Although there are currently many anti-glaucomatous drugs formulated without preservatives, preservative agent such as benzalkonium chloride (BAK) is still frequently used in intraocular pressure lowering eyedrops. Preservatives used in topical eye drops may cause ocular surface disorders, including superficial punctate keratitis, corneal erosion, conjunctival allergy, conjunctival injection, and anterior chamber inflammation. While the toxic effects of BAK could be negligible for a short-term exposure, chronic or repeated eye drop use can have dose-dependent toxic effects. After repeated instillations, BAK penetrates healthy eyes and is detected in both ocular surface structures and deeper tissues, such as the trabecular meshwork (TM) and optic nerve. Eye drop preservatives may cause long-term trabecular degeneration and increased outflow resistance. Therefore, investigating protective agents against BAK-induced TM damage may improve the treatment and prevention of glaucoma.

Ribonuclease 5 which is the fifth member of RNase and is also called angiogenin (ANG), is known to undergo nuclear
translocation and stimulate rRNA transcription to perform its various activities.\textsuperscript{7} ANG is associated with cancer and neurological disease through angiogenesis and activating gene expression that suppresses apoptosis.\textsuperscript{8,10} ANG is highly concentrated in normal tear fluid that has pooled overnight and helps maintain corneal avascularity. It is suggesting that ANG plays a physiological role which is separate from its angiogenic role under normal ocular surface conditions.\textsuperscript{11} In addition, it has been reported that ANG can be a candidate survival booster for transformed human TM cell lines with possible mechanisms such as activated Akt-mediated signals for nitric oxide production, and TM remodeling by regulating the production of matrix metalloproteinase (MMP)-1 and -3, and rho-kinase.\textsuperscript{12}

However, recombinant human ANG (Rh-ANG) is expensive to apply in large quantities to research, even though ANG has a protective effect against the toxicity of BAK in TM. Therefore, we developed a plant-derived ANG fusion protein using molecular farming technique and evaluated its effect in TM cells. Molecular farming uses plant transformation to generate transgenic plants that can produce therapeutically valuable proteins.\textsuperscript{13} Plant production systems provide the opportunity to generate antibodies on a very large scale. In addition, molecular farming using a transgenic plant expression system may prove to be a less expensive, large-scale alternative for existing fermentation-based production systems.\textsuperscript{14}

The possibility of exploiting plants for the production of recombinant functional full-size antibody was first demonstrated in transgenic tobacco with its successful expression in 1989.\textsuperscript{15} Since that time, the biopharmaceutical industry has produced recombinant proteins for use as protective subunit vaccines, diagnostic and therapeutic antibodies, hormones, cytokines and many other proteins for medical or industrial application in plants.\textsuperscript{16} The purpose of the present study was attempted to introduce molecular farming technology to the ophthalmology field, and to explore the ANG’s possibility in protecting TM cell lines.

Materials and Methods

\textbf{Plant-derived ANG-FcK protein development}

cDNA fragments encoding the human ANG fused Fc region of immunoglobulin G-tagged endoplasmic reticulum retention signal, KDEL (ANG-FcK), was cloned into a pBI121 plant expression vector. The gene was inserted with the alfalfa mosaic virus untranslated leader sequence (AMV) of RNA4 under the control of the cauliflower mosaic virus 35S promoter into the vector. The ANG-FcK gene expression cassette was transferred as a HindIII-EcoRI fragment into the plant binary vector pBI121. Agrobacterium-mediated plant transformation was conducted using the vector to generate transgenic tobacco (Nicotiana tabacum) lines expressing ANG-FcK.

Transgenic plant leaf tissue (100 mg) was homogenized in 300 μL of 1× phosphate buffer saline (PBS). Plant extracts were resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was incubated in blocking solution (5% [w/v] skim milk [Fluka] in 1× TBS, 0.05% [v/v] Tween 20 [TBST]), followed by anti-ANG antibody (1:250, Abcam Inc., Cambridge, MA, USA) as the primary antibody and anti-mouse immunoglobulin G2a Fc fragment (1:3,000, Jackson ImmunoResearch Labs, West Grove, PA, USA) conjugated to horseradish peroxidase as the secondary antibody to detect ANG-FcK. The signal was detected using SuperSignal (Pierce, Rockford, IL, USA) chemiluminescence substrate. Rh-ANG (R&D Systems, Minneapolis, MN, USA) was used as a positive control.

To purify plant-derived ANG-FcK, tobacco plant leaves were homogenized using an HR2094 aluminum blender (Philips, Seoul, Korea) on ice with extraction buffer (37.5 mM Tris-HCl pH 7.5, 50 mM NaCl, 15 mM EDTA, 75 mM sodium citrate, and 0.2% sodium thiosulfate). After centrifugation at 8,800 × g for 30 minutes at 4°C, the suspension was filtered through a Miracloth (Biosciences, La Jolla, CA, USA) and its pH was adjusted to 5.1 by the addition of acetic acid (pH 2.4). The solution was centrifuged at 10,200 × g for 30 minutes at 4°C. The pH was brought to 7.0 by
the addition of 3 M Tris-HCl, and ammonium sulfate was added to 8% saturation. After centrifugation at 8,800 × g for 30 minutes at 4°C, the precipitate was discarded and ammonium sulfate was added to the supernatant to 40% saturation. After overnight incubation at 4°C, the solution was centrifuged, the pellet was resuspended in extraction buffer to 1/10 of the original volume, and the final solution was then centrifuged at 10,200 × g for 30 minutes at 4°C. The supernatant was filtered through a 0.45-mm filter and loaded onto a HiTrap Protein A column (Pharmacia, Uppsala, Sweden). Soluble protein extract was applied to a protein A column (GE Healthcare, Piscataway, NJ, USA). Elutes of plant-derived ANG-FcK protein were dialyzed against 1× PBS buffer. Aliquots were frozen in liquid nitrogen and stored at -80°C for the glycosylation analysis.

**Culture of primary human TM cells and chemical treatment**

Primary human TM (HTM) cells were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). HTMs were cultured in TM Cell Medium (Cat No. 6591) containing 2% FBS, TM cell growth supplement (Cat. No. 6592), and 1% penicillin/streptomycin solution in an incubator at 37°C, 5% CO₂ and the medium was changed every 2 days. When cells grew to 95% confluence, they were passaged using standard trypsinization techniques.

For protein and RNA prep, HTM cells were cultured in six-well plates for 2 days. They were washed twice with PBS. The medium of confluent HTM cells was changed to serum-free TMEM 4 hours before treatment. Cells were treated with Rh-ANG (5 μg/mL) or Ang-FcK (5 μg/mL) for 23 hours, and then co-treated 0.001% BAK for 10 minutes. Medium was replaced with serum-free TMEM, following by incubation at 37°C, 5% CO₂ for 1 hour.

**Cell viability assay**

HTM cells were cultured in 96-well plates for 1 day. The medium of confluent HTM cells was changed to serum-free TMEM 4 hours before treatment. HTM cells were incubated in the presence or absence of Rh-ANG (5 μg/mL) or ANG-FcK (5 μg/mL) 23 hours after seeding. They were co-treated with 0.0001%, 0.0005%, 0.001%, and 0.002% BAK for 10 minutes and media were changed to serum-free TMEM. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. In brief, MTT was dissolved in PBS at 5 mg/mL. MTT was added to each well (10 μL per 100 μL of medium), and plates were incubated at 37°C for 2 hours in dark conditions. The medium was replaced with 100 μL of dimethyl sulfoxide, and absorbance was measured at 570 nm using a Spectramax microplate photometer (Molecular Devices, Sunnyvale, CA, USA).

**Immunocytochemical analyses of MMP-1 and α-SMA**

HTM cells were seeded at 5 × 10⁴ cells/cm² on poly-L-lysine-coated 12-mm coverslips. HTM cells cultured for 2 days were washed twice with PBS and fixed with 4% paraformaldehyde for 20 minutes. Non-specific binding was blocked using 10% normal goat blocking solution in PBS (pH 7.4) for 1 hour at room temperature, followed by overnight incubation with anti-MMP-1 or α-SMA antibody (1:100 in PBS containing 1% normal goat serum, 0.1% Triton X-100). Slides were incubated with secondary antibody conjugated to Alexa Fluor™ 488 (green) or Alexa Fluor™ 568 (red) (1:400 in PBS with 5% normal goat serum) for 2 hours at room temperature. Flat-mounts were placed on glass slides with the HTM cell layer facing up and mounted in Fluoroshield™ mounting medium with DAPI. The slides were examined using a confocal microscope (LSM700 META, Carl Zeiss, Jena, Germany).

**Western blot analysis**

Total cell lysates were prepared by lysing HTM with PRO-PREP (iNtRON Biotechnology, Seoul, Korea). The total protein concentration was measured using a spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA). Individual protein samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF; Millipore Corporation). The membrane was blocked with 5% non-fat milk in tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5, 150 mM NaCl). Primary antibodies against MMP-1 (Bioworld Technology, Inc., St. Louis Park, MN, USA), β-actin (Sigma-Aldrich), and
α-SMA (EMD Millipore) diluted in TBS-T (1:1,000) containing 5% BSA were applied to the PVDF membrane and incubated overnight at 4°C. The secondary antibodies were diluted in TBS (1:2,000), applied to the membrane, and incubated for 1 hour at room temperature. After each step, the PVDF membrane was washed four times with TBS-T (0.1% Tween 20 in TBS buffer) for 10 minutes. The protein signal was visualized using an Enhanced Chemiluminescence Western Blotting Detection Kit (Pierce Biotechnology, Inc.).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured HTM cells using TRizol reagent (Invitrogen-Gibco) according to the manufacturer’s instructions. After washing with 75% ethanol, the final RNA was eluted in 30 μL of distilled diethylpyrocarbonate-treated water. Total cDNA was synthesized using a cDNA synthesis kit (Takara Bio Inc., Otsu, Japan). qRT-PCR was performed using SYBR Premix Ex Taq (Takara). SybrGreen fluorescence was quantified using the CFX96™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) and an appropriate standard curve was obtained from autonomous qRT-PCR assay reactions. Each sample and the positive control were analyzed by triplicate qRT-PCR.

Statistical analysis

Cells at passage 3-5 were used for all experiments. The results of cell viability was expressed as means ± standard errors, and normality and equal variances in groups were tested. The mRNA expression was normalized to the expression level of GAPDH and calculated using the following equation: Fold change = $2^{-\Delta\Delta CT}$. Unpaired t-test was used to compare the data, and the probability level for statistical significance was set at 5%. Data were recorded and analyzed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Expression and purification of ANG-FcK in transgenic plants

Randomly selected transgenic plants were selected and their expression was confirmed (Fig. 1A). Western blot analysis with anti-human Fc fragment antibody was conducted to confirm the expression of ANG-FcK in plants. Positive control, the Rh-ANG protein band was detected at approximately 15 kDa and the ANG-FcK was detected at approximately 44 kDa. No band was observed in the non-transgenic plant. ANG-FcK was purified from leaves harvested from transgenic tobacco plants. The protein A column purification yielded an average of 2 mg of plant-derived ANG-FcK per kg of fresh leaves from high protein expressing line. SDS-PAGE analysis of the purified ANG-FcK revealed one major band (44 kDa) (Fig. 1B).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Development of angiogenin fusion protein (ANG-FcK). (A) Expression of ANG-FcK in randomly selected transgenic plants. (+), positive control, recombinant human angiogenin (Rh-ANG); (-), non-transgenic tobacco plant leaf extract. #1167–1187, transgenic plant line number. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis results for purified ANG-FcK. #1–8, purified protein fraction number; Column through, plant extracts passed through a column.
Cellular toxicity induced by BAK in HTM cells

The MTT assay showed a cell viability decrease with BAK concentration, and this decrease of viability was even greater with concentration of 0.002%, reaching less than 40% (Fig. 2A). The lowest concentration (0.0001%) showed no difference with the control. BAK 0.001% with a cell viability decrease of about 70% was used for the other test, because our aim was making degeneration in TM by chronic exposure to low concentration of BAK. The morphological analysis of HTM cells showed that administration of BAK reduced TM cell density and cell shape (Fig. 2B). Both Rh-ANG and ANG-FcK had little effect on the cell viability in HTM cells.

Figure 2. Cellular toxicity induced by benzalkonium chloride (BAK). (A) In a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the viability of human trabecular meshwork cells decreased with the BAK concentration, and this decrease reached about 70% for 0.001% BAK. (B) BAK reduced the human trabecular meshwork cell density and affected cell shape. Rh-ANG = recombinant human angiogenin; ANG-FcK = plant-derived angiogenin fusion protein.

![MTT assay showing cell viability decrease with BAK concentration](image)

Figure 3. Immunocytochemical analyses of matrix metalloproteinase-1 (MMP-1) and alpha smooth muscle actin (α-SMA) in human trabecular meshwork cells. The expression of MMP-1 and α-SMA were increased by benzalkonium chloride (BAK) treatment; however, that were preserved by the single angiogenin (ANG) treatment. The expression of α-SMA was decreased by co-treatment with ANG compared to the single BAK treatment. All images were magnified 200 times. Veh = vehicle for sham-treated control; DAPI = diamidino phenylindol; Rh-ANG = recombinant human angiogenin; ANG-FcK = plant-derived angiogenin fusion protein.
Effects of BAK and ANG on MMP-1 and α-SMA in HTM cells

Single ANG treatment showed no significant change however, increased MMP-1 and α-SMA expression were obvious in response to the BAK treatment by immunocytochemistry (Fig. 3). The combination of ANG and BAK induced more MMP-1 expression and lesser α-SMA expression compared to the single BAK treatment. Expression pattern of MMP-1 and α-SMA in response to the ANG-FcK was similar to that of Rh-ANG with or without BAK. Expression levels of MMP-1 at the protein and mRNA were significantly augmented in BAK treatment groups compared to single ANG and control groups, and the expression in combination ANG with BAK was higher than the single BAK treatment group, but it was not significant statistically (Fig. 4A, B). Expression of α-SMA were significantly increased in BAK treatment groups compared to single ANG and control groups at the protein and mRNA levels. The expression of α-SMA in the single BAK treatment was about 2.2 times ($p < 0.01$) and that in the combination treatment of ANG and BAK were 1.5 to 1.8 times ($p < 0.01$) more than control, and the differences between single BAK and combination with ANG were significant ($p < 0.01$) also (Fig. 4A, C).

Discussion

In this study, we developed ANG-FcK, using molecular farming technique, and examined its defenses against the toxicity of BAK exposure on TM cell lines. Expressions of MMP-1 and α-SMA were increased through BAK treatment in human TM cell lines, and co-treatment with ANG induced more MMP-1 expression and significantly less remarkable α-SMA expression compared to the single BAK treatment, suggesting that ANG prevents fibrosis. Treatment of the ANG-FcK in the TM cell lines showed similar response to that of Rh-ANG, indicating that ANG-FcK has important practical applications as a protecting substance.

The isolation and purification of proteins from plant biomass is one of salient factors in the production of therapeutic proteins by transgenic plants on the molecular level. Fusion of Fc region of immunoglobulin G and KDEL brought on greater molecular weight of the ANG-FcK
(44 kDa) over Rh-ANG (15 kDa), and that might have improved yields by enhancing protein stability and facilitating purification. Purifying plant-derived ANG-FcK yielded an average of 2 mg per kg of fresh leaves. With a cost of approximately 1,000 U.S. dollars per 250 μg of Rh-ANG, 1 kg of transgenic plants is worth approximately 8,000 U.S. dollars of conventional protein. To the best of our knowledge, this is the first study to apply molecular farming techniques in ophthalmology, and our production of recombinant ANG may be beneficial to this field.

Increased MMP-1 and α-SMA expressions in human TM cell lines have been observed in response to the BAK treatment, and these findings imply that BAK causes epithelial mesenchymal transition-like phenomenon and myofibroblast like phenotype change in TM. This epithelial mesenchymal transition like phenomenon results in the abundant expression of fibronectin and activation of motility in TM cells, and the cells switch to a myofibroblast-like phenotype which strengthens simultaneously both their actin cytoskeleton and their directly associated extracellular matrix. Overall, the changes cause an increase in TM rigidity and resistance to aqueous humor outflow.

Combination use of ANG and BAK have been showed more MMP-1 and significantly less noticeable α-SMA expression compared to the single BAK treatment, and these findings support that ANG protects from the fibrosis and myofibroblast like phenotype changes induced by BAK. The protective mechanism of ANG could be explained by the regulation of MMPs that initiate the turnover of extracellular matrix in the TM, and necessary to maintain outflow facility. In human TM cells, MMP-1 was upregulated by the BAK exposure and the levels of MMP-1 expression were elevated with ANG. These results were in close agreement with previous study showed that MMP-1 expression were defective in primary glaucomatous TM cells and augmented by ANG treatment over the course of time. MMP activation by ANG may induce cytoskeletal changes in TM cells, to probably defend against the expression of α-SMA as for a fibrogenic marker.

Our study has its limitations. First, we used TM cells from a commercial source and did not characterize them thoroughly. There is strong evidence in the field that the TM cells from this commercial source are not a pure population. Second, effects of BAK on the TM and ANG’s protective mechanism have not been demonstrated and in vivo studies are needed for more comprehensive data when managing glaucoma. Third, extra in vitro experiments are necessary to investigate the protective mechanism of ANG against BAK. Previous studies showed that ANG may activate Akt-mediated signals for nitric oxide production and TM remodeling by regulating MMP and rho-kinase. Finally, further research on ANG’s effect on retinal ganglion cells may clarify its function and improve its clinical effectiveness. Because glaucoma is an ocular neurodegenerative disease characterized by the progressive death of retinal ganglion cells, the importance of ANG enrichment in normal motor neurons has been observed in studies on amyotrophic lateral sclerosis, a fatal neurodegenerative disease.

Conclusions

In conclusion, ANG’s protective effect on TM may involve an anti-fibrotic function against the exposure to single toxic substance such as BAK. Plant-derived ANG-FcK’s protective effect is similar to that of Rh-ANG, and it is a promising candidate for an alternative eye drop additive. Future studies should focus on ANG’s detailed defense mechanism and potential applications in glaucoma management.

Acknowledgement

This report was supported by the National Research Foundation of Korea (2017R1C1B5018031).

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국문초록

 섬유주세포에서 MMP-1과 α-SMA에 대한 식물생명공학 기법으로 합성한 안지오제닌의 효과

목적: 안압하강제 및 안약 보존제로서 널리 이용되는 벤زال코니움은 사용량에 비례하는 독성을 나타낼 수 있다. 안지오제닌의 다양한 기능이 녹내장 치료에 이용될 수 있다는 기존 보고들에 근거하여, 벤잘코니움으로 손상된 사람 섬유주세포에서 식물생명공학 기법으로 합성한 안지오제닌의 효과를 알아보고자 한다.

대상과 방법: 상용화된 재조합 사람 안지오제닌(Rh-ANG)에 면역글로불린 G의 Fc 부분과 KDEL을 결합하여 식물로부터 안지오제닌(ANG-FcK)을 합성하였다. 면역화학 분석을 통해 단백질과 mRNA 수준에서 섬유주세포에서의 MMP-1과 α-SMA 발현에 대해 확인하였다. 독성 자극으로 벤잘코니움을 이용하였고, 두 가지 안지오제닌(Rh-ANG, ANG-FcK)을 보호물질로 이용하였다.

결과: 벤잘코니움에 노출된 섬유주세포에서 MMP-1과 α-SMA 발현이 유의하게 증가하였다. 안지오제닌을 벤잘코니움과 병용 투여한 결과 MMP-1 발현은 증가하는 양상이었으나, α-SMA 발현은 벤잘코니움 단독 노출에 비해 유의하게 감소하는 양상으로 나타났다. 두 가지 안지오제닌(Rh-ANG, ANG-FcK)의 발현 양상은 큰 차이가 없었다.

결론: 식물생명공학 기법으로 합성한 안지오제닌의 섬유주세포에 대한 효과는 상용화된 안지오제닌과 비슷하였고, 항섬유화 기전과 연관이 있을 것으로 보이며, 녹내장 치료에 이용될 수 있는 가능성을 시사한다.