea as an active electrode (Mayo Corporation, Inazawa, Japan), and a needle reference electrode was inserted subcutaneously above the neck. A grounded stainless steel clip electrode was placed on the tail. Body temperature was maintained at 37°C with a heating pad. The ERG was recorded from eyes using the Stand-Alone Ganzfeld System (SG-2002; LKC Technologies, Inc., Gaithersburg, MD, USA). Responses were amplified 10,000 times and band-pass filtered from 0.3 to 500 Hz using a Bio-Amplifier (ML135; ADInstruments, New South Wales, Australia). The amplified signals were stored in the PowerLab Data Acquisition System (ML820; ADInstruments) with digitized 4000 Hz sampling. A limited number of waveforms (2-32) for each response were averaged considering the proper time interval to avoid exceeded stimulus light adaptation. The ERG was recorded under dark adaptation with increasing stimulus intensity: -4.98, -3.69, -2.64, -1.97, -0.74, and 0.41 log cd·s/m². All intensities were measured by a calibrated photometer (IL1700; International Light Technologies, Inc., Peabody, MA, USA) and an optical detector (SED033/T/R; International Light Technologies, Inc.). After ERG, the mice were killed and the eyecups were obtained in the same manner. Cryostat sections were stained with hematoxylin and eosin and observed by light microscopy.
Statistical Analysis
All results were expressed as the means ± SEM. The values were analyzed using the Fisher’s exact test, the 1-way ANOVA, or the unpaired t-test. \( P < 0.05 \) was considered significant.

RESULTS

Suppression of Fluorescein Leakage from CNV by Intravitreal Injection of tPA
One week after laser photocoagulation, pathologically significant leakage (grade 3) developed at most laser spots in mice injected with PBS and 4 IU of tPA; however, there were significantly \( (P < 0.01) \) fewer spots in eyes injected with 40 IU of tPA (Fig. 1).

Reduction of CNV Volume by Intravitreal Injection of tPA
One week after laser photocoagulation, intravitreal injection of tPA reduced the CNV volume in a dose-dependent manner. The CNV volume in the group injected with 40 IU of tPA was 89,793 ± 19,440 \( \mu \)m\(^3\), which was significantly \((P < 0.05)\) smaller than 228,017 ± 50,080 \( \mu \)m\(^3\) in the group injected with 4 IU of tPA and 264,273 ± 65,431 \( \mu \)m\(^3\) in the group with PBS. However, there was no significant difference in the CNV volume between groups injected with 4 IU of tPA and PBS (Fig. 2).

Immunohistochemistry
Immunostaining showed that intravitreal injection of tPA (40 IU) reduced the fibrin/fibrinogen expression and CD31-positive cells at the site of laser application (Fig. 3).

ERG and Histology
Electroretinography and histology were performed 7 days after laser photocoagulation to investigate any toxicity related to tPA (Fig. 4). Electroretinography showed that the a-waves (photoreceptor function) and b-waves (inner retinal function) in the tPA-injected eyes did not decrease significantly compared to those in the PBS-injected eyes. No histologic differences in hematoxylin and eosin staining were seen between the tPA-injected eyes and the PBS-injected eyes.

DISCUSSION
We investigated the efficacy of tPA on experimental laser-induced CNV in mice and showed that intravitreal injection of tPA significantly suppressed laser-induced CNV. Both doses of tPA used in this study were equivalent to those used clinically to displace submacular hemorrhages secondary to AMD.
Choroidal neovascularization has been reported to develop in fibrin in exudative AMD. Tissue plasminogen activator is a serine protease that catalyzes the conversion of inactive plasminogen into plasmin, a broadly acting enzyme that can degrade a variety of extracellular matrix proteins and activate metalloproteinases and growth factors. In our clinical pilot study, combination therapy with intravitreal ranibizumab and...
FIGURE 2. The impact of tPA on CNV volume. (A) Representative microscopic images of CNV in mice treated with PBS or 4 or 40 IU of tPA. Scale bars: 100 μm. (B) The CNV volume decreases significantly after intravitreal injection of 4 and 40 IU of tPA, compared to that in the PBS group. The data are expressed as the means (solid square) ± SEM (open circles: individual values). **P < 0.01, *P < 0.05 (1-way ANOVA).

FIGURE 3. The impact of tPA on expression of fibrin/fibrinogen and CD31 in an experimental CNV model. (A–D) Representative images of hematoxylin-eosin staining and immunohistochemical analyses of fibrin/fibrinogen and CD31 in the PBS-treated group and (E–H) the tPA 40 IU-treated group. (F–H) The tPA-treated group has lower fibrin/fibrinogen (red and arrows) and CD31 (green) expression compared to (B–D) the PBS group. Scale bars: 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
tPA caused marked regression of subretinal fibrinous tissue in type 2 CNV even in cases refractory to previous repeated intravitreal ranibizumab injections. In the current study, tPA monotherapy reduced expression of fibrin/fibrinogen and CD31 at the site of laser injury. These results suggested that fibrin may have an important role in CNV development and that fibrinolysis by tPA may suppress CNV development and progression.

However, the role of endogenous tPA in angiogenesis is complicated. Rakic et al. reported expression of tPA in CNV membranes excised surgically from human eyes with exudative AMD and murine laser-induced CNV. Another study reported that the levels of intraocular tPA were associated with proliferative diabetic retinopathy. Urokinase-type plasminogen activator (uPA) and tPA are essential for degrading the basement membrane of the endothelial cells as the primary step in angiogenesis. However, plasminogen activator inhibitor-1 (PAI-1) also is a key regulator of angiogenesis by maintaining the extracellular matrix for sprouting of endothelial cells in the subsequent steps of angiogenesis. Ratel et al. showed that VEGF-mediated angiogenesis involves a complex interplay between the matrix metalloproteinases and plasmin-mediated proteolytic systems in a three-dimensional fibrin matrix model. Lambert et al. reported that PAI-1 exhibited dose-dependent pro- and antiangiogenic effects in a laser-induced CNV model of control and PAI-1-deficient mice. Thus, the PA/PAI-1 system regulates angiogenesis by elaborate balancing of the expression of PA and PAI-1 in each step. Therefore, inhibition and overexpression of PA or PAI-1 might exhibit antiangiogenic effects in vascular remodeling, tissue regeneration, and tumoral growth. In the current study, fibrinolysis with excess dosing of tPA may impair the PA/PAI system, resulting in inhibition of CNV development.

Previous studies have shown that endothelial cell migration is not impaired severely by inhibition of endogenous plasminogen, uPA, or tPA in fibrin gels; the aortic ring model; and a model of tumoral angiogenesis induced by implantation of malignant keratinocytes. Those results may have occurred as the result of a variety of enzymes involving uPA, tPA, and other metalloproteinases that work complementarily to execute

**FIGURE 4.** Histology and ERGs from eyes with and without injection of tPA. (A) Hematoxylin-eosin stained sections show that tPA-treated eyes are not histologically different from PBS-treated eyes. Scale bars: 50 μm. (B) A representative ERG record from PBS-treated eyes and eyes treated with 40 IU of tPA. (C) The ERG shows that a-waves (photoreceptor function) and b-waves (inner retinal function) from tPA-treated eyes are not significantly reduced compared to those from PBS-treated eyes. N.S., not significant.
angiogenesis. Therefore, a reasonable therapeutic strategy is that fibrin has an important role as a foothold in adhesion, and migration of endothelial cells is lysed by exogenous tPA.

Although tPA has been used clinically as an adjuvant for displacing submacular hemorrhages and has no serious adverse effects on the retina, potent retinal toxicity has resulted from intravitreal tPA in rabbit and cat eyes. Although, in the current study, ERG recordings and histologic analyses showed that 40 IU of tPA, which is equivalent to the dose used clinically, did not induce remarkable retinal toxicity in mice, there may be a trend of the decrease of a-waves. This issue should be addressed carefully in the future study. Nevertheless, tPA already has been used clinically, showing no serious adverse effects.

These results supported the idea that tPA may have a potential advantage as an adjuvant therapy not only for displacing submacular hemorrhages but also for treating CNV. Although further investigations are needed to determine the safety of tPA on the retina, a new therapeutic strategy using tPA as an adjuvant for exudative CNV may be beneficial.

In summary, the current study showed that exogenous tPA reduced the expression of fibrinogen/fibrin at the site of laser-induced CNV in mice and suppressed CNV. Fibrin may have a pivotal role as an alternative extracellular matrix that bridges the loosened intercellular space around highly permeable vessels and provides a scaffold for endothelial cells to migrate and proliferate. Fibrinolysis by exogenous tPA may disrupt the fibrin scaffold and possibly interfere with migration and proliferation and the subsequent steps in angiogenesis. Tissue plasminogen activator may have a potential role as an adjuvant therapy for CNV secondary to AMD.

**Acknowledgments**

Supported by a 2012 Grant-in-Aid for Scientific Research No. 25462758 from Japan Society for the Promotion of Science.

Disclosure: D. Ozone, None; T. Mizutani, None; M. Nozaki, None; M. Ohbayashi, None; N. Hasegawa, None; A. Kato, None; T. Yasukawa, None; Y. Ogura, None

**References**


Tissue Plasminogen Activator Suppresses CNV


