Inhibition of hypoxia-inducible factor-1α and vascular endothelial growth factor by chrysin in experimental choroidal neovascularization

Ji Hun Song

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Sung Chul Lee

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Ji Hun Song

June 2016

This certifies that the Doctoral Dissertation of Ji Hun Song is approved.

Thesis Supervisor: Sung Chul Lee

Thesis Committee Member#1: Sung Soo Kim

Thesis Committee Member#2: Ho Min Lew

Thesis Committee Member#3: Hyun Chul Chung

Thesis Committee Member#4: Jong Eun Lee

The Graduate School
Yonsei University

June 2016

ACKNOWLEDGEMENTS

I would like to express my gratitude to professor Sung Chul Lee for all the guiding and encouragements during the process of this thesis.

I also cannot leave out professor Sung Soo Kim, Ho Min Lew, Hyun Chul Chung, and Jong Eun Lee for the professional advices they gave me.

I thank my family and my friends for their continuous support and would also like to thank all the staff and residents in Ajou University Department of Ophthalmology for their full-hearted help.

I dedicate the fruits of this research to my beloved wife Sun Ha, my children Yun Ha and Chan Young for their endless trust in me.

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ABSTRACT

Inhibition of hypoxia-inducible factor 1α and vascular endothelial growth factor by chrysin in experimental choroidal neovascularization

Ji Hun Song

Department of Medicine
The Graduate School, Yonsei University

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Purpose: To determine the effects of a single intravitreal injection of chrysin to inhibit angiogenesis in an experimental rat model of choroidal neovascularization (CNV).

Methods: A diode laser was used to break Bruch's membrane in Brown Norway rats. One week later after laser photocoagulation, each rat was injected intravitreally with 5 μl of 15 mg/ml chrysin in the right eye and same amount of solvent solution in the left eye. The formation of CNV was evaluated according to the size and intensity of dye leakage by fluorescein angiography (FA) at 2 weeks. The effect of chrysin on CNV was assessed by FA score and histology. The expression level of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in retina/choroid complex was measured in both chrysin-treated eyes and control eyes.

Results: In chrysin-treated eyes, the size and intensity of fluorescein dye leakage from CNV lesions decreased significantly compared with those in the control group (Mean CNV score, 2.34 ± 0.10 vs. 2.97 ± 0.09, $p < 0.05$ by unpaired t test). When each photocoagulated lesion was categorized into low or high leakage groups, the number of low leakage lesions was significantly greater in chysin-treated than in control eyes ($p < 0.05$, by Pearson chi-square test). The relative risk of control eyes developing high leakage lesions compared with chrysin-treated eyes was 2.03 (95% confidence interval, 1.46-2.83). The mean CVN thickness of chrysin-

treated eyes was significantly thinner than that of control eyes (33.90 ± 0.89 μm vs. 38.50 ± 0.99 μm, $p < 0.05$ by unpaired t test). The mean HIF-1α and VEGF level in chrysin-treated eyes were significantly lower than those in control eyes ($p < 0.05$ in each protein analysis, by unpaired t test). Conclusion: Chrysin inhibited effectively laser-induced CNV in a rat model and downregulated HIF-1α and VEGF. Further studies are warranted to evaluate its potential as a candidate substance of therapy for wet age-related macular degeneration and other CNV associated vision-threatening conditions.

Key words: Choroidal neovascularization, Chrysin, Hypoxia-inducible factor, Intravitreal injection, Vascular endothelial growth factor.

Inhibition of hypoxia-inducible factor 1α and vascular endothelial growth factor by chrysin in experimental choroidal neovascularization

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The Graduate School, Yonsei University

(Directed by Professor Sung Chul Lee)

I. INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss among people older than 50 years of age in the developed conturies.[1] Choroidal neovascularization (CNV) in AMD represents an advanced stage of the disease, and is characterized by the abnormal ingrowth of new vessels under the macula. CNV can also occur in other conditions, including angioid steaks, pathologic myopia, and various inflammatory diseases.[2] The previous treatment modalities for CNV such as laser photocoagulation, photodynamic therapy, and macular translocation resulted in poor visual outcome.[3-5] CNV develops as a result of pathologic angiogenesis, including proteolysis of the extracellular matrix, migration and proliferation of endothelial cells, and synthesis of new matrix components.[6] The development of pharmacologic inhibitors of angiogenesis has thus focused on the inhibition of vascular endothelial growth factor (VEGF) and proteolytic enzyme such as matrix metalloproteinase (MMP).[7-10] Although new therapies using vascular endothelial growth factor (VEGF) antibodies such as bevacizumab, ranibizumab, and aflibercept bring paradigm shift to the treatment of AMD, these treatments also have some limitations.[11-13]

Flavonoids are natural compounds widely distributed in many fruits and vegetables. It is used as healthy dietary supplements or herbal remedies. Some flavonoids have been shown to inhibit the migration and the proliferation of endothelial cells and tube capillary formation in vitro.[14] Flavonoids are also very

safe and has a low toxicity, and thus become good candidates for chemo-prophylactic agents.

Chrysin (5,7-dihydroxyflavone) is a flavonoid substance naturally present in many fruits and vegetables (Figure 1). Because chrysin has been proved to have multiple biological activities such as anti-inflammatory, anti-oxidative, and anticancer effects in recent studies, it has become an attractive element for therapeutic modalities in many fields of medical research.[15-17] Although the exact mechanisms of this biologic activities of chrysin are not fully understood, it is recently found that chrysin inhibits VEGF transcriptional activation, which is regulated by hypoxia-inducible factor (HIF)-1α, in human tumor tissue. Chrysin is also shown to inhibit angiogenesis in nude mice.[18]

In the previous proof-of-concept study, the effects of chrysin on experimental CNV has been tested preliminarily in a small number of animals, and it has founded that chrysin significantly inhibited angiographic leakage of experimental CNV.[19] In this study, it has been further investigated that the inhibitory effects of intravitreally injected chrysin on angiogenesis and HIF-1α/VEGF expression in a rat model of laser-induced CNV.

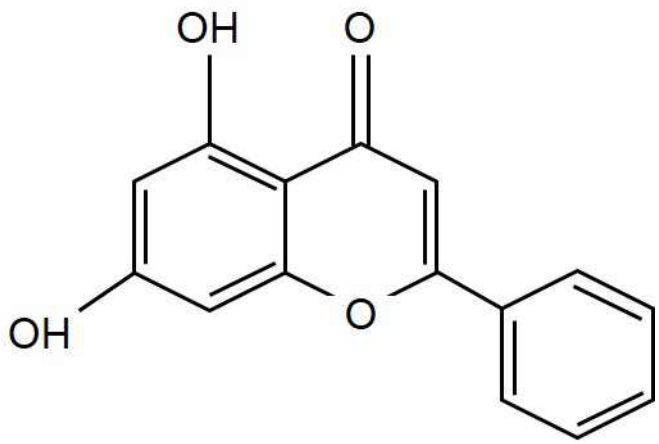

Figure 1. Chrysin (5,7-dihydroxyflavone) molecular structure. Chemical name of chrysin is 5,7-dihydroxy-2-phenyl-4H-1-benzopyran-4-one.

II. MATERIALS AND METHODS

1. Materials

Chrysin of >96% purity and dimethyl sulfoxide (DMSO) of ≥99.9% were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sodium fluorescein was from Alcon laboratories, Inc. (Fort Worth, TX, U.S.A.). Primary antibodies for immunofluorescence technique were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and secondary antibodies were purchased from Invitrogen (Carlsbad, CA, U.S.A.).

2. Laser-induced experimental choroidal neovascularization

The study protocol was approved by the Ajou University Ethics Committee for Animal Experiments. Male Brown Norway (BN) rats (n=20) between seven and nine weeks of age, weighing 200-250 g, were used in this study, in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research. The rats were anesthetized by intramuscular injection of 1:1 mixture of tiletamine / zolazepam (10mg/kg) and xylazine (5mg/kg) for all procedures. The techniques and laser parameters of making experimental CNV were identical to those in our previous study.[19] They are as follows. The pupils were dilated with 1% tropicamide and 2.5% phenylephrine. The fundus was visualized with slide cover glass and with 2.5% hydroxypropyl methylcellulose solution (Methocel: Ciba Vision, Wessling, Germany). Using a frequency-doubled, diode-pumped, solid state laser (VISULAS 532s; Carl Zeiss Meditech Inc., Dublin, CA, U.S.A.) with a wavelength of 532 nm, a spot size of 100 ㎛, 100 ms exposure, and 150 mW power, a pattern of six or seven lesions was concentrically placed at approximately equal distance around the optic disc of both eyes of each rat. Rupture of Bruch's membrane was defined by the presence of acute vapor bubbles. Lesions with subretinal hemorrhage that interfered with evaluation, and adjacent merged lesions were excluded from evaluation.

3. Intravitreal administration of chrysin and control vehicle

After 1 week of laser treatment, all BN rats were anesthetized, and 5 μl volume of chrysin solution (15mg/ml in the injection buffer of DMSO and Balanced Salt Solution) was injected intravitreally in the right eye using a 30 gauge needle. The concentration of chrysin and the concentration of DMSO in the injection buffer was approximately 60 mM and 0.5%, respectively. The same volume of solvent solution was injected intravitreally in the left eye of each rat as control.

4. Fluorescein angiography

Two weeks after laser treatment, fluorescein angiography (FA) was performed using a confocal scanning laser ophthalmoscope (Heidelberg Retinal Angiograph 2, Heidelberg Engineering, Heidelberg, Germany) to evaluate CNV development and activity. The procedures of taking angiogram were identical to those in our previous report.[19] Each rat was injected intravenously with 0.3 ml of 10% fluorescein sodium through the tail vein, and both early (<2 min) and late (>7 min) phase angiogram photographs were taken. The formation of CNV was evaluated according to the size and intensity of dye leakage. This scoring system was adopted and modified from the previously reported study.[20] The lesions were considered “leaky” if hyperfluorescence was observed during early phase angiography and if the size and intensity of leakage increased during late phase. Each photocoagulated lesion was classified according to the fluorescein leakage as grade 1 (minimum leakage or staining of tissue with no leakage); grade 2 (small but evident leakage); grade 3 (moderate intensity and medium size (<1/2 disc diameter) leakage); grade 4 (large evident leakage) (Figure 2). Two examiners judged the scores in a masked fashion; if the two did not agree for a particular lesion, the higher score was used in the analysis. To further analyze the relationship between chrysin treatment and degree of angiographic leakage, each photocoagulated lesion was categorized into low (Grades 1 and 2) or high (Grades 3 and 4) leakage groups.

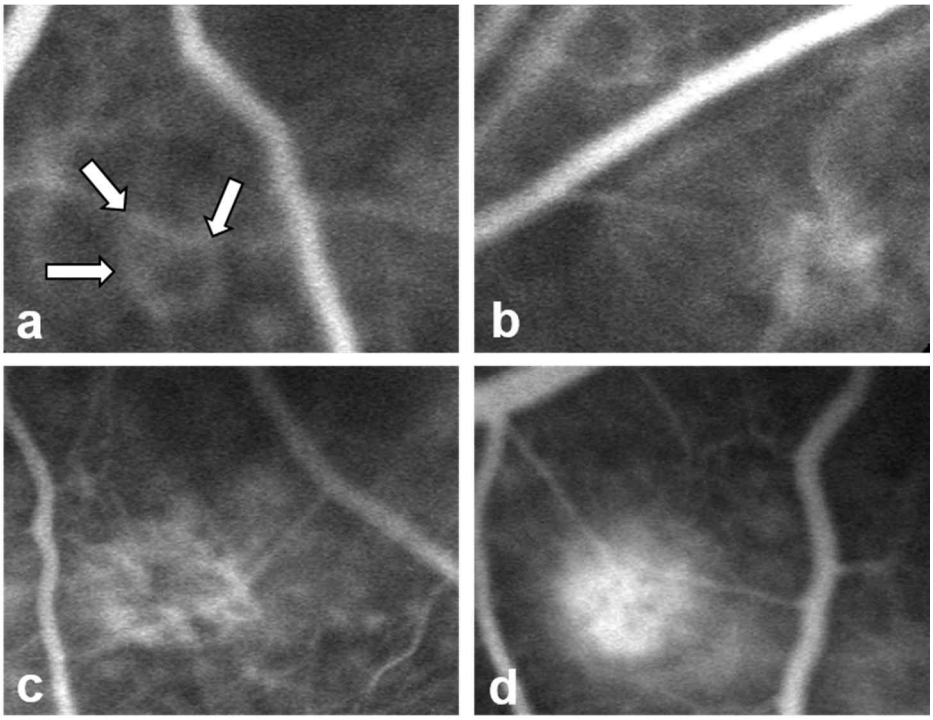

Figure 2. Standard examples of choroidal neovascularization (CNV) grade on fluorescein angiography after laser treatment in rat retina. Each photocoagulated lesion was classified from 1 to 4 according to the size and the intensity of fluorescein dye leakage. (a) Grade 1, minimum leakage or staining of tissue with no leakage (arrows); (b) Grade 2, small but evident leakage; (c) Grade 3, moderate intensity and medium sized (<1/2 disc diameter) leakage; (d) Grade 4, large evident leakage.

5. Histological analysis and immunofluorescence

Two days after angiography, five rats were euthanized and histological analysis was performed. Whole body perfusion fixation was performed with 4% paraformaldehyde fixative before enucleation. Eyes were enucleated and fixed in 4% paraformaldehyde for 6 hours. The anterior segment was removed, and the

eye cup samples were dehydrated and embedded in paraffin. Serial 5 ㎛ sagittal sections were prepared to include all the lesions, and stained with hematoxylin and eosin (H&E). The sections were assessed via light microscopy (Carl Zeiss, Jena, Germany) to determine the effect of chrysin on CNV growth. The maximal CNV thickness was measured from the outer border of pigmented choroidal layer to the highest point of CNV membrane, using the central section of each CNV lesion. Additionally, sections were analyzed by immunofluorescence (IF) technique using a mouse monoclonal HIF-1α antibody and a rabbit polyclonal anti-rat VEGF antibody as primary antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA, U.S.A.). Alexa 555 for HIF-1α and Alexa 488 for VEGF were used as secondary antibody (Invitrogen, Carlsbad, CA, U.S.A.).

6. Quantification of HIF 1α and VEGF in retina and choroid

Fifteen rats were used for the measurement of chorioretinal concentration of HIF 1α and VEGF. Ten days after intravitreal chrysin injection and seventeen days after laser treatment, rats were euthanized and eyeballs were enucleated. The globes were kept on ice until further process of deep freezing and dissection, and then frozen in liquid nitrogen. The anterior segment was removed and frozen globes were cut into halves through optic nerve. The remnantal eye cup samples were further dissected into vitreous, retina-choroid, and sclera under a microscope. The retina/choroid complex was sonicated indirectly in lysis buffer (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 15 minutes. HIF-1α and VEGF levels in the supernatant were detected by ELISA kit (R&D systems, Minneapolis, MN, U.S.A.) and normalized to total protein.

7. Statistical analysis

CNV scores, CNV thickness, and concentration of HIF 1α and VEGF were expressed as mean ± standard error (SE) and compared using unpaired Student's t tests. The chi-square test was used to analyze categorized groups. All analyses were performed using SPSS statistical software (version15.0; SPSS Inc., Chicago, IL, U.S.A.). A p value less than 0.05 was considered statistically significant.

III. RESULTS

1. Mean CNV Grade score

The total number of analyzable CNV lesions in chrysin-treated eyes (n = 20) was 124, and that in control eyes (n = 20) was 128. Table 1 shows the number of CNV lesions belonging to each angiographic Grade in both groups. The mean CNV Grade score, which is calculated per lesions (n=124 in chrysing-treated group, n=128 in control group), of chrysin-treated group was significantly lower than that of control group (2.34 ± 0.10 vs. 2.97 ± 0.09, $p < 0.05$ by unpaired t test) (Figure 3).

Table 1. Number of choroidal neovascularization (CNV) lesions and mean CNV score of chrysin-treated and control eyes

	CNV Grade					Mean CNV
	1	2	3	4	Total	Grade score[1]
Chrysin-treated eyes	37	30	29	28	124	2.34 ± 0.10
Control eyes	19	15	45	49	128	2.97 ± 0.09
p* value						< 0.05

[1] Mean ± standard error (SE).

* Unpaired Student's t test

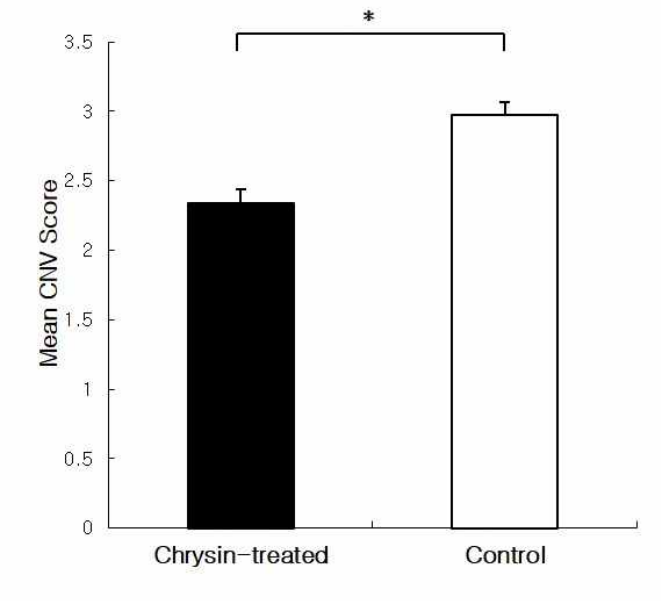

Figure 3. Mean choroidal neovascularization (CNV) Grade score of chrysin-treated and control eyes 1 week after treatment. The mean CNV Grade score of chrysin-treated group was significantly lower than that of control group (*$p < 0.05$ by unpaired t test).

2. Analysis of categorized groups

When each analyzable CNV lesion was categorized into either low (Grade 1 and 2) or high (Grade 3 and 4) leakage group at 1 week after intravitreal treatment, the number of CNV lesions categorized into low leakage group was significantly higher in chrysin-treated eyes than in control eyes (*$p < 0.05$ by Pearson chi-square test). The relative risk of control group for developing high leakage lesions was 2.03, compared with chrysin-treated group (95% confidence interval, 1.46-2.83) (Table 2 and Figure 4).

Table 2. The number of CNV lesions in each categorized group

	Low leakage group	High leakage group	Total
Chrysin-treated eyes	67	57	124
Control eyes[1]	34	94	128
p* value			< 0.05

[1] Relative risk (control/chrysin-treated) = 2.03
95% confidence interval, 1.46-2.83
*Pearson chi-square test

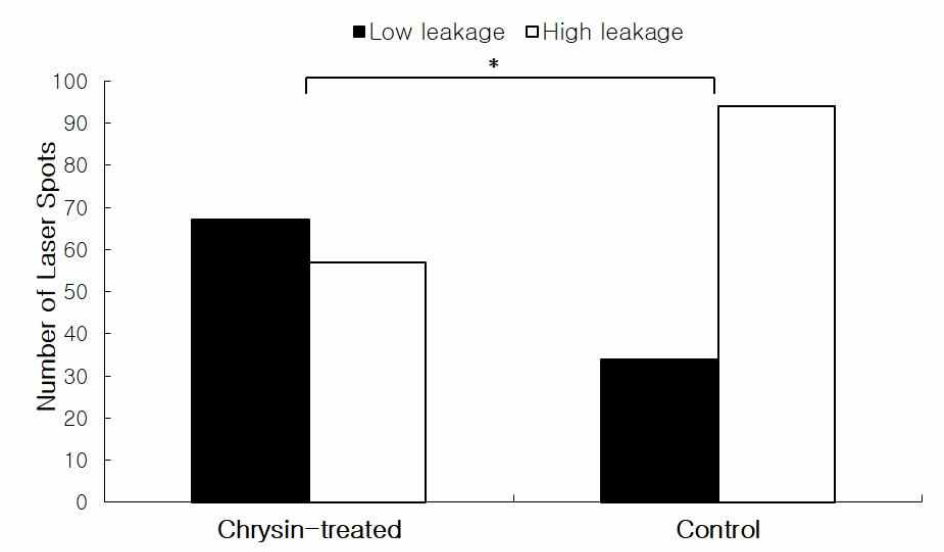

Figure 4. The number of low and high leakage choroidal neovascularization (CNV) lesions in chrysin-treated and control group. When each CNV lesion was categorized into either low (Grades 1 and 2) or high (Grades 3 and 4) leakage groups, the number of low leakage CNV lesions was significantly higher in chrysin-treated group than in control group (*$p < 0.05$ by Pearson chi-square test).

3. Effect of intravitreal chrysin on experimental CNV

Nine days after intravitreal treatment, retinal sections were analyzed using H&E and IF staining (Figure 5). H&E stained sections through the center of CNV lesions (Figure 5a and 6b) show that CNV thickness of chrysin-treated group was significantly smaller than that of control eyes. IF analysis revealed that HIF 1α and VEGF fluorescence was significantly less identified in the retina/choroid complex of chrysin-treated eyes, while more fluorescence was consistently found in the retina/choroid of control eyes (Figure 5c-f). The mean CNV thickness was significantly thinner in chrysin-treated eyes (n = 5) than in control eyes (n = 5) (33.90 ± 0.89 μm vs. 38.50 ± 0.99 μm, $p < 0.05$ by unpaired t test) (Figure 6).

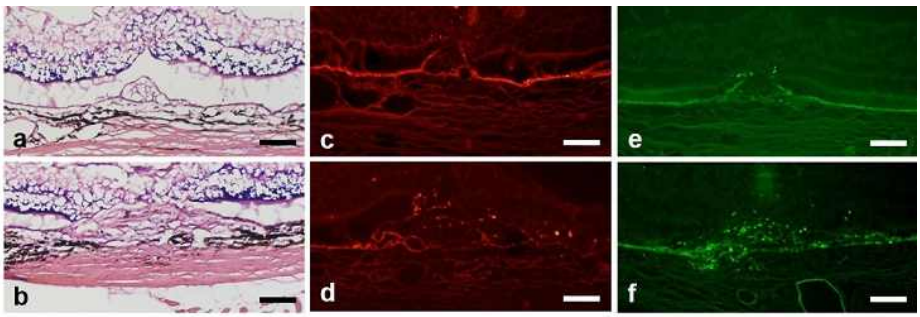

Figure 5. Hematoxylin and eosin (H&E) and immunofluorescence (IF) staining of laser-induced choroidal neovascularization (CNV) lesions. Typical samples of H&E stained sections show that chrysin-treated eyes (a) had much smaller CNV lesions than control eyes (b). IF technique using a mouse monoclonal hypoxia-inducible factor (HIF)-1α antibody and a rabbit polyclonal anti-rat vascular endothelial growth factor (VEGF) antibody demonstrates minimal fluorescence in chrysin-treated eyes (c and e), while more abundant fluorescence in control eyes (d and f). Scale bar = 50 μm.

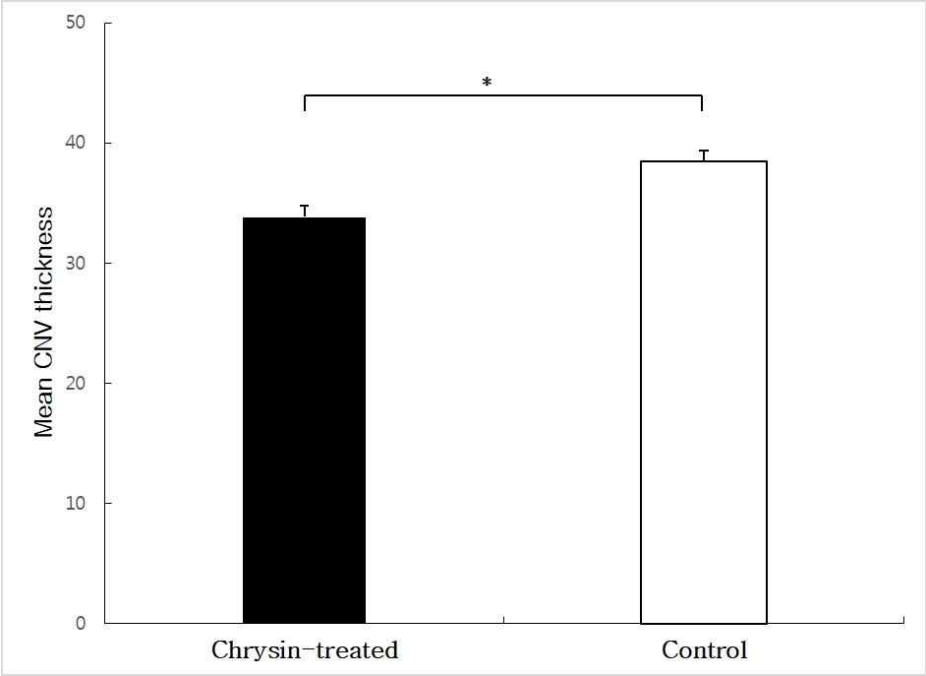

Figure 6. Thickness of experimental choroidal neovascularization (CNV) in chrysin-treated and control eyes. Nine days after intravitreal chrysin treatment, CNV thickness was compared between chrysin-treated group (n=5) and control group (n = 5). The mean thickness of CNV in chrysin-treated group was significantly smaller than that in control group (*$p < 0.05$ by unpaired t test).

4. HIF-1α and VEGF levels in the retina/choroid complex

The mean HIF-1α level in chrysin-treated eyes (n = 15) was significantly lower than that in control eyes (n = 15) (29.13 ± 1.97 vs. 46.40 ± 1.15, pg/mg total protein, $p < 0.05$ by unpaired t test) (Figure79a). The mean VEGF level in the retina/choroid complex was also diminished significantly in chrysin-treated eyes (n = 15) than in control eyes (n = 15) (97.93 ± 3.47 vs. 124.80 ± 5.13, pg/mg total protein, $p < 0.05$ by unpaired t test) (Figure 7b).

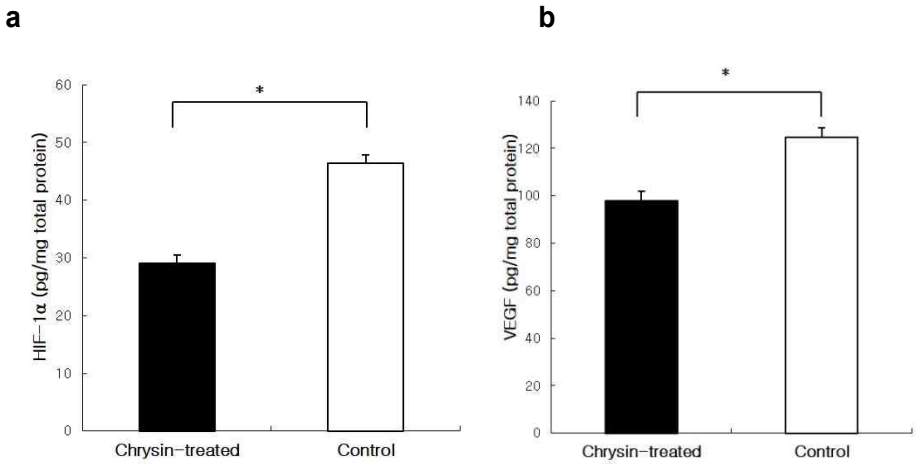

Figure 7. The hypoxia inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) levels in chrysin-treated and control eyes. HIF-1α and VEGF protein were measured by ELISA and normalized to total protein. Number of eyes in each group was 15. (a) The HIF-1α level in the retina/choroid complex was significantly lower in chrysin-treated eyes than in control eyes. (b) The VEGF level in chrysin-treated eyes was also significantly diminished compared with that of control eyes. (*$p < 0.05$ by unpaired t test).

IV. DISCUSSION

AMD is commonly a bilateral condition affecting older age groups and causes severe visual impairment.[21] CNV is the typical feature of neovascular form of AMD, and moreover, various diseases of retina and choroid can also accompany CNV as a secondary manifestation. Anti-VEGF agents is currently the standard-of-care therapy for this form of AMD. This treatment can maintain vision in most of the patients, but still have some drawbacks. Because patients need monthly clinic visits and frequent retreatments, current intravitreal injection therapy of anti-VEGF agents costs a lot of expense and patients' time.

Although the underlying pathogenesis of the development of CNV is not fully

elucidated, VEGF, a diffusible cytokine that promotes angiogenesis and vascular permeability, has been shown to play a key role and there is much evidence that the development of CNV is promoted by increased expression of VEGF.[22,23] VEGF expression is found to be increased not only in AMD patients but also in laser-induced CNV in animal model.

Transcription of VEGF gene is mainly regulated by HIF-1. HIF-1 is a heterodimeric transcription factor composed of 2α and 2β subunits. It binds to the hypoxia response element of the VEGF promotor region and activates VEGF gene transcription.[24] HIF-1α-medicated VEGF expression can be induced by growth factors, oncogenes and hypoxia.[25] Overexpression of HIF-1 has been found in various human cancer tissues and its level of activity correlates with angiogenesis and tumorigenicity.[26] Antiangiogensis therapies for the treatment of CNV have thus targeted the HIF-1α/VEGF system.

Dietary intake of antioxidants and some other supplements was shown to slow progression to advanced AMD.[27,28] Plant-based dietary intervention including flavonoids has been found to be effective for modifying cancer risk and preventing other chronic diseases.[15,29] Flavonoids also regulate cell proliferation and in vitro angiogenesis in tumor tissue, as well as endothelial cell function in laser-induced CNV.[30,31]

Chrysin has been previously studied as an antitumor agent.[32] In our previous study using a small number of rats, chrysin proved to inhibit angiographic leakage in laser-induced CNV.[19] Based on that result, we further investigated the inhibitory effects of intravitreally injected chrysin on angiogenesis. The effect of chrysin on HIF-1α and VEGF expression was also evaluated to investigate the hypothesis that intravitreal injection of chrysin may decrease HIF-1α and VEGF levels within the laser-induced CNV lesions.

In the present study, it was demonstrated that chrysin-treated eyes showed significantly less leakage than control eyes on fluorescein angiography. CNV thickness was significantly reduced and less immunofluorescence in neovascular tissue was detected in chrysin-treated eyes compared with control eyes, suggesting that chrysin inhibits the development and growth of CNV.

Furthermore, expression levels of HIF-1α and VEGF in the retina/choroid complex were significantly diminished by intravitreal chrysin treatment.

In vitro studies of tumor tissue, chrysin is shown to inhibit VEGF gene transcription by inhibiting HIF-1α expression. Level of HIF-1α is regulated by protein synthesis and degradation, and chrysin has an effect on both processes. Phosphatidylinositol 3-kinase/AKT signaling pathway has an important role in the expression of HIF-1α. Chrysin was revealed to inhibit phosphorylation of AKT, and thus suppress HIF-1α expression through AKT signaling. Chrysin was also found to reduce the HIF-1α half-life and stability. The prolyl hydroxylation of HIF-1α at oxygen-dependent degradation domain (ODD) is critical in the regulation of HIF-1α within steady state level. Chrysin promotes prolyl hydroxylation of ODD of HIF-1α. It facilitates ubiquitination and thereafter promotes proteasome degradation of HIF-1α. Lastly, binding of HIF-1α with heat shock protein (HSP) 90 stabilizes HIF-1α. Chrysin inhibits HIF-1α binding to HSP 90, resulting in interfering with the interaction between HIF-1α and HSP 90. This also promotes degradation of HIF-1α.[18]

One of the limitations of this study is the relatively high concentration of chrysin intravitreally injected. The dose of chrysin used was 15 mg/ml, corresponding to a concentration of about 60mM, because chrysin is verified to have a poor bioavailability. This is beyond a sufficient dose, and other similar small molecule inhibitors for inhibition of CNV were usually used at concentrations of about 10 μM-1mM. The effect of different doses of chrysin on CNV needs to be further investigated in future study.

With higher concentration than the usual, 0.5% concentration of DMSO was used in the buffer, because of the poor solubility of chrysin. There is a possibility that this high percentage of DMSO itself could have a suppressive effect on CNV. However, the same concentration and amount of DMSO solution was also injected to the control group and statistical analysis could disclose the differences of therapeutic effect between the two groups. Chrysin-treated eyes demonstrated a significantly lower mean CNV Grade score, the lower relative risk for developing high leakage lesions, the thinner CNV membranes, and the lower

levels of HIF-1α and VEGF than control eyes.

Another limitation of this study is a possible bias that may have arisen in the procedure of scoring CNV Grade. I decided CNV Grade score based on fluorescein angiography, and the fine scoring of CNV Grade was sometimes confoundable while a presence of CNV lesion could be easily determined. I made greater effort to minimize this bias by two examiners judging the scores in a masked fashion and using the higher score on disagreement.

This results suggest that chrysin inhibits lase-induced experimental CNV in animal model and also present that the in vivo evidence that chrysin suppresses HIF-1α/VEGF expression in CNV lesion for the first time. This results provide the possibilities of using chrysin as an therapeutic agent for neovascular AMD and other CNV-associated conditions. Furthermore, prevention of CNV development is considered to be more important than before because number of patients with neovascular AMD is growing faster and AMD is anticipated to become more serious health-care burden owing to progression of population aging in the westernized countries. Current strategies for the prevention of AMD such as the use of some dietary supplements proved to reduce risk of progression to advanced AMD, however, additional preventive strategies are still needed.[27,28] The present results suggest that chrysin, a common and readily available natural flavonoid, may be a candidate for not only pharmacologic treatment but also preventive measures of CNV in AMD and other CNV-associated diseases.

V. CONCLUSION

Chrysin inhibited effectively the angiographic leakage of laser-induced CNV in a rat model and downregulated HIF-1α and VEGF in the retina/choroid complex of experimental CNV. Further studies are warranted to evaluate its potential as a candidate substance of therapy for neovascular age-related macular degeneration and other CNV associated vision-threatening conditions.

REFERENCES

1. Fine SL, Berger JW, Maguire MG, Ho AC. Age-related macular degeneration. N Engl J Med 2000;342:483-92.

2. Weber ML, Heier JS. Choroidal Neovascularization Secondary to Myopia, Infection and Inflammation. Dev Ophthalmol 2016;55:167-75.

3. Moisseiev J, Alhalel A, Masuri R, Treister G. The impact of the macular photocoagulation study results on the treatment of exudative age-related macular degeneration. Arch Ophthalmol 1995;113:185-9.

4. Mandal N, Chisholm IH. Identifying the proportion of age related macular degeneration patients who would benefit from photodynamic therapy with verteporfin (Visudyne). Br J Ophthalmol 2002;86:118-9.

5. Macular translocation. American Academy of Ophthalmology. Ophthalmology 2000;107:1015-8.

6. Rakic JM, Lambert V, Munaut C, Bajou K, Peyrollier K, Alvarez-Gonzalez ML, et al. Mice without uPA, tPA, or plasminogen genes are resistant to experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 2003;44:1732-9.

7. Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338-46.

8. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina 2002;22:143-52.

9. Kvanta A, Shen WY, Sarman S, Seregard S, Steen B, Rakoczy E. Matrix metalloproteinase (MMP) expression in experimental choroidal neovascularization. Curr Eye Res 2000;21:684-90.

10. Takahashi T, Nakamura T, Hayashi A, Kamei M, Nakabayashi M, Okada AA, et al. Inhibition of experimental choroidal neovascularization by overexpression of tissue inhibitor of metalloproteinases-3 in retinal pigment

epithelium cells. Am J Ophthalmol 2000;130:774-81.

11. Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419-31.

12. Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med 2011;364:1897-908.

13. Yuzawa M, Fujita K, Wittrup-Jensen KU, Norenberg C, Zeitz O, Adachi K, et al. Improvement in vision-related function with intravitreal aflibercept: data from phase 3 studies in wet age-related macular degeneration. Ophthalmology 2015;122:571-8.

14. Trochon V, Blot E, Cymbalista F, Engelmann C, Tang RP, Thomaidis A, et al. Apigenin inhibits endothelial-cell proliferation in G(2)/M phase whereas it stimulates smooth-muscle cells by inhibiting P21 and P27 expression. Int J Cancer 2000;85:691-6.

15. Birt DF, Pelling JC, Nair S, Lepley D. Diet intervention for modifying cancer risk. Prog Clin Biol Res 1996;395:223-34.

16. Lapidot T, Walker MD, Kanner J. Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells in vitro. J Agric Food Chem 2002;50:7220-5.

17. Cho H, Yun CW, Park WK, Kong JY, Kim KS, Park Y, et al. Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives. Pharmacol Res 2004;49:37-43.

18. Fu B, Xue J, Li Z, Shi X, Jiang BH, Fang J. Chrysin inhibits expression of hypoxia-inducible factor-1alpha through reducing hypoxia-inducible factor-1alpha stability and inhibiting its protein synthesis. Mol Cancer Ther 2007;6:220-6.

19. Song JH, Kim YH, Lee SC, Kim MH, Lee JH. Inhibitory Effect of Chrysin (5,7-Dihydroxyflavone) on Experimental Choroidal Neovascularization in Rats. Ophthalmic Res 2016;56.

20. Sagara N, Kawaji T, Takano A, Inomata Y, Inatani M, Fukushima M, et al. Effect of pitavastatin on experimental choroidal neovascularization in rats.

Exp Eye Res 2007;84:1074-80.

21. Bressler NM. Age-related macular degeneration is the leading cause of blindness. Jama 2004;291:1900-1.

22. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1996;37:855-68.

23. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest Ophthalmol Vis Sci 1996;37:1929-34.

24. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604-13.

25. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002;277:38205-11.

26. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5.

27. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. Arch Ophthalmol 2001;119:1417-36.

28. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. JAMA 2013;309:2005-15.

29. Hollman PC, Katan MB. Dietary flavonoids: intake, health effects and bioavailability. Food Chem Toxicol 1999;37:937-42.

30. Zou Y, Chiou GC. Apigenin inhibits laser-induced choroidal neovascularization and regulates endothelial cell function. J Ocul Pharmacol Ther 2006;22:425-30.

31. Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, et al. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Res 1997;57:2916-21.

32. Zhang T, Chen X, Qu L, Wu J, Cui R, Zhao Y. Chrysin and its phosphate ester inhibit cell proliferation and induce apoptosis in Hela cells. Bioorg Med Chem 2004;12:6097-105.

ABSTRACT(IN KOREAN)

실험적 맥락막신생혈관에서 chrysin에 의한 hypoxia-inducible factor 1α와 vascular endothelial growth factor의 억제 효과

<지도교수 이 성 철>

연세대학교 대학원 의학과

송 지 훈

연구배경 및 목적: Chrysin은 자연상태에서 존재하는 flavonoid 물질로서 항염증, 항산화 및 항암 효과 등을 포함한 다양한 생물학적 활성을 가진 것으로 알려졌다. 본 연구에서는 쥐에서 실험적으로 유도된 맥락막신생혈관(choroidal neovascularization, CNV)에서 유리체강 내로 단일 주사된 chrysin에 의한 신생혈관형성 억제효과에 대하여 알아보고자 하였다.

연구방법: Diode laser를 이용하여 Brown Norway rat의 양안에 브루크막 파열을 유도하였다. 레이저 치료 1주 후 우안에는 15 mg/ml 농도의 chrysin 용액 5 μl를, 좌안에는 동일 용량의 용매 용액을 유리체강 내로 1회 주사하였다. 레이저 치료 2주 째 형광안저혈관조영술을 이용하여 검사 상 나타나는 형광물질 누출의 크기와 강도에 따라 CNV의 발생을 평가하였다. CNV에 대한 chrysin의 효과는 형광안저혈관조영술 및 조직학적 검사를 통해 확인하였으며, chrysin 투여를 받은 처치군과 용매 만을 투여받은 대조군 각각에서 맥락막/망막 복합체 내의 hypoxia-inducible factor-1α (HIF-1α)와 vascular endothelial growth factor (VEGF)의 발현수준을 측정하여 비교하였다.

결과: CNV로부터 발생하는 형광누출의 크기와 강도는 대조군에 비하여 처치군에서 유의하게 감소하였다(Mean CNV score, 2.34 ± 0.10 vs. 2.97 ± 0.09, $p < 0.05$ by unpaired t test). 레이저 치료로 발생된 병변 각각을 저누출군 또는 고누출군으로

분류하였을 때, 대조군에 비하여 처치군에서 저누출군에 속하는 병변의 수가 유의하게 더 많았다($p < 0.05$, by Pearson chi-square test). 처치군에 비하여 대조군에서 레이저에 의해 발생된 병변이 고누출군에 속할 상대 위험도는 2.03 (95% confidence interval, 1.46-2.83)이었다. 평균 CNV 두께는 대조군에 비하여 처치군에서 유의하게 작았다(33.90 ± 0.89 μm vs. 38.50 ± 0.99 μm, $p < 0.05$ by unpaired t test). 맥락막/망막 복합체 내의 HIF-1α와 VEGF의 발현 정도 역시 대조군에 비하여 처치군에서 유의하게 낮게 나타났다($p < 0.05$ in each protein analysis, by unpaired t test).

결론: Chrysin은 쥐에서 실험적으로 유발된 CNV를 억제하였으며 HIF-1α와 VEGF의 발현을 하향조절하는 것으로 확인되었다. 향후 습성연령관련황반변성을 포함한 CNV와 연관되어 심각한 시력저하를 유발하는 망막질환들의 치료를 위한 후보물질로서 chrysin에 대한 추가적인 연구가 필요할 것으로 생각된다.

핵심되는 말 : 맥락막신생혈관, chrysin, hypoxia-inducible factor, 유리체강내 주사, vascular endothelial growth factor