·Basic Research·

Suppression of laser-induced choroidal neovascularization by intravitreal injection of tristetraprolin

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Abstract

• AIM: To examine the effect of intravitreal adenoviral vector-mediated tristetraprolin (Ad-TTP) on VEGF mRNA expression in a rat model of laser-induced choroidal neovascularization.

• METHODS: Ad-TTP was prepared using a commercial kit. Retinal laser-induced photocoagulation (10 spots per eye) was performed on rats in this experimental choroidal neovascularization (CNV) model. Rats were divided into four groups: control (single intravitreal injection of balanced salt solution, *n*=10), laser-induced CNV (photocoagulation only, *n*=20), laser-induced CNV plus Ad-TTP injection (photocoagulation plus a single intravitreal Ad-TTP injection, *n*=20) and Ad-TTP injection only (*n*=10). Changes in choroidal morphology were evaluated in ten rats in the laser only and the laser plus Ad-TTP groups. Two weeks after laser injury, the size of CNV was calculated by perfusion with high-molecular-weight fluorescein isothiocyanate (FITC)-dextran. VEGF mRNA expression in retina-choroid tissue from ten rats in each group was measured by reverse transcription polymerase chain reaction (RT-PCR).

• RESULTS: Two weeks after treatment, the area of laser-induced CNV was reduced by approximately 60% in the rats given the Ad-TTP injection compared with that in the laser-only group. There was a tendency toward decreased VEGF mRNA expression in the Ad-TTP injection groups.

• CONCLUSION: A single intravitreal injection of Ad-TTP significantly suppressed CNV size in this experimental laser-induced CNV model. Ad-TTP injection also decreased VEGF mRNA expression compared with that in the laser-induced CNV group. The present study is meaningful as the first study to investigate the effect of tristetraprolin delivered *via* intravitreal injection.

• **KEYWORDS:** laser-induced choroidal neovascularization; tristetraprolin; adenoviral vectors
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INTRODUCTION

Anti-vascular endothelial growth factor (VEGF) antibodies are widely used to suppress choroidal neovascularization (CNV)[1,2]. Ranibizumab and bevacizumab are monoclonal anti-VEGF antibodies, which inhibit VEGF by binding to a VEGF receptor [3]. The anti-angiogenic effect of anti-VEGFs has been confirmed by extensive research. However, anti-VEGFs have several limitations: multiple injections are required and some patients do not respond[4,5]. New approaches to therapy are needed to overcome these limitations.

Experimental CNV model have been used to test new drugs[6,7]. Laser photocoagulation selectively ablates the photoreceptor outer segments, retinal pigment epithelium, choriocapillaris, and anterior choroid [8-12]. The subsequent wound response includes the ingrowth of fibroblasts, retinal pigment epithelium, and vascular endothelial cells to form a defined neovascular lesion. Honda *et al* [13] found that laser photocoagulation of 6 spots per eye (wavelength, 532 nm; power, 90 mW; spot diameter, 100 μm) induced CNV in animal models. Previous studies characterized these infiltrating cell types and identified the expression of VEGF and other proteins typically associated with new blood vessel growth[8-11]. Edelman and Castro[12] reported that the method of fluorescein isothiocyanate (FITC)-dextran labeling of CNV was reproducible and quantifiable, and concluded that it may accelerate the discovery of therapeutic interventions to treat CNV. Recently, Honda *et al* [13] reported a laser-induced CNV model (wavelength, 532 nm; power, 90 mW; spot diameter, 100 μm), which they used to evaluate the ability of

liposomal SU5416 (an angiogenesis inhibitor) to inhibit the development of experimental CNV in rats. However, this setting is not suitable for humans. For these reasons, present study used the laser-induced CNV model using FITC-dextran labeling for evaluating the effect of tristetraprolin (TTP).

Expression of VEGF is regulated through both transcriptional and post-transcriptional mechanisms [14]. VEGF transcription is activated by growth factors and hypoxia[15-17]. The induction of VEGF is transient and returns to baseline levels *via* post-transcriptional regulation [18]. VEGF transcripts contain AU-rich elements (AREs) within their 3′ untranslated regions (3' UTR), which determine mRNA stability [19]. ARE-mediated post-transcriptional regulation is facilitated by trans-acting ARE-binding proteins, which form stable complexes with the 3' UTR and regulate the stability of VEGF mRNA[20-22]. Therefore, the relative abundance of these ARE-binding proteins determines the levels of VEGF transcripts[8]. The human antigen-R (HuR) protein promotes the stabilization of VEGF mRNA, and overexpression of HuR results in increased VEGF expression [21,23]. Conversely, B-related factor 1 (BRF1) and TTP destabilize VEGF mRNA and decrease expression of the VEGF gene product[22,24]. TTP is a 34 kDa member of the three conserved cysteine residues and one histidine residue (CCCH) class of tandem zinc finger proteins and is expressed in various tissues [25]. TTP was first shown to interact with the ARE from TNFα mRNA, and its list of known and likely mRNA targets continues to grow[26]. Several studies used diverse cell and/or tissue models to show that TTP inhibits VEGF production by destabilizing VEGF mRNA, and that reduction of TTP expression may be responsible for the increase in VEGF levels observed in various human cancers[27-30]. Adenoviruses (AV) are the most common vectors used in human cancer studies, especially for cancer gene therapy strategies based on intratumoral injections [31]. In addition, AV has been widely used for gene transfer to the eye [32-35]. A number of reports showed delayed retinal degeneration in rd mice (Homozygous for the retinal degeneration 1 mutation, Pde6brd) following the subretinal delivery of AV and AV-encapsulated vectors carrying a gene encoding either phosphodiesterase β-subunit (β-PDE) or proto-oncogene in follicular B cell lymphoma 2 (Bcl-2), or the intravitreal delivery of an adenoviral (AV) vector carrying a gene encoding ciliary neurotrophic factor (CNTF)[32-35]. AV are easy to manipulate, and they have a relatively good safety profile and a greater survival rate than other therapeutic vectors[31].

The aim of the present study was to examine the effects of intravitreal administration of adenoviral vector-mediated tristetraprolin (Ad-TTP), and its influence on VEGF mRNA expression in a rat model of experimental laser-induced CNV.

MATERIALS AND METHODS

Animals Sexually mature white male Sprague-Dawley (SD) rats (200-250 g; Samtaco, Osan, Korea) were maintained in cages under 12h light and 12h dark conditions. A total of 60 rats were divided into four groups: control (10 rats), laser-induced CNV (20 rats) only, laser-induced CNV followed by Ad-TTP injection (20 rats) and Ad-TTP injection without laser treatment (10 rats). The morphologic changes following laser treatment were evaluated in ten rats in the laser-only group and the laser-treated plus Ad-TTP injection group. All experiments adhered to the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmologic and Vision Research and the Gyeongsang National University guidelines for care and use of laboratory animals[36].

Preparation of Adenoviral Vector and Tristetraprolin An adenoviral vector harboring TTP was prepared using the ViraPower™ Adenoviral Expression System kit (Invitrogen, Carlsbad, CA, USA).The kit includes a destination vector for cloning the DNA sequence of interest and the 293A cell line used to facilitate production of the adenovirus. The ViraPower™ Adenoviral Expression System is suitable for *in vivo* gene delivery applications [37,38]. Many groups have successfully used AV to express a target gene in a multitude of tissues, including skeletal muscle, lung, heart, and brain [36,38-40]. The published methods and the recommended protocol were followed [41]. Briefly, the adenoviral expression clone containing the TTP-mCherry DNA (used to visualize the location of Ad-TTP) was generated. Next, the 293A producer cell line was transfected with the adenoviral expression clone and the adenovirus-containing cells were harvested by washing them off the culture plate using a tissue culture pipette and transferring them to a sterile, capped tube. After harvesting the adenovirus-containing cells and media, a crude viral lysate was prepared using several freeze/thaw cycles followed by centrifugation. The adenovirus was then amplified by infecting 293A producer cells with the crude viral lysate. The titer of the initial viral stock obtained from the 293A cells generally ranged from 1×10^7 to 1×10^8 plaque-forming units (pfu)/mL. Amplification allowed production of a viral stock with a titer ranging from 1×10^8 to 1×10^9 pfu/mL.

Induction of Choroidal Neovascularization Using a Laser Rats were anesthetized by intraperitoneal injection of 30mg/kg ketamine (Yuhan Ketamine, Yuhan Corporation) and 2.5 mg/kg xylazine (Yuhan Rompun, Yuhan Corporation). Laser-induced CNV models were induced by a widely used method [13,42]. No dilating medications were used. Laser photocoagulation (wavelength, 532 nm; power, 90 mW; spot diameter, 100 μm) was performed in 10 spots per eye (surrounding the optic disc) using a slit delivery system (Carl Zeiss) [13,42]. Laser treatment was performed in a clockwise direction, maintaining the same distance from the disc as

much as possible. This was performed on only one eye because intravitreal anti-VEGF may affect the fellow eye by systemic effects.

Intravitreal Injection of Adenoviral Vector –mediated Tristetraprolin After photocoagulation, the rats were divided randomly into a laser-induced CNV group (20 rats) and a laser-induced CNV plus Ad-TTP injection group (20 rats). Ad-TPP was then injected into the vitreal cavity. The anesthetized rats were immobilized and a drop of ofloxacin was administered into the eye to avoid infection. The eyes were slightly protruded by applying pressure around the orbit with a pair of forceps and a cotton ball, and stabilized by holding the conjunctivae with fine forceps. Each rat received a single 10 μL intravitreal injection through the pars plana using a 30 gauge needle. The rats in the laser-induced CNV group were given an injection of balanced salt solution (BSS). Only one eye per animal was injected. To evaluate the location of Ad-TTP, we performed double imaging of Ad-TTP-mCherry (red) and CNV (green).

Visualizing and Auantifying Choroidal Neovascularization Two weeks after intravitreal injection and laser treatment, blood flow was visualized by vascular perfusion with high-molecular-weight [2×10^6 mW (2-MMW)] FITC-dextran (Sigma-Aldrich Corp, St Louis, Missouri) as described by D'Amato *et al*[43] and Honda *et al*[13]. Animals were anesthetized by intraperitoneal injection with 30 mg/kg ketamine (Yuhan Ketamine, Yuhan Corporation) plus 2.5 mg/kg xylazine (Yuhan Rompun, Yuhan Corporation). Then, the left ventricle of the heart was perfused with 70 mL of lactated Ringer's solution followed by 20 mL of lactated Ringer's solution containing 10% (wt/vol) gelatin and 5 mg/mL FITC-dextran to visualize CNV. After perfusion, the eyes were enucleated within one minute, and fixed in 4% paraformaldehyde for next 30min. Then, RPE-choroid-sclera flat mounts were made by hemisecting the eye. Radial cuts were performed to permit the tissue to be flattened onto a microscope slide with the RPE side facing up. Flat mounts were prepared with Permount™ mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

The specimens were then examined under a fluorescence microscope (Olympus DSU, Japan) equipped with an FITC-detecting filter unit. The CNV areas were photographed and images were captured with a charge-coupled device camera. Quantitative analysis of the CNV areas was performed using the NIH Image J program. The FITC-dextran-perfused vessels in areas of neovascularization were quantified by a clinician experienced in delineating fibrovascular membranes[8]. Double imaging of Ad-TTP-mCherry (red) and CNV (green) was performed to evaluate the location of Ad-TTP in the laser-treated eyes.

Reverse Transcriptase Polymerase Chain Reaction Ten eyes of ten rats from each experimental group were used to evaluate the VEGF mRNA level of retina-choroid tissue. The rats were sacrificed 14d after laser and/or Ad-TTP treatment and the eyes were rapidly enucleated. The cornea and lens were removed promptly from the enucleated eyeballs and the retina-choroid was collected. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis using an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Primers were synthesized on the basis of the human cDNA sequences for VEGF and GAPDH deposited in the NCBI data bank. The sequences of the primers used for polymerase chain reaction (PCR) were as follows: VEGF forward 5'-CACTGTGAGCCTTGTTCAG-3', VEGF reverse 5'-AAG AGTCTCCTCTCCCTTCA-3'; and *GAPDH* forward, 5'-TG CCGCCTGGAGAAACCTGC-3', *GAPDH* reverse, 5'-TGAG AGCAATGCCAGCCCCA-3'(Bioneer Corporation, Daejeon, South Korea). The amplification conditions were as follows: 25 cycles of 94℃ for 30s, 55℃ for 30s, and 72℃ for 1min. After amplification, PCR products were electrophoresed on an agarose gel, stained with ethidium bromide, and then visualized with a UV transilluminator. The amplified DNA fragments were 308 and 172 bp for VEGF and *GAPDH*, respectively. The band intensities were assessed using Sigma Gel software (Jandel Scientific, San Rafael, CA, USA).

Statistical Analysis All data were expressed as the mean ± standard error of mean (SEM). Data were analyzed by analysis of variance and the t-test. P values<0.05 was considered to be statistically significant.

RESULTS

Two weeks after treatment, CNV was observed in the RPE-choroid-sclera flat mounts as hyperfluorescent areas overlying the laser lesions. The area of CNV (0.39±0.05 AU; area unit) in rats that were given Ad-TTP was suppressed approximately 60% compared with that in the rats treated with laser-induced CNV (0.40±0.46 AU) but not Ad-TTP (1.0±1.13 AU; Figure 1). Ad-TTP-mCherry was located at the sites of CNV lesions (Figure 2).

The expression of VEGF mRNA [as determined by reverse transcription-PCR (RT-PCR)] is shown in Figure 3. The VEGF bands were stronger in the laser-induced CNV group than in the control group (P=0.03). The mean VEGF mRNA expression was suppressed in the laser-induced CNV plus Ad-TTP group (1.49±0.58 AU) compared with in the laser-induced CNV group, without statistical significance (2.24±0.33 AU, P=0.06; Figure 3). VEGF mRNA expression levels in the eyes of rats that received intravitreal injections of Ad-TTP without laser treatment (0.65±0.36 AU) was also supressed compared with those in the control group, without statistical significance (1.0 AU, P=0.09; Figure 3).

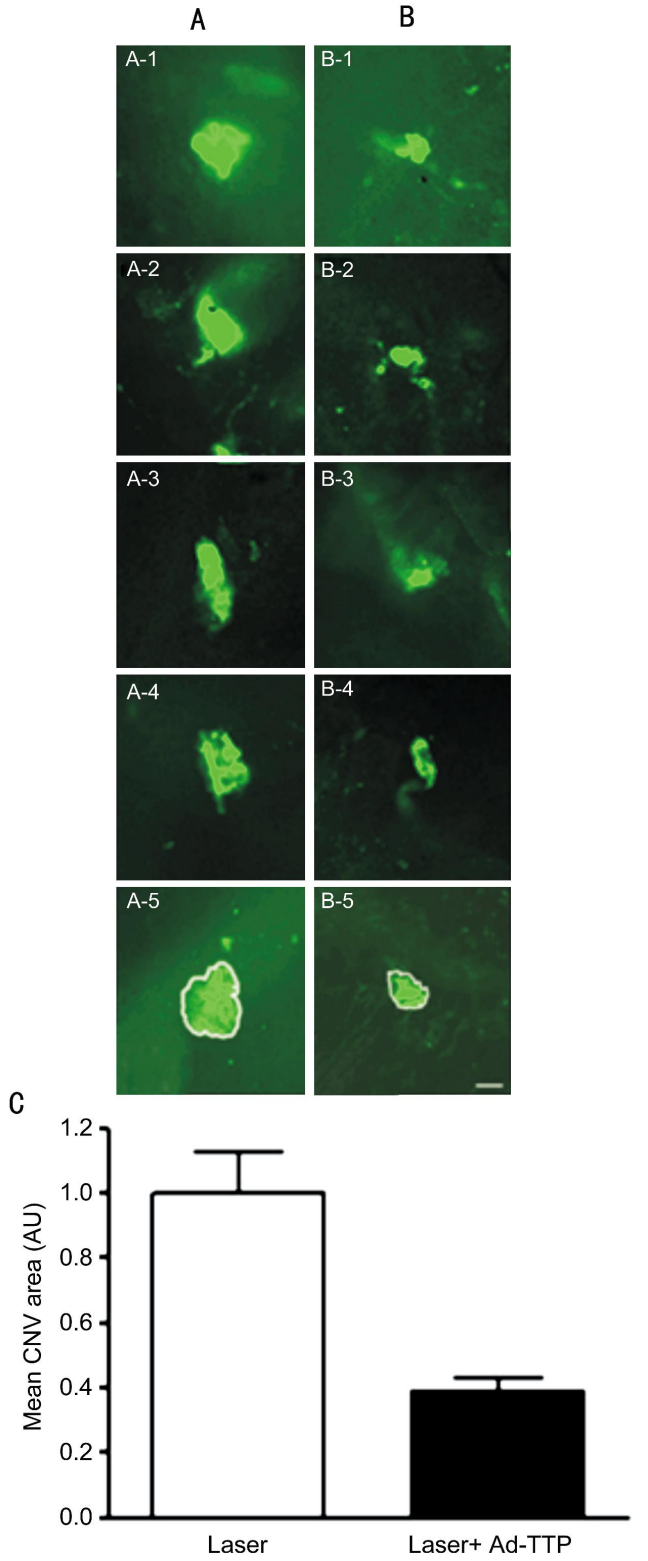

Figure 1 FITC-dextran angiography in the laser-induced CNV group (CNV is indicated by the green fluorescence). The white lines were used to calculate CNV size using the NIH Image J program. Bar, 50μm A: Laser-induced CNV in rats without Ad-TTP injection; B: Laser-induced CNV plus Ad-TTP injection group; C: Two weeks after laser treatment and intravitreal injection, the area of CNV was reduced by approximately 60% in animals given an Ad-TTP injection (0.39±0.05 AU; area unit *vs* 1.0±1.13 AU). AU: Area unit; CNV: Choroidal neovascularization; TTP: Tristetraprolin.

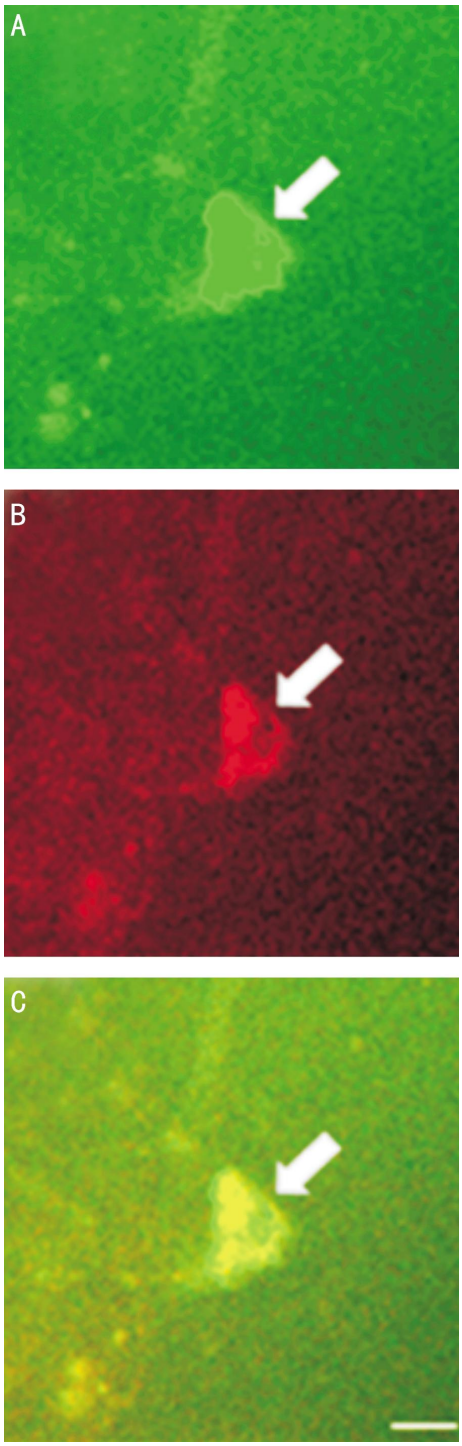

Figure 2 Ad-TTP-mCherry (red) and CNV (green) double labeling. Ad-TTP combined with red fluorescein protein (RFP). Ad-TTP-mCherry expression in a CNV lesion in a rat from the TTP-adenovirus treatment group was shown Localization of CNV (A) and Ad-TTP (B) 14d after intravitreal injection. After merging both labels (C). Arrows indicate regions of choroidal neovascularization. Bar, 50 μm.

DISCUSSION

Many studies have used laser-induced CNV models with different laser settings, and they have all produced reproducible and useful results [6,7,12,44,45]. Recently, Honda *et al* [13] used a laser-induced (wavelength, 532 nm; power, 90 mW; spot diameter, 100 μm) CNV model to examine the

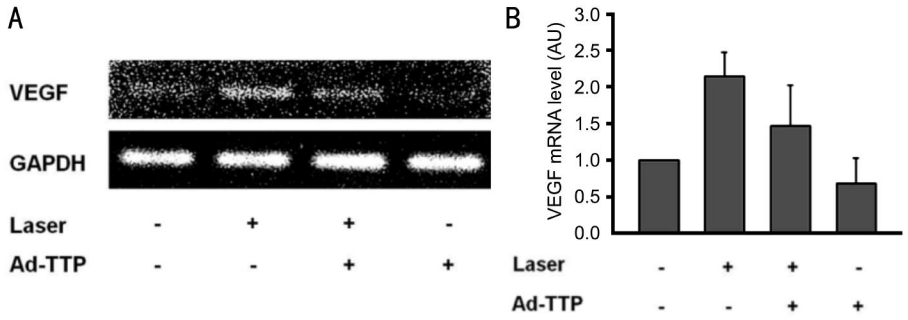

Figure 3 RT-PCR of VEGF mRNA and quantitative analysis of the RT-PCR results A: Expression of VEGF mRNA 14d after laser treatment was shown thourgh RT-PCR. VEGF mRNA expression was lower in the laser-induced CNV plus Ad-TTP injection group than that of the laser-induced CNV group; B: Quantitative RT-PCR analysis revealed that VEGF mRNA levels in the laser-induced CNV plus Ad-TTP injection group were lower than those in the laser-induced CNV group, but the difference was not statistically significant. VEGF mRNA levels in the laser-induced CNV group and laser-induced CNV plus Ad-TTP injection group were significantly higher than those in the control group. VEGF mRNA levels in the Ad-TTP injection only group was lower than those in the control group, but the difference was not significant.

efficacy of liposomal SU5416 as an angiogenesis inhibitor. Present study used the same method to make laser-induced CNV model. The reproducibility and usefulness of laser-induced CNV models can be confirmed by angiography using 2-MMW FITC-dextran and fluorescent microscopy as in the present study.

Honda *et al* [13] used liposomes to deliver the drug *via* intravitreal injections. Interest in intravitreal administration of drugs (as a way to improve the treatment of diseases of the posterior segment of the eye or edematous maculopathies) has increased over the last two decades. Intravitreal administration allows the drugs to reach high concentrations in the vitreous humor and avoids adverse effects associated with systemic administration [46]. However, many drugs are rapidly cleared from the vitreous humor; therefore, to achieve and to maintain effective therapy, repeated administrations are necessary. Unfortunately, frequent intravitreal injections increase the risk of endophthalmitis, damage to the lens, retinal detachment, and vitreal hemorrhage [46]. Thus, new drug delivery methods, such as liposomes and viral vectors, have been developed to improve the quality of intravitreal drug administration. Liposomes are easily injectable and facilitate reduced toxicity and increased residence time of drugs administered directly into the eye[46,47]. They also protect peptides and nucleic acids from degradation *in vivo*[46]. However, present study used an adenovirus vector as the gene delivery method. Adenovirus vectors have many advantages[31,37]. Yan *et al* [48] reported the usefulness of AV in an experimental study of CNV, which was inhibited by adenoviral-vectored pigment epithelium-derived factor (PEDF). Systemic delivery of adenoviral vectors results in rapid physiological responses that include activation of innate immunity, the induction of cytokines, inflammation, transient liver toxicity, and thrombocytopenia [49]. To overcome these limitations, several strategies have been utilized, such as targeting specific organs, engineering viral envelopes, switching serotypes, or modifying the transgene cassette[50,51]. Intravitreal injection may overcome these limitations because the inner eye does not contain well-developed lymphatics. Thus, it is necessary to study whether delivery *via* liposomes or adenoviral vectors is more appropriate for maximizing the effect of TTP.

Injection of 0.01 mL (10 μL) of drug into the vitreal cavity is commonly performed in rat studies. This dose was sufficient to effectively suppress the size of the CNV in the present study. However, the present study did not evaluate the most effective TTP concentration. Further studies are needed to answer these questions. Askou *et al* [52], reported that choroidal neovascularization in mice following adenoviral vector-delivered anti-VEGF shRNA was significantly reduced by 48%. Honda *et al* [13] reported that the area of CNV observed in rats 2wk after the intravitreal injection of liposomal SU5416 suppressed CNV 33% compared with the placebo group. Their study also induced CNV by laser photocoagulation and then liposomal SU 5416 was injected immediately after the laser. Their study, and also ours, could not perform enucleation after waiting for CNV formation, since iatrogenical CNV can spontanelusly resolve with time without treatment[12]. The present study showed that the extent of CNV in the Ad-TTP injection group was reduced by approximately 60% compared with that in animals that did not receive an Ad-TTP injection. Present study did not compare Ad-TTP with other widely used anti-VEGF drugs. Nevertheless, the result of this study that Ad-TTP reduced the size of laser-induced CNV was very meaningful.

VEGF mRNA levels in both laser-treated groups were significantly higher than the control group. The VEGF mRNA level was lower in the laser-induced CNV plus Ad-TTP than in the laser-only group; however, the difference was not significant. The reason for this may be suppressed

VEGF production during the wound healing process 2wk after laser treatment. Thus, subsequent studies should address VEGF levels shortly after treatment and at extended periods after laser treatment.

The present study showed that a single intravitreal injection of Ad-TTP significantly suppressed size of CNV in an experimental laser-induced CNV model. Ad-TTP injection also decreased the level of VEGF mRNA in the laser-induced CNV plus Ad-TTP injection group compared with that in the laser-induced CNV group. This suggests that TTP may be useful for treating CNV in clinical practice.

This study has some limitations. Firstly, the most appropriate injection dose and frequency should be determined. Also the duration of the single injection effect and same effect in other animal need to be researched. Additional study must be needed to find the solutions to these problems. Although something to do still remained, the present study is meaningful as the first study to investigate the effect of TTP delivered *via* intravitreal injection.

In conclusion, single intravitreal injection of Ad-TTP significantly suppressed CNV size in experimental laser-induced CNV model. Ad-TTP injection also decreased VEGF mRNA expression laser-treated plus Ad-TTP injection group compared with that in the laser-induced CNV group. Therefore, this study is meaningful as it is the first study to investigate the effect of TTP delivered via intravitreal injection.

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