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Inhibition of Corneal Neovascularization with the Combination of Aflibercept and Plasmid Pigment Epithelium-Derived Factor-Synthetic Amphiphile INTeraction-18 Vector

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ABSTRACT

Aflibercept, a recent VEGF inhibitor, is a recombinant fusion protein consisting of portions of human VEGF receptors 1 and 2 extracellular domains, fused to the Fc portion of human IgG1 formulated as an iso-osmotic solution for intravitreal administration. In this study, we have estimated that the combination of aflibercept and plasmid pigment epithelium-derived factor-synthetic amphiphile INTeraction-18 (p-PEDF-SAINT-18) has a favorable antiangiogenic effect on corneal neovascularization (NV). Four groups (Group A: 0 µg + 0 µg, B: 0. 1µg + 0.1 µg, C: 1 µg + 1 µg, D: 10 µg + 10 µg) of aflibercept + p-PEDF-SAINT-18 were prepared and implanted into rat subconjunctival substantia propria 1.5 mm from the limbus on the temporal side. The 2 µg of p- bFGF-SAINT-18 were prepared and implanted into the rat corneal stroma 1.5mm from the limbus on the same side. Inhibition of NV was observed and quantified from day 1 to day 90. 18-kDa bFGF and VEGF protein expression was analyzed through biomicroscopic examination, western blot analysis and immunohistochemistry. No inhibition activity for normal limbal vessels was noted. Subconjunctival injection by a combination of aflibercept and p-PEDF-SAINT-18 successfully inhibited corneal NV. Successful genes of bFGF and PEDF were expressed through western blot analysis; immunohistochemistry staining showed mild immune response for HLA-DR. We conclude that the combination of aflibercept and p-PEDF-SAINT-18 may have more potent and prolonged antiangiogenic effects, making it possible to reduce the frequency of subconjunctival aflibercept administration, while maintaining relatively high safety and low toxicity.

Introduction

Neovascularization (NV) or angiogenesis (formation of new blood vessels) is a severe complication of ischemic ocular diseases such as neovascular glaucoma, diabetic retinopathy, branch and central

retinal vein occlusion, and retinopathy of prematurity. Corneal NV is a sight-threatening condition that can develop in response to inflammation, hypoxia, trauma, or limbal stem cell deficiency [1,2]. Several natural and synthetic angiogenesis inhibitors have shown beneficial effects in experimental animal studies, such as topical corticosteroids, suleparoide (heparan sulfate) [3], thalidomide [4], suramin [5], genistein [6], avastin [7] and aflibercept [8] have been effective in inhibiting corneal NV [9,10]. However, the pathogenesis of corneal NV has yet to be fully defined; further investigation is needed to determine whether the anti-angiogenic therapy is an effective treatment against actively growing or established corneal angiogenesis.

Vascular endothelial growth factor (VEGF), a potent and specific mitogen for vascular endothelial cells, is a critical mediator of corneal NV. Animal studies have shown that VEGF overexpression is sufficient to induce corneal NV in the eye [11,12], whereas inhibition reduces this effect [13]. Anti-VEGF has been associated with inhibition of iris and corneal NV and with suppression of the formation of new retinal vessels in primates [14-16]. Several antiangiogenic compounds that target VEGF and VEGF receptors are currently being developed, and some are available as tumor therapies.

Aflibercept, a recent VEGF inhibitor, is a recombinant fusion protein consisting of portions of human VEGF receptors 1 and 2 extracellular domains fused to the Fc portion of human IgG1, formulated as an iso-osmotic solution for intravitreal administration. In an experimental model, aflibercept's equilibrium disassociation constant (Kd, inversely related to binding affinity) for VEGF-A165 was 0.49 pM, compared with 9.33 pM and 88.8 pM for experimental native VEGFR1 and VEGFR2, respectively [17]. Aflibercept binds to all VEGF-A isoforms, to VEGF-B, and to placental growth factor (PlGF) [17,18]. Furthermore, it has been demonstrated to have a higher affinity to VEGF-A than ranibizumab and bevacizumab [19]. Aflibercept has specifically been designed for use in the eye to treat neovascular (wet) macular degeneration and other retinal diseases, and is approved in this use by the FDA. Aflibercept has been designated for sequestering and antagonizing VEGF and to prevent blood vessel formation. Several studies have also reported that aflibercept has antiangiogenic properties [20].

PEDF, a 50-kDa glycoprotein initially isolated from the conditioned media of retinal pigment epithelial (RPE) cells, has demonstrated neurotrophic, neuronotrophic, neuroprotective, gliastatic, anti-tumorigenic and anti-vasopermeability properties [21,22]. A possible role for PEDF in the regulation of ocular NV has been suggested, as the molecule has been detected in the vitreous and the aqueous humor, and as it was shown to be one of the most potent known anti-angiogenic proteins found in humans [21,23]. It is noteworthy that PEDF inhibits VEGF-induced proliferation and migration of microvascular endothelial cells [24] and bFGF-induced capillary morphogenesis of endothelial cells [25-28] through Fyn [29]. Matsui T. *et al.* [30] have reported that a PEDF-derived peptide inhibits corneal angiogenesis by suppressing VEGF expression. In addition, PEDF is the most potent natural

inhibitor of angiogenesis in the mammalian eye. The following is a model modified from those of Aplin *et al.* [25] and Conway *et al.* [27], which demonstrated that the binding of VEGF with its receptors results in the self-phosphorylation (P) of the receptors, which in turn leads to a series of intracellular events that trigger angiogenesis. In this case, integrins are associated with protein tyrosine phosphatase (PTP). However, the activation of integrins in adhering cells may activate protein kinases, thereby triggering a series of downstream events that inhibit the angiogenesis induced by VEGF. The binding of PEDF with integrins, directly and/or indirectly via ECM, leads to conformation changes of the integrin or collagen-integrin complexes, causing phosphatases to dissociate and then reassociate with the VEGF receptors. This PEDF-integrin complex may also inactivate the protein kinases. The dephosphorylation of receptors or their downstream factors stops signal transduction, thereby blocking angiogenesiss [28].

We previously showed that SAINT-18 was capable of directly delivering genes to the ocular surface by way of subconjunctival injection, and it delivered sustained, high levels of gene expression in vivo that inhibited angiogenesis. This effect may last longer than that of aflibercept alone, because the PEDF protein could be detected on day 90 after transfection of p-PEDF-SAINT-18. In this study, we were interested in understanding the antiangiogenic effect of the combination of aflibercept and p-PEDF-SAINT-18 on corneal NV

Material and Methods
Animals
Male Sprague-Dawley rats (300-350 g; NSC Animal Center, Taiwan) were used in this study. All protocols and the treatment of animals were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocol in this study has been reviewed and approved by Institutional Animal Care and Use Committee (IACUC), and the committee recognizes that the proposed animal experiment follows the Animal Protection Law by the council of Agriculture, Executive Yuan, R.O.C. and the guidelines as shown in the Guide for the Care and Use of Laboratory Animals as promulgated by the Institute of Laboratory Animal Resources, National Research Council, U.S.A.

Naked DNA vector
The bFGF and PEDF expression vectors pCMV-GFP, pCMV-bFGF and pCMV-PEDF were kindly provided by Dr M. H. Tai (Department of Medical Research, Kaohsiung Veterans' General Hospital, Kaohsiung City, Taiwan.). The plasmid was purified commercially by Clone-E Therapeutics Inc. (Kaohsiung, Taiwan) and was endotoxin free. DNA was produced according to the proprietary process established by Clone-E Therapeutics Inc. The *E coli* DH5α cells were purchased from Bioresource Collection & Research Center (Hsinchu, Taiwan). They carried the plasmid that was grown in an ampicillin-containing medium in a 5-L fermentor. The fermentation broth was subjected to a series of purification steps including complete cell lysis, anion exchange, and gel filtration chromatography. The purified plasmid was dialyzed

against a formulation buffer (Saline, pH 7.0). It was quantified using UV absorbance. Agarose gel analysis showed primarily supercoiled plasmids with a small amount of nicked plasmid.

P-DNA-SAINT-18 Complex Preparation

The SAINT-18 (1-methyl-4- (cis-9-dioleyl) methylpyridinium-chloride) delivery system (Synvolux Therapeutics B.V.) was based on a cationic pyridinium head group, and shows excellent biocompatibility. The molecular structure of SAINT-18 was purchased from Synvolux Therapeutics B.V. Each vial was "ready to use" and contained 2 mL SAINT-18 in water at a concentration of 0.75 mM. Before use, the SAINT-18 was vortexed thoroughly to minimize micelle-size, thereby increasing complexing efficacy. The relative amounts of DNA to carrier for SAINT-18/DNA (1µl of SAINT-18 per 1 µg DNA phosphate) were allowed to form at room temperature. To minimize the loss experienced with liquid reagents during transfection into corneal tissue, the complex was partially dried via SpeedVac (SpeedVac Model SC110 + VLP120 Oil Vacuum Pump, Savant Instruments, Inc., Farmingdale, NY) at ambient temperature for 60-90 min after the plasmid bFGF (2 µg) and PEDF (0 µg (the substitute was purified water), 0.1 µg, 1 µg, or 10 µg) had been complexed with SAINT-18. The partially dried form of the complex was drawn out of the eppendorff tube by curettage and prepared for immediate implantation into the corneal pocket.

Corneal pocket assay

All surgical procedures were performed using sterile techniques. The corneal pocket implantation performed in this study was a modification of the technique that has been described previously [31]. The rats were placed under general anesthesia with 3% isoflurane in an O_2/room air mixture (1:1). As additional topical anesthesia, 0.4% benoxinate hydrochloride (Novesin, Ciba Vision, Hettlingen, Switzerland) was applied to the corneal surface. The eyes were proptosed by grasping the temporal limboconjunctival epithelium with a jeweler's forceps, and a 30°-45° fan-shaped central-peripheral corneal intrastromal lamellar pocket (middle stroma depth; the distance of inlet was 0.7-1.0 mm; the distance of radius was 1.5-2.0 mm) was dissected with a surgical blade (Paragon No. 11, Maersk Medical, and Sheffield, UK) and an ophthalmic slit knife (Alcon Inc., Fort Worth, TX, USA). The pocket was extended 1.5 mm from the limbus. After the p-bFGF-SAINT-18 gene complex (2µg) was implanted into the corneal stromal pocket in each eye using forceps and a blade, 0 + 0 µg, 0.1 + 0.1 µg, 1 + 1 µg and 10 + 10 µg of aflibercept and p-PEDF-SAINT-18 were delivered immediately to the subconjunctival tissue 1.5 mm from the limbus via the indwelling cannula using a Hamilton syringe. Topical antibiotic ointment (0.3% gentamycin; Alcon Cusi, Spain) was applied to the corneal surface to reduce irritation and prevent infection.

Visualization and quantification of corneal NV

Examinations were made with a dissecting microscope and results were photographed. While the rats were under anesthesia, the eyes were proptosed and the maximum vessel length and width in the NV region were measured with calipers. Photographs obtained during corneal angiogenesis assay were taken at a resolution of 640 480 pixels using a digital CoolPix 995 camera (Nikon, Japan). The operator was masked to the treatment group from which each cornea was derived. Areas containing blood vessels were traced on a computer monitor (FT Data Systems, Stanton, CA, USA). The area within the trace was calculated with image analysis software (Enhance 3.0; MicroFrontier, Des Moines, IA, USA) and was reported in square millimeters, with determinations confirmed at ×40 magnifications. Three independent observers conducted masked assessments.

Analysis of bFGF, VEGF and PEDF protein expression by western blot after transfection

On day 20 and 90 after transfection, the rats in each group were killed with an overdose of thiopental sodium and the fresh corneas were removed along the contour of the NV using scissors. These specimens were homogenized by sonication in ice-cold lysis buffer (50 mM Tris, [pH 7.5], 150 mM NaCl, 2% Triton X-100, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin), and then centrifuged at 50,000 g for 30 min at 4°C. The protein content of the supernatant was determined using the Bio-Rad Protein Assay system. An equal volume of sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol, 50 mM Tris-HCl [pH 7.2]) was added to the sample. Proteins were separated by electrophoresis (NuPAGE Electrophoresis; Invitrogen, San Diego, CA, USA) on 15% SDS-polyacrylamide gels at 120 V for 90 min. They were then transferred to a polyvinylidene difluoride membrane (0.45 µM pore size; Immobilon-P, Millipore) in a transfer buffer (50 mM Tris-HCl, 380 mM glycine, 1% SDS, 20% methanol) at 50 V for 60 min. The membrane was blocked with 5% nonfat dry milk in Tween-20 and Tris-buffered saline (TTBS; 0.1% Tween-20, 20 mM Tris-HCl, 137 mM NaCl, pH 7.4) for 60 min at room temperature. The membrane was then incubated with anti-bFGF (purified anti-human FGF-basic antibody; BioLegend, San Diego, CA, USA), anti-PEDF antibody (anti-human serpin F1/PEDF antibody; R&D Systems, inc., USA) and anti-VEGF (Santa Cruz Biotechnology inc. CA.USA) for 90 min at room temperature. Each blot was washed three times for 10 min in TTBS, blocked with 5% nonfat dry milk in TTBS, and then incubated with horseradish-peroxidase-conjugated secondary antibody (1:1000; Transduction Laboratories) for 1 h at room temperature. Antibody labeling was detected through chemiluminescence (ECL, Amersham). Colored molecular-weight standards were run in parallel on each gel. The housekeeping gene α-tubulin was used as the control.

Histological examination

On day 20 and 90 after transfection in the group D, the rats were killed with an overdose of thiopental sodium; their eyes were then enucleated, fixed in paraformaldehyde, and frozen at -70 °C in OCT compound. Corneas were excised and cryostatically cut into 3-8-µm sections for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Excised corneal tissues for IHC were fixed in Bouin's solution and embedded in paraffin. After the different sections had been cut (3 µm) and mounted on slides coated with poly-L-lysine, they were immunostained

(ImmunoBlot, Invitrogen) with anti-rat HLA-DR antibody (Santa Cruz Biotechnology Inc., CA, USA).

Statistical analysis

Repeated measures ANOVA (SPSS version 10.0 for Windows; SPSS Inc., Chicago, IL, USA) and the Bonferroni post hoc test were used to analyze differences in the length and area of corneal NV between pairs of groups. Length and area were measured every three days from day 0 to day 90. The values for each measure were recorded as separate variables, and were defined as within-subject factors. The assigned groups were defined as between-subject factors. A value of $p \leq 0.05$ was considered statistically significant.

Results

Biomicroscopic examinations of corneal NV

Gross examination in our rat corneal model seemed to show no interference in the wound healing process or in corneal limbal deficiency induced by aflibercept + p-PEDF-SAINT-18 (Figure 1A). Another control, i.e., 2 μg p-GFP-SAINT-18, was evaluated and compared (Figure 1B). Forty-eight rats (48 eyes) were divided equally into three experimental groups (Group B: 0.1 μg + 0.1 μg, C: 1 μg + 1 μg, D: 10 μg + 10 μg of aflibercept + p-PEDF–SAINT-18) and one control group (Group A: 0+0 μg of aflibercept + p-PEDF–SAINT-18; the substitute was purified water). The inhibition of NV in the 1 μg p-PEDF–SAINT-18 alone and 1 μg aflibercept alone was shown on day 20 (Figure 2E,F). Biomicroscopic examinations revealed that the corneal epithelium healed within 24 h of surgery. Corneal edema and limbal injection were noted in all corneas. Limbal vessels began sprouting into the cornea on postoperative day 3. Corneal NV was induced dose-dependently via 2 μg p-bFGF-SAINT-18 complex, and NV reached a maximum on days 18-21 in the control group, and then continued to regress. The NV response was intense, localized, and reproducible. Maximal growth of NV is shown in Figure 2. Compared with the control group, there was significant inhibition of NV in groups C and D.
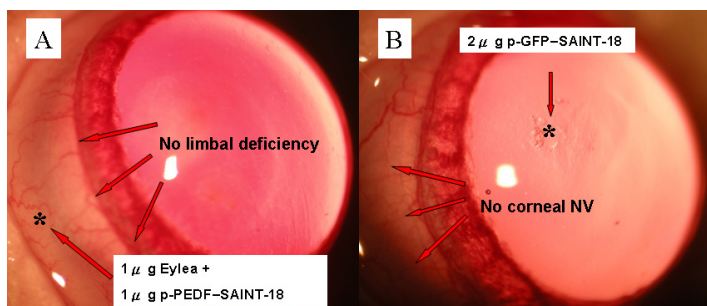
Figure 1: The results of corneal limbal deficiency were evaluated on day 20. **(A)** Limbal deficiency test: 1 μg + 1 μg of aflibercept + p-PEDF–SAINT-18; No inhibition activity for normal limbal vessels was noted. **(B)** Control group: 2 μg p-GFP; No limbal vessels began sprouting into the cornea.

However, the standard deviation was high (e.g., group C, 6621± 2728× 10^{-4} mm; group D, 1932 ± 950× 10^{-4} mm) on day 20 after the transfection. Data from the four groups were compared using repeated measures ANOVA. The results of the length data were $F = 1230.15$ ($p < 0.001$ between the control and either group C or D, but $p > 0.05$ between the control and group B). The results of the area data were $F = 306.80$ ($p < 0.001$ between the control and either group C or D, but $p > 0.05$ between the control and group B) (Figure 3). The inhibition of NV in the treatment with 1 μg of aflibercept alone was greater than that in the treatment with 1 μg of p-PEDF-SAINT-18 alone ($p < 0.05$). However, on day 90, p-PEDF–SAINT-18 showed a similar antiangiogenic effect to that of bevacizumab, suggesting that the antiangiogenic effect of p-PEDF-SAINT-18 may last longer than that of aflibercept, but be less potent during the early period of the treatment. In addition, we also evaluated the antiangiogenic effect among the three groups (1 μg of aflibercept alone, 1 μg of p-PEDF–SAINT-18 alone, and 1 μg of aflibercept + 1 μg of p-PEDF–SAINT-18). The results for the length data were $F = 568.12$ ($p < 0.001$ between 1 μg of aflibercept alone and 1 μg of p-PEDF-SAINT-18 alone). Moreover, the results for the length data were $F = 565.44$ ($p < 0.001$) between 1 μg of p-PEDF-SAINT-18 alone and 1 μg of aflibercept + 1 μg of p-PEDF-SAINT-18 but $F = 152.03$ ($p > 0.05$) between 1 μg of aflibercept alone and 1 μg of aflibercept +1 μg of p-PEDF–SAINT-18. The results for the area data were $F = 126.32$ ($p < 0.001$) between 1 μg of aflibercept alone and 1 μg of p-PEDF–SAINT-18 alone. Importantly, the results for the area data were $F = 125.28$ ($p < 0.001$) between 1 μg of p-PEDF–SAINT-18 alone and 1 μg of aflibercept +1 μg of p-PEDF–SAINT-18, and $F = 85.14$ ($p < 0.05$) between 1 μg of aflibercept alone and 1 μg of aflibercept +1 μg of p-PEDF–SAINT-18 (Figure 3).

Western blot analysis

On day 20 after transfection, the rats in each group were killed with an overdose of Pentothal, and corneal samples were removed along the contour of NV. The α-tubulin band was used as the control for normalization. The bFGF bands (molecular weight, approximately 18 kDa) were detected in protein from the groups treated with 2μg p-bFGF, using rabbit anti-human bFGF polyclonal antibody (Figure 4A). The PEDF bands (molecular weight, approximately 50 kDa) produced with 0 μg, 0.1 μg, 1 μg and 10 μg of the PEDF-encoding plasmid was visualized. This band is also noted in the 0 μg PEDF group (endogenous PEDF). Levels of VEGF protein were also estimated via by western blot analysis on day 20 and 90 (Figure 4B, 4C).

Histology

Twenty days after administration, successful gene expression of p-GFP-SAINT-18 within the cornea was histologically evaluated (Figure 5A). A vast number of capillaries appeared in the corneal stroma, running from the limbal blood vessels up to the p-bFGF-SAINT-18 implant (Figure 5B). The inflammation could be detected through the presence of macrophages and other inflammatory cells such as lymphocytes; however, mild immune response of HLA-DR was shown through IHC staining (Figure 5C).
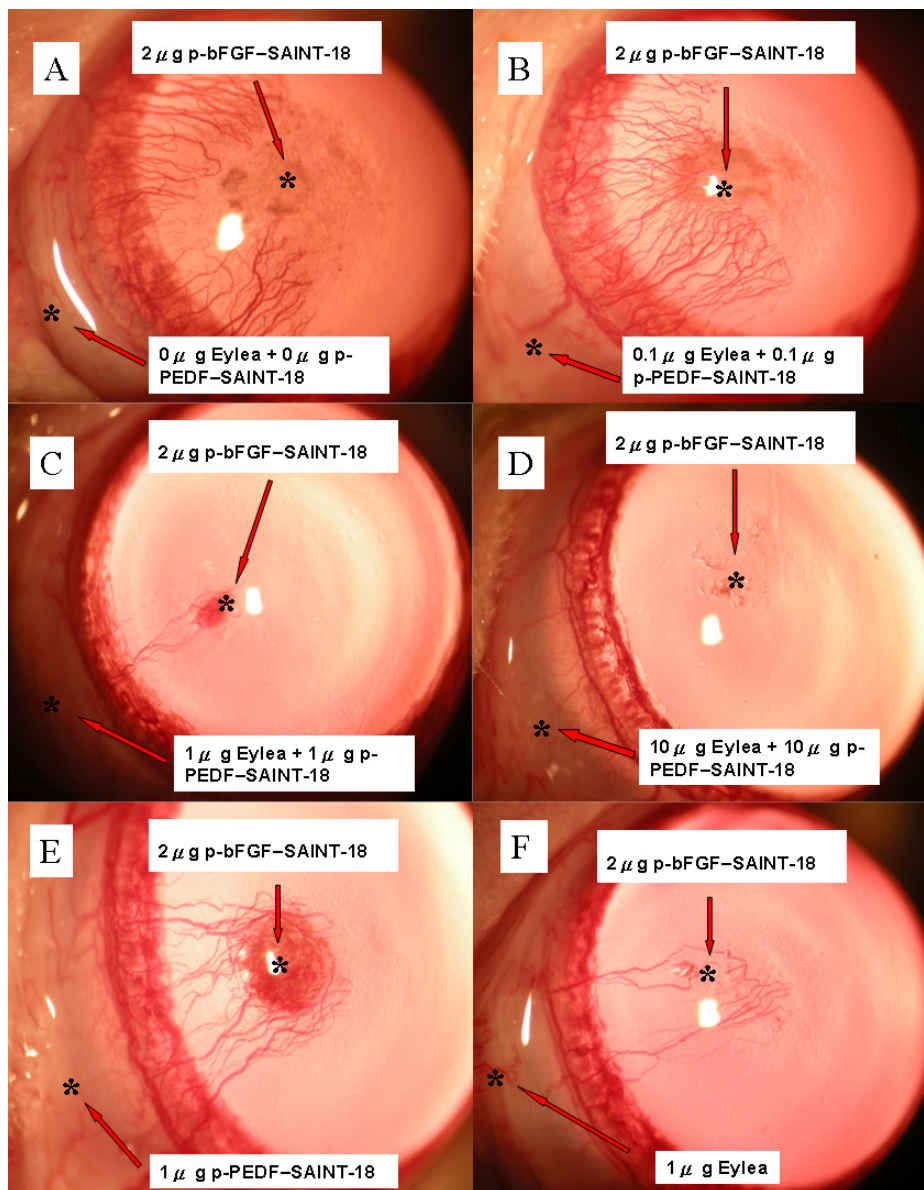
Figure 2: Slit-lamp photographs of Sprague-Dawley rat corneas showing corneal NV for the four experimental groups on day 20. (**A**) 0 μg + 0 μg of aflibercept + p-PEDF-SAINT-18, respectively (control group; the substitute was purified water); (**B**) 0.1 μg + 0.1 μg of aflibercept + p-PEDF-SAINT-18, respectively; (**C**) 1 μg + 1 μg of aflibercept + p-PEDF–SAINT-18, respectively; (**D**) 10 μg + 10 μg of aflibercept + p-PEDF–SAINT-18, respectively; (**E**) Subconjunctival administration of 1 μg of p-PEDF–SAINT-18 alone; (**F**) Subconjunctival administration of 1 μg of aflibercept alone. ***** 2 μg of the p-bFGF–SAINT-18 complex.

Discussion

Ocular NV diseases are major contributors to blindness around the world. Angiogenesis in the eye may be the result of an imbalance between stimulatory growth factors and endogenous inhibitors, which presumably results from the elevated expression of local angiogenic factors. Several growth factors are used for inducing corneal NV, such as VEGF and bFGF [32]. VEGF and its receptors play a vital role in normal and pathologic angiogenesis. It is an important signaling protein that promotes several steps of angiogenesis, including proliferation, proteolytic activity, differentiation, and endothelial cell migration [33]. The bFGF that was utilized in this study has been used extensively in corneal angiogenesis models. It is widely expressed in developing and adult tissues during cellular differentiation, angiogenesis, mitogenesis, and wound repair.

Recent studies have shown that intravitreous aflibercept effectively inhibits VEGF in experimental models or clinical trials [34-36]. However, repeated administration or repeated injections with other modalities may be required for better effect, because aflibercept has a short half-life. In rabbit models, the vitreous half-life of aflibercept is 94.1 hours (3.92 days). This is shorter than that of bevacizumab (6.99 days), and longer than that of ranibizumab (2.51 days) [37]. We have observed that the transgene, PEDF, can be stably expressed for over 3 months [38]. For this reason, we hypothesized that group D in this study would show the
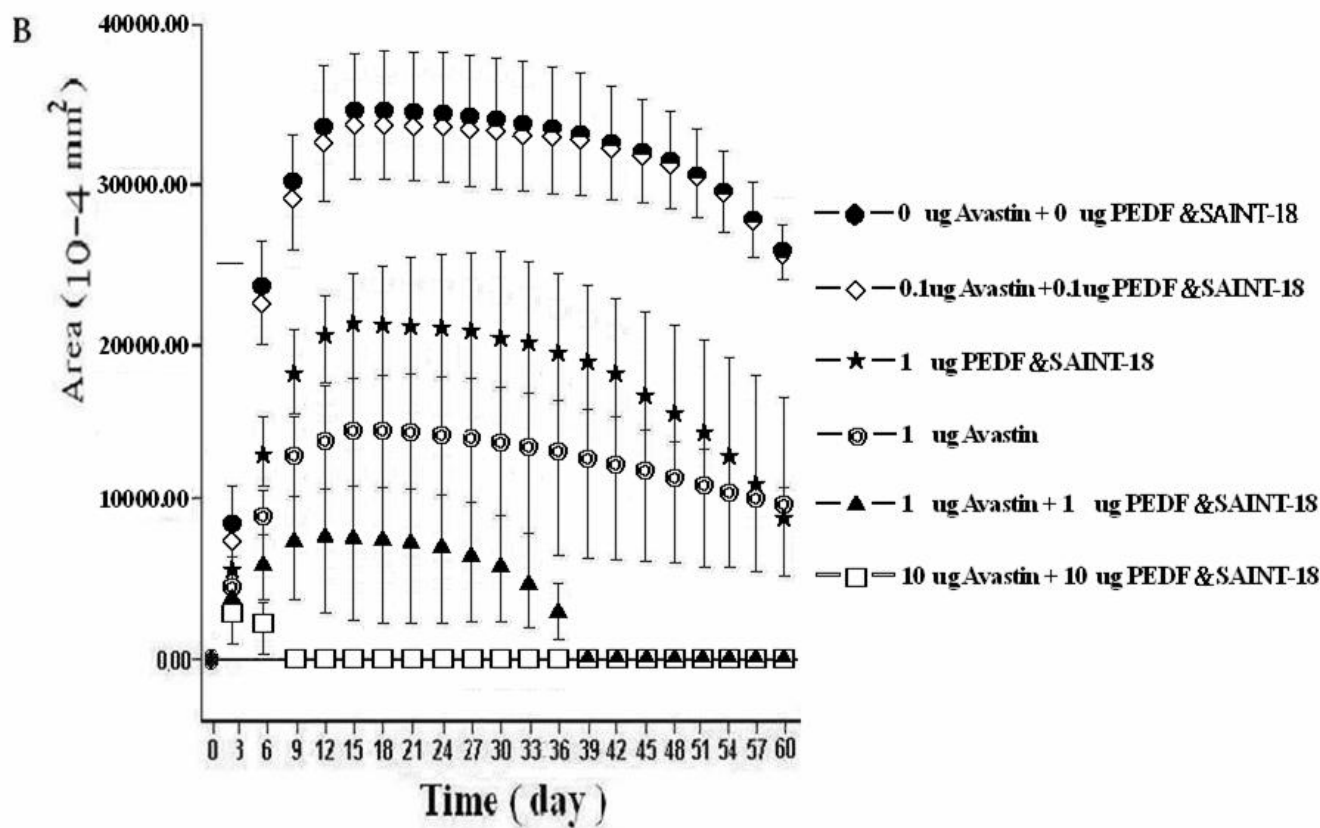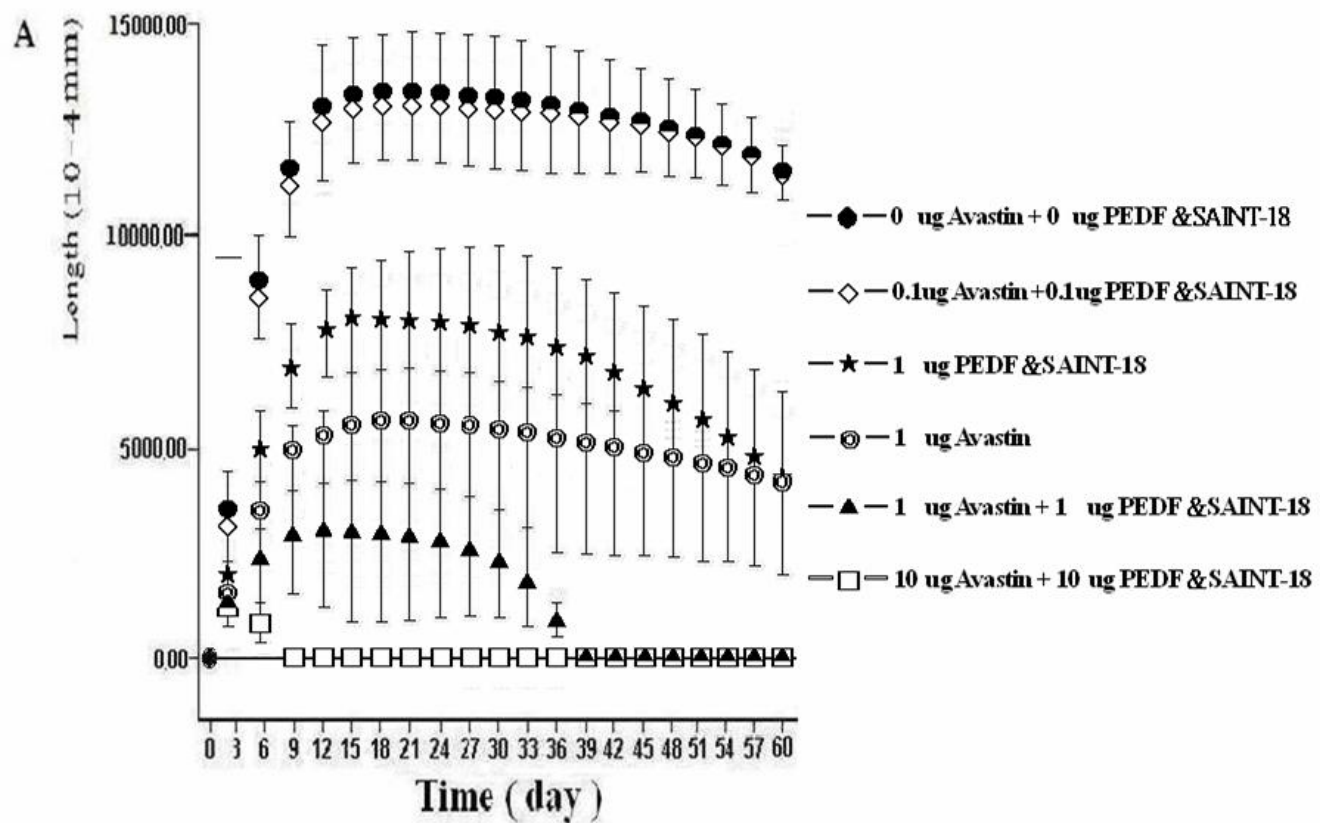
Figure 3: The mean length **(A)** and area **(B)** of corneal NV. Angiogenesis induced by 2 μg p-bFGF–SAINT-18, with six different groups treated via subconjunctival injection. Vertical bars denote standard deviation of the mean.
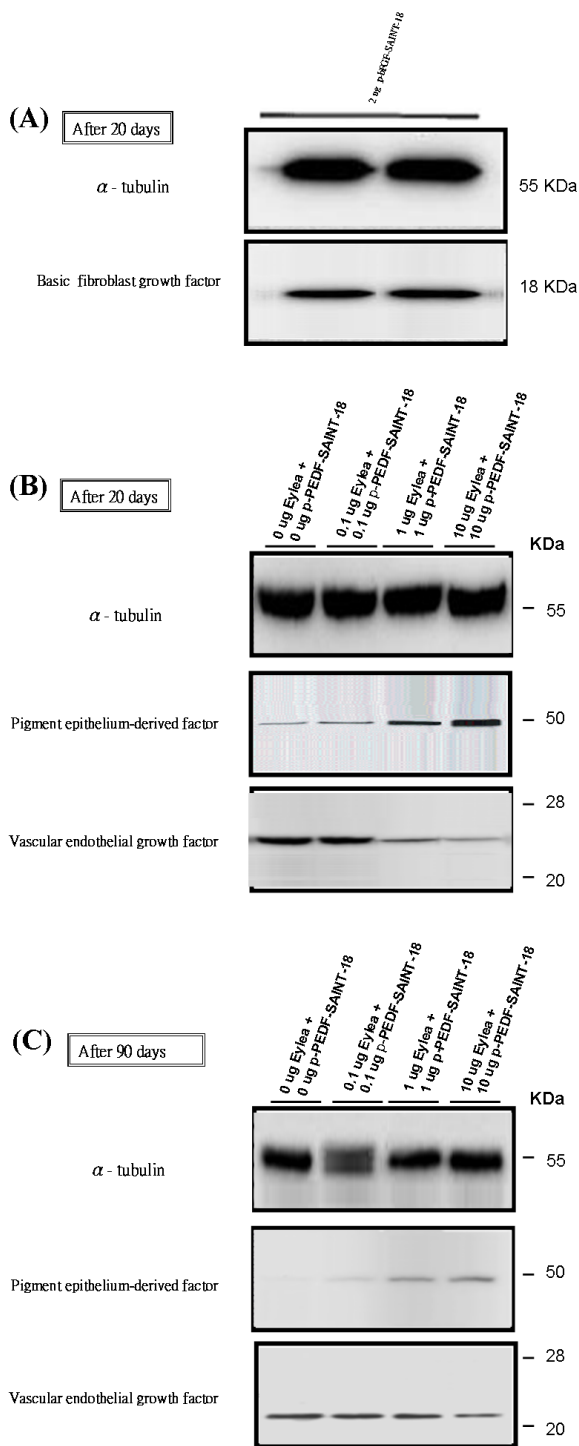
Figure 4: Levels of bFGF, VEGF and PEDF protein were estimated by western blot analysis. Visualization of the α-tubulin band was used as the control for normalization. **(A)** BFGF bands (molecular weight, approximately 18 kDa) were produced with 2 μg p-bFGF-encoded plasmids after day 20. **(B)** Visualization of the PEDF bands (molecular weight, about 50 kDa) was produced with 0 μg, 0.1 μg, 1 μg, and 10 μg PEDF-encoding plasmids, and this band was also noted in the 0 μg PEDF group due to endogenous PEDF. Inhibition of VEGF expression was more significant in the 10μg + 10 μg of aflibercept + p-PEDF–SAINT-18 group. **(C)** On day 90 after administration, the PEDF and VEGF levels within the corneal and subconjunctival substantia propria were determined.

greatest regression of corneal NV, because combined treatment with aflibercept and p-PEDF-SAINT-18 could block various proangiogenic growth factors. This would have greater effect through two mechanisms: First, aflibercept demonstrated higher binding affinity for VEGF-A isoforms and greater potency in vitro than ranibizumab or bevacizumab [17]. Second, it has been found to offer a more prolonged and potentially more potent anti-VEGF effect in wet age-related macular degeneration than both bevacizumab and ranibizumab [39].

If intravitreal aflibercept can penetrate the retinal surface and the entire retinal thickness to induce regression of corneal NV [40-43], subconjunctival aflibercept administration will probably permeate and penetrate the ocular surface to induce regression of corneal NV and modulate the neovascular process. Compared with subconjunctival injection, other traditional methods, such as (1) Intracameral and intravitreous injections are more invasive to the eye and more advanced techniques are needed. (2) Topical application includes difficulty of formulation, low water solubility, and low stability in solution, with consequent susceptibility to loss of bioactivity during long-term storage. Also, long-term topical administration of anti-VEGF may induce epitheliopathies and descemetocele [44,45]. Further, the risk of systemic side effects may be higher with topical administration than with injection into the subconjunctivae. Although conjunctival blood vessels do not form a tight junction barrier [46], subconjunctival injection is also an effective mode of administration for intraocular neovascular diseases. Based on the reasons above, subconjunctival injection is to be considered rather than other traditional applications. However, subconjunctival injection may result in minor complications such as subconjunctival hemorrhage, thinning, or erosive changes to the conjunctiva and/or sclera.

In our study, subconjunctival injections of four different doses of aflibercept + p-PEDF–SAINT-18 complex were given immediately after corneal implantation of 2 μg p-bFGF–SAINT-18 complex. The Bonferroni test indicated significant differences in length and area of NV between group A (the control) and groups C and D. In agreement with this finding, the inducing intensity of corneal NV by 2 μg p-bFGF-SAINT-18 in the cornea was weaker than the inhibition of 1 μg aflibercept + 1ug p-PEDF-SAINT-18 in the conjunctiva. In addition, we examined the antiangiogenic effect of subconjunctival aflibercept alone, and of p-PEDF-SAINT-18 alone; we determined their action durations compared with control group. The results revealed significant differences between the control and the aflibercept alone/p-PEDF-SAINT-18 alone groups ($p<0.05$) (Figure 3); the inhibitory effects of corneal NV by 1 μg aflibercept + 1ug p-PEDF–SAINT-18 were more than 2 μg p-bFGF–SAINT-18, but no significant difference was found with 1 μg aflibercept. During the early period of subconjunctival administration, aflibercept showed more significant antiangiogenic effects than p-PEDF-SAINT-18 did. These effects of aflibercept were similar to the results reported in previous studies of different animal models [47,48]. On day 90, p-PEDF-SAINT-18 showed similar antiangiogenic effect to aflibercept, suggesting that the antiangiogenic effect of p-PEDF-SAINT-18 may last longer than
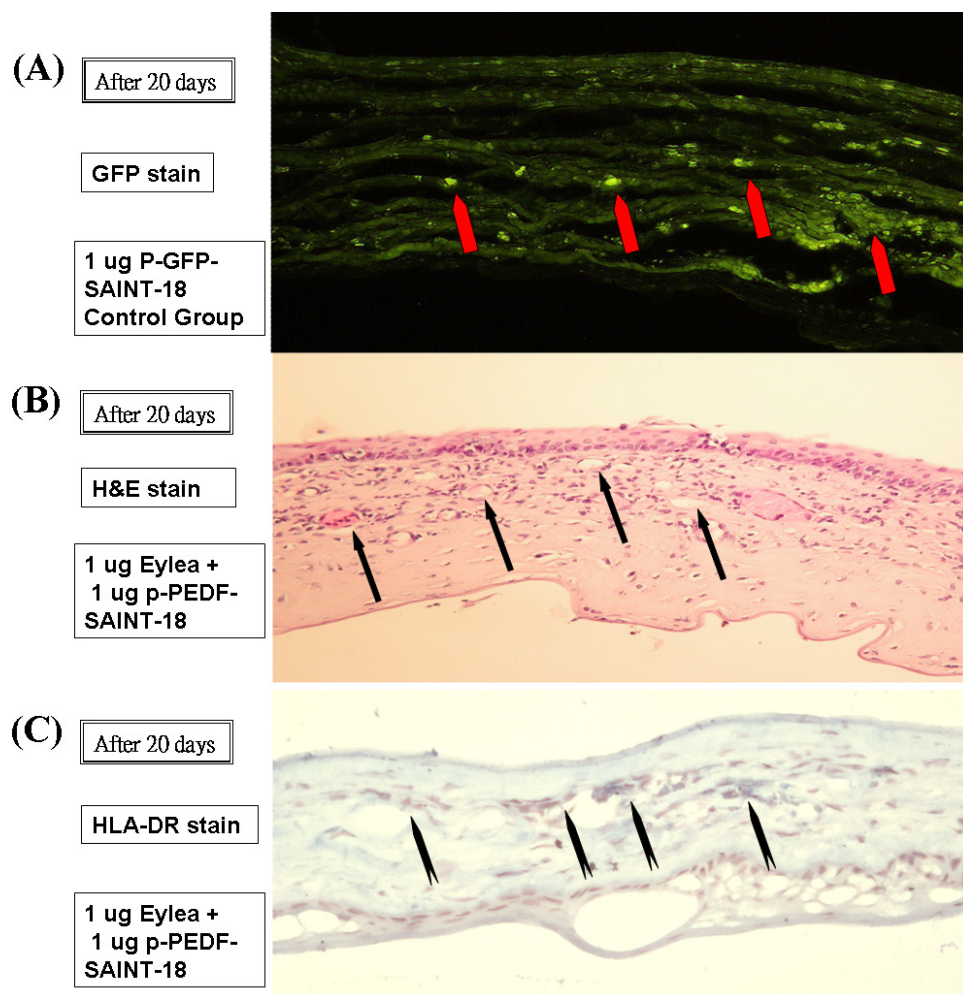
Figure 5: The tissue was histologically evaluated after administration after day 20. **(A)** GFP was expressed within the corneal intrastromal layer and keratocytes after implantation of p-GFP–SAINT-18; no vascular lumen was noted. **(B)** The intervening stroma displayed cells, edema, a mononuclear inflammatory response, and numerous vascular lumens (indicated by arrows) after hematoxylin and eosin staining in the corneal and subconjunctival substantia propria (100× magnification). **(C)** Inflammation was detected by the presence of macrophages and other inflammatory cells such as lymphocytes through immune staining (anti-rat HLA-DR antibody) in the corneal and subconjunctival substantia propria (100x magnification).

that of aflibercept, but may be less potent during the early period of treatment. One of the limitations of this study was its short duration. Nonetheless, we observed that the transgene PEDF was stably expressed for over 4 months, with a potential for longer periods of time, which remains to be studied. The expression of the gene is usually confined to the vicinity of the injection site. Concern about possible side effects of the therapeutic gene over the long term may be quelled upon termination of the gene expression after disease recovery; the long-term effects require further evaluation.

To our knowledge, this is the first study to evaluate the inhibitory effect of the combination of aflibercept and p-PEDF-SAINT-18 vector aflibercept on corneal NV. The results seem to be an effective and safe method for the treatment of corneal NV. The minimal incidence of complications and negative side effects promise future progress for the treatment, and its adoption into broader clinical practice. Further clinical studies are required to evaluate the efficacy, dosage, and safety of the drug in every type of corneal NV.

Conclusions

In conclusion, we found that the combination of aflibercept and p-PEDF-SAINT-18 may have potent and prolonged antiangiogenic effects, making it possible to reduce the frequency of administration of subconjunctival aflibercept alone, and have the advantages of relatively high safety and low toxicity.

Acknowledgments

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