Inhibition of choroidal neovascularization in mice by systemic administration of the multikinase inhibitor, sorafenib

Eun Jee Chung

Department of Medicine

The Graduate School, Yonsei University

Inhibition of choroidal neovascularization in mice by systemic administration of the multikinase inhibitor, sorafenib

Directed by Professor Hyoung Jun Koh

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

Eun Jee Chung

June 2009

This certifies that the Doctoral Dissertation of Eun Jee Chung is approved.

Thesis Supervisor: Hyoung Jun Koh

Thesis Committee Member#1: Joon Haeng Lee

Thesis Committee Member#2: Kyung Ah Park

Thesis Committee Member#3: Kook In Park

Thesis Committee Member#4: Min Goo Lee

The Graduate School

Yonsei University

June 2009

ACKNOWLEDGEMENTS

I would first like to thank God for giving me the privilege of becoming a physician, and the opportunity to further my education in the field of ophthalmology.

I thank professor Hyoung Jun Koh for all his guidance and encouragement not only during my years of fellowship but throughout the years of this degree. His passion and effort in academic and clinical fields of retina have always been an inspiration to me. Also I am grateful to professor Joon Haeng Lee, Kyung Ah Park, Kook In Park, and Min Goo Lee for their teaching and advice in writing of this thesis.

I dedicate the fruits of this research to my beloved family, without whom this work could not have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Inhibition of choroidal neovascularization in mice by systemic administration of the multikinase inhibitor, sorafenib

Eun Jee Chung

Department of Medicine

The Graduate School, Yonsei university

(Directed by Professor Hyoung Jun Koh)

Choroidal neovascularization (CNV) is known to be the leading cause of irreversible vision loss in patients with age-related macular degeneration. A variety of preclinical and clinical studies suggest that vascular endothelial growth factor (VEGF) is a central player in pathologic neovascularization in the eye. Although VEGF clearly has a central role in the development of neovascular diseases, other growth factor pathways, including those that

signal through additional receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptors (FGFRs), have also been implicated in neovascularization and ocular diseases.

Sorafenib (Nexavar®, Bay-43-9006, Bayer Schering Pharma, Germany) is a novel multikinase inhibitor that was recently approved by the Food and Drug Administration for the treatment of renal cell carcinoma. In addition to Raf kinases, several other kinases, including VEGFR2, VEGFR3 and PDGFR-β, are inhibited by sorafenib.

This study is to explore the anti-angiogenic properties of sorafenib in an animal model of CNV.

Sorafenib or vehicle was administered orally to female C57BL/6 mice at the onset (day 0) of experiments. CNV was induced by laser photocoagulation the following day. After 14 days, mice were perfused with fluorescein-labeled dextran, and the area of CNV was measured on choroidal flat mounts by

image analysis. In some groups of mice, treatments were started 7 days after the laser photocoagulation to determine the effect of the agent on established CNV. Expression of phosphorylated extracellular signal-regulated kinase (p-ERK) in choroidal tissues was measured by Western-blot analysis to demonstrate the kinase-inhibitory effect of sorafenib in intracellular signaling pathways involved in CNV formation.

Sorafenib significantly reduced the extent of CNV in a dose-dependent manner. The area of CNV was reduced by 43% in the 30-mg·kg^{-1}·day^{-1} group and by 61% in the 60-mg·kg^{-1}·day^{-1} group compared with vehicle-treated controls (both $P < 0.0001$). Oral administration of sorafenib also caused significant regression of established CNV. The area of CNV was reduced by 59% in the 30-mg·kg^{-1}·day^{-1} group and by 66% in the 60-mg·kg^{-1}·day^{-1} group compared with both baseline and control measurements ($P < 0.0001$). The expression of p-ERK in choroidal tissues was increased within 1 day of laser

photocoagulation and remained elevated for 2 weeks. The expression of p-ERK was suppressed by sorafenib.

In conclusion, the current study showed that oral administration of the multikinase inhibitor sorafenib significantly suppressed the development of laser-induced CNV and caused regression of established CNV in mice. Sorafenib interferes with multiple pro-angiogenic receptor tyrosine kinases, including VEGFR 2, PDGFR-β and Raf kinase, and thus holds promise for the treatment of CNV in clinical settings.

Key words: antiangiogenesis; choroidal neovascularization; extracellular signal-regulated kinase; sorafenib

Inhibition of choroidal neovascularization in mice by systemic administration of the multikinase inhibitor, sorafenib

Eun Jee Chung

Department of Medicine

The Graduate School, Yonsei university

(Directed by Professor Hyoung Jun Koh)

I. INTRODUCTION

Choroidal neovascularization (CNV) is known to be the leading cause of irreversible vision loss in patients with age-related macular degeneration.[1] The pathogenesis of CNV is multifactorial, involving RPE (retinal pigment epithelium) alterations, ruptures of Bruch's membrane and pathologic angiogenesis. Although a large number of growth factor pathways have been implicated in angiogenesis, a variety of preclinical and clinical studies suggest that vascular endothelial growth factor (VEGF) is a central player in

pathologic neovascularization in the eye and elsewhere in the body.[2] Elevated levels of VEGF mRNA and protein have been found in ocular tissues and fluids from patients with CNV and in laser-induced CNV animal models.[3-7]

VEGF signals through a family of tyrosine kinase receptors that includes Flt-1(VEGFR1) and KDR (VEGFR2/Flk-1). VEGF binding to KDR causes receptor dimerization and autophosphorylation on several tyrosine residues in the KDR cytoplasmic domain.[8] Activation of KDR in this manner leads to signaling through a variety of pathways, including the Raf/MEK/ERK pathway, which is involved in endothelial cell proliferation.[9-13]

Although VEGF clearly has a central role in the development of neovascular diseases, other growth factor pathways, including those that signal through additional receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and fibroblast growth factor receptors (FGFRs), have been implicated in neovascularization and ocular diseases.[14-16] Some studies have

demonstrated more potent decreases in angiogenesis with the use of anti-angiogenic agents that inhibit multiple tyrosine kinase receptors, and inhibition of PDGF-B signaling has been reported to enhance the efficacy of anti-VEGF therapy in multiple models of ocular neovascularization.[17-19]

Sorafenib (Nexavar®, Bay-43-9006, Bayer Schering Pharma, Germany) is a novel multikinase inhibitor that was recently approved by the Food and Drug Administration for the treatment of renal cell carcinoma. In addition to Raf kinases, several other kinases, including VEGFR2, VEGFR3 and PDGFR-β, are inhibited by sorafenib.[20] The fact that sorafenib has inhibitory activities against a number of kinases implicated in angiogenesis, including VEGFR2, PDGFR-β and Raf, indicates that this drug is an inhibitor of angiogenesis. In this study, we have explored the anti-angiogenic properties of sorafenib in an animal model of CNV.

II. MATERIAL AND METHODS

1. Drug

Sorafenib tablets were ground in a mortar and dissolved in Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethanol) as a 4X stock solution that was freshly prepared every 4 days. The final 1X dosing concentration was prepared by diluting with sterile water immediately prior to administration to mice. The solution was administered orally by gavage.

2. Preventive treatment of laser-induced CNV

Mice were treated in accordance with the Association for Research in Vision and Ophthalmology guidelines for the use of animals in research. Choroidal neovascularization was induced by laser photocoagulation-induced rupture of Bruch's membrane, as described previously.[21] Briefly, 9–10-week-old female C57BL/6 mice were anesthetized with intraperitoneal zoletil (21 mg/kg,

Zoletil 100; Virbac, Carros, France) and xylazine (7 mg/kg) and their pupils were dilated with 0.5% tropicamine and 0.5% phenylephrine (Mydrin-P, Santen, Osaka, Japan). To generate burns, three bursts of 532 nm diode laser photocoagulation (75-μm spot size, 0.1-second duration, 120 mW) were delivered to each retina using the slit-lamp delivery system of Visulas 532s (Carl Zeiss, Jena, Germany), and using a handheld cover slip as a contact lens to view the posterior pole of the retina. Only burns that produced a bubble, indicating rupture of Bruch's membrane, were included in the study.

The mice were randomized into three groups: (1) placebo, 150 μl/day of vehicle via p.o. gavage; (2) sorafenib, 30 $mg \cdot kg^{-1} \cdot day^{-1}$ via p.o. gavage; and (3) sorafenib 60 $mg \cdot kg^{-1} \cdot day^{-1}$, via p.o. gavage. Vehicle and sorafenib treatment started on day 0 (i.e., 1 day before laser photocoagulation).

Two weeks after rupture of Bruch's membrane, the sizes of CNV lesions were measured on choroidal flat mounts by an investigator blinded with

respect to treatment group.[22] Mice used for the flat-mount technique were deeply anesthetized and perfused with 1 ml phosphate-buffered saline containing 50 mg/ml fluorescein-labeled dextran ($2X10^6$ average molecular weight; Sigma, St. Louis, MO), as previously described.[23] The mice were euthanized humanely, and eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts (four or five) were made from the edge to the equator and the eyecup was flat mounted in Aquamount with the sclera facing down. Flat mounts were examined by fluorescence microscopy using an Axioplan 2 microscope (Carl Zeiss, Jena, Germany), and images were digitized using an Axiocam camera and Axiovision version 4.5 image capture software. MetaMorph (version 4.6r5) Professional Image Analysis software (Universal Imaging Corp.; Downingtown, PA) was used to measure the total area of CNV associated

with each burn, with the operator blinded with respect to treatment group.

3. Treatment of established CNV

To determine whether sorafenib was effective against established CNV, mice with Bruch's membrane ruptures at three locations in each eye were divided into four groups. In one group, mice were perfused 7 days after rupture of Bruch's membrane to measure the baseline size of CNV present at 7 days. The other three groups received 30 $mg \cdot kg^{-1} \cdot day^{-1}$ or 60 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o. sorafenib or vehicle starting on day 7 after rupture of Bruch's membrane. On day 14, the mice were perfused with fluorescein-labeled dextran and the CNV area at each Bruch's membrane rupture site was measured on choroidal flat mounts.

4. Histologic procedures

A subset of mice (n=2 for each group) was sacrificed and the eyes were enucleated and processed for histopathology analysis. For histopathologic evaluation, the cornea was perforated by a needle to allow better penetration of the fixative and the intact eye was placed in 4% paraformaldehyde overnight and paraffin embedded using standard techniques. The eyes were sectioned into 5-μm-thick slices, stained with hematoxylin and eosin, and mounted on glass slides.

5. Western-blot analysis of the choroid and RPE layer

ERK phosphorylation was semiquantitatively evaluated by Western-blot analysis of the choroid and RPE layer from laser-treated and untreated C57BL/6 mice (n=3 mice for each group at each time point). Briefly, the vitreous and retina were removed, and the choroid and RPE layer were pooled

in 100 µl of lysis buffer (Pro-prep™ Protein Extraction Solution, iNtRON Biotechnology, Korea) and homogenized using a Precellys 24-bead–based homogenizer (Bertin technologies, France). The samples were then cleared by microcentrifugation (14,000 rpm, 15 minutes, 4°C) and protein concentrations were determined by the Bradford assay (BioRad laboratories, Munich, Germany). Fifteen micrograms of protein per sample were electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad) and blocked with a 3% BSA solution. Phosphorylated ERK (p-ERK) and total ERK were detected using a phospho-specific ERK 1/2 (pThr202/pTyr204) antibody (Cell Signaling, Danvers, MA) and a p44/42 mitogen-activated protein kinase antibody (Cell Signaling), respectively. After washing with Tris-buffered saline (TBS)-Tween 0.05%, blots were incubated with the respective secondary peroxidase-labeled antibody for 1 hour at room temperature. The blots were then washed four times with TBS-Tween 0.05%

and processed for chemiluminescence detection of the immunoreactive proteins by Western-blot analysis using a peroxidase substrate (Lumigan™ PS-3; Lumigen, Inc., Southfield, MI). The densities of immunoreactive bands were measured using ImageJ for Windows (NIH, Bethesda, MD). p-ERK protein expression levels in laser-treated and untreated mice were normalized to the corresponding levels of total ERK protein and expressed as arbitrary units. Three independent experiments were evaluated.

6. Statistical analyses

Data were analyzed using a linear mixed model that accounts for possible correlations in measurements from the same mice. Dunnett's adjustment was made for multiple comparisons. The level of statistical significance was set at $P < 0.05$.

III. RESULTS

1. Quantitative assessment of laser-induced CNV after oral administration of sorafenib

Sorafenib treatment (p.o.) for 2 weeks did not induce any significant systemic adverse effects (such as weight loss, severe infection, death) and recipient mice maintained normal appetite and activity levels. Sorafenib treatment caused a significant reduction in the extent of CNV. An analysis of choroidal flat mounts showed that the mean area of CNV produced by laser treatment was 0.048 ± 0.014 mm^2 in the control (placebo-treated) group, 0.027 ± 0.011 mm^2 in the 30-$mg \cdot kg^{-1} \cdot day^{-1}$ group, and 0.018 ± 0.006 mm^2 in the 60-$mg \cdot kg^{-1} \cdot day^{-1}$ group (Fig. 1).

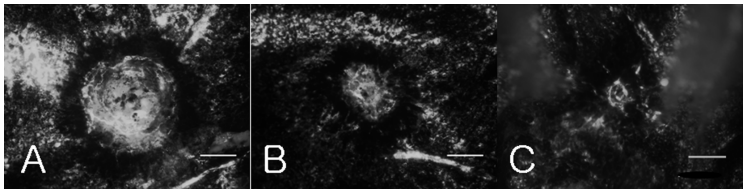

Figure 1 A, B, C. Oral administration of sorafenib suppresses the development of choroidal neovascularization (CNV) at Bruch's membrane rupture sites. Representative choroidal flat mounts after perfusion with fluorescein-labeled dextran 2 weeks after laser photocoagulation. Sorafenib (or vehicle) was delivered daily by oral gavage beginning 1 day prior to laser

treatment. (A) Control group, (B) 30 mg·kg^{-1}·day^{-1} sorafenib, (C) 60 mg·kg^{-1}·day^{-1} sorafenib. Scale bar represents 100 μm.

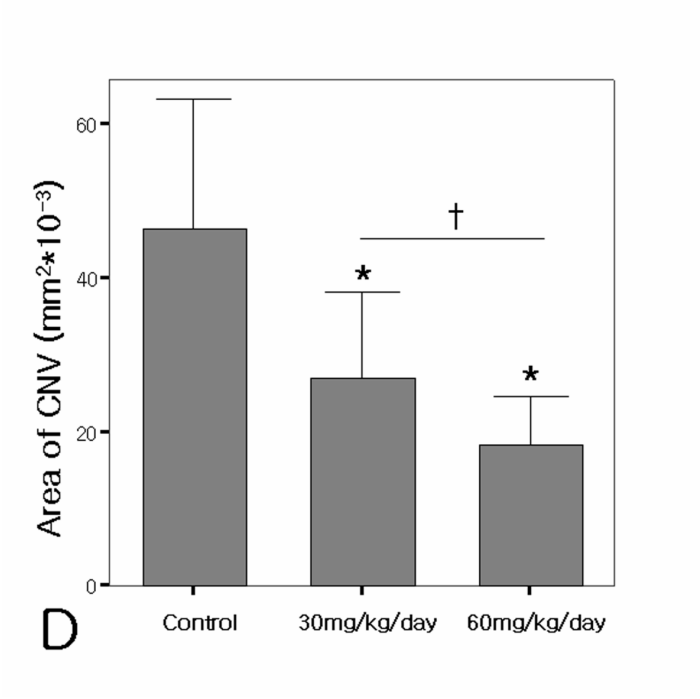

Figure 1 D. Image analysis confirmed that there significantly less CNV in mice that received sorafenib compared with those that received vehicle. *P < 0.0001 compared with vehicle control. †P = 0.0349 when 30 mg·kg^{-1}·day^{-1} and 60 mg·kg^{-1}·day^{-1} sorafenib treatment groups were compared.

This translated into a 43 % decrease in CNV area in the low-dose group and a 61 % decrease in the high-dose group compared with controls (P < 0.0001 at

each dose; linear mixed model). The mean area of CNV was also significantly smaller in the 60-mg·kg^{-1}·day^{-1} group than in the 30-mg·kg^{-1}·day^{-1} treatment group ($P = 0.0349$).

2. Effect of sorafenib on established CNV

Fourteen days after laser treatment, mice that had received sorafenib exhibited a significant reduction in CNV area on choroidal flat mounts compared with those seen in 7-day baseline eyes ($P < 0.0001$ for each dose; linear mixed model) and in the control (placebo-treated) group ($P < 0.0001$ for each dose), indicating a regression of CNV. The mean area of CNV was 0.051 ± 0.019 mm^2 in 7-day baseline eyes, 0.050 ± 0.018 mm^2 in the control (placebo-treated) group, 0.021 ± 0.007 mm^2 in the 30-mg·kg^{-1}·day^{-1} group, and 0.017 ± 0.004 mm^2 in the 60-mg·kg^{-1}·day^{-1} group, as indicated by an analysis of choroidal flat mounts (Fig. 2).

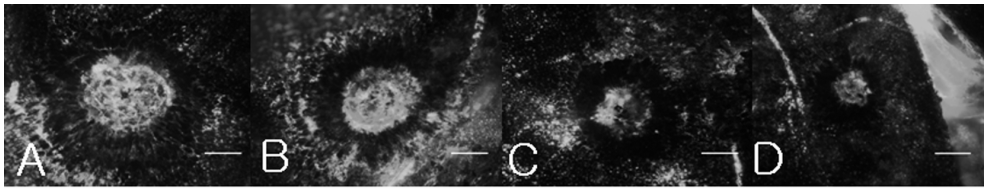

Figure 2 A, B, C, D. Oral administration of sorafenib resulted in the regression of established choroidal neovascularization (CNV) at Bruch's membrane rupture sites. Representative choroidal flat mounts after perfusion with fluorescein-labeled dextran 2 weeks after laser photocoagulation. Sorafenib (or vehicle) was delivered daily by oral gavage beginning 7 days after laser treatment. (A) 7-day baseline group, (B) Control group, (C) 30 $mg \cdot kg^{-1} \cdot day^{-1}$ sorafenib, (D) 60 $mg \cdot kg^{-1} \cdot day^{-1}$ sorafenib. Scale bar represents 100 μm.

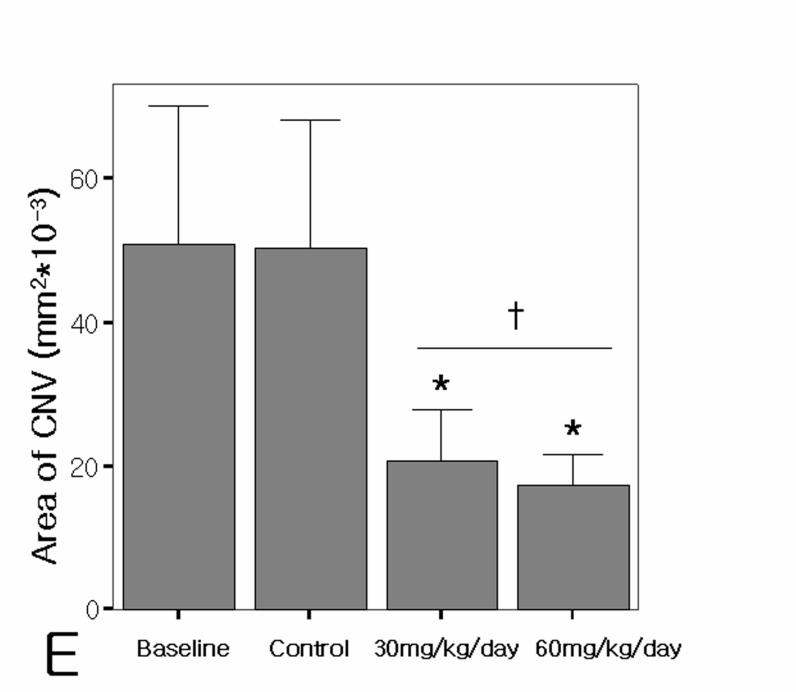

Figure 2 E. Image analysis confirmed that there was significantly less CNV in mice that received sorafenib compared with 7 day baseline eyes and those that received vehicle (E). *P < 0.0001 compared with both baseline measurement and vehicle control. †P = 0.777 when 30 $mg \cdot kg^{-1} \cdot day^{-1}$ and 60 $mg \cdot kg^{-1} \cdot day^{-1}$ sorafenib treatment groups were compared.

This could be translated into a 59 % decrease in CNV area in the low-dose group and a 66 % decrease in the high-dose group compared with both baseline and control measurements. Although the mean CNV area was

reduced in a dose-dependent manner, the difference between the 30-mg·kg^{-1}·day^{-1} and the 60-mg·kg^{-1}·day^{-1} group was not significant in a regression model ($P = 0.777$).

3. Histologic evaluation of laser burns

Histopathology analysis confirmed that CNV lesions in sorafenib-treated mice were smaller in diameter and had thinner centers compared with those in control animals. There was a discontinuity in Bruch's membrane in the area of each laser burn in both control and treatment groups. At the site of the laser spots, areas of fibrovascular tissue consisting of vessel lumen were observed. Control mice treated with vehicle showed larger lesion areas consisting of fibrovascular tissue, RPE cells, and pigment clumps compared with mice that received sorafenib 1 day before laser injury (Fig. 3).

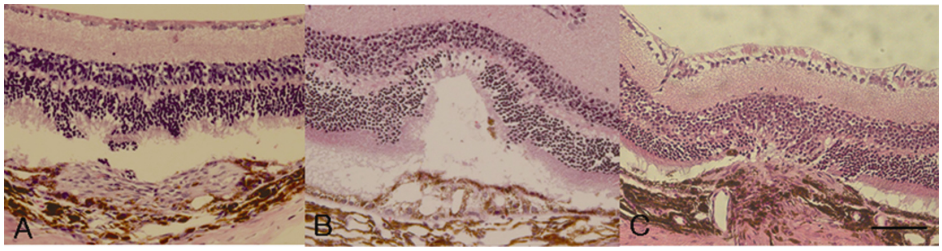

Figure 3. Hematoxylin-eosin-stained light micrograph of choroidal neovascularization (CNV) lesions 2 weeks after laser injury. Sorafenib (or vehicle) was delivered daily by oral gavage beginning 1 day prior to laser treatment. Each photograph shows the center of CNV lesions. (A) Control group, (B) 30 $mg \cdot kg^{-1} \cdot day^{-1}$ sorafenib, (C) 60 $mg \cdot kg^{-1} \cdot day^{-1}$ sorafenib. Scale bar represents 100 μm.

4. Phosphorylated-ERK expression after laser photocoagulation and inhibition of CNV via p-ERK inhibition

Activation of the Raf/MEK/ERK kinase pathway in endothelial cells is necessary for angiogenesis. This signaling pathway also acts downstream of both VEGF and PDGF, so we investigated whether sorafenib treatment inhibited ERK phosphorylation in our CNV model. At days 1, 7 and 14 after laser injury, the expression level of p-ERK relative to that of total ERK

increased compared with baseline levels. In laser-treated mice receiving sorafenib, the relative expression of p-ERK was reduced at all time points (Fig. 4).

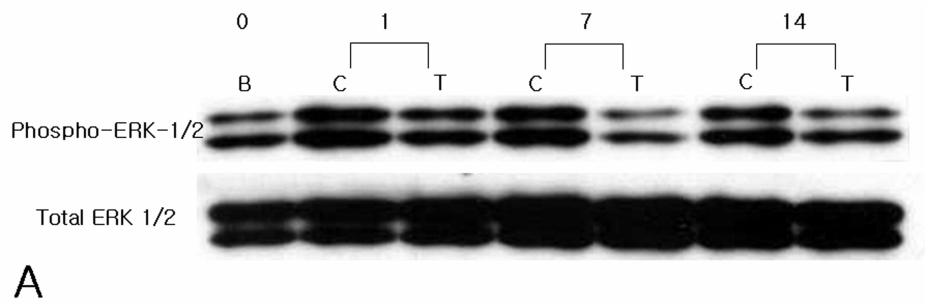

Figure 4A. p-ERK expression in the choroid and RPE layer of mice from the non-treated group (without laser injury), the control group (vehicle-treated after laser injury) and the treatment group (60-mg·kg^{-1}·day^{-1} sorafenib-treated after laser injury) at days 1, 7 and 14 after laser injury. Western-blot analysis revealed that p-ERK expression increased 2 weeks after laser photocoagulation and was suppressed by sorafenib treatment. B: baseline without laser treatment; C: vehicle-treated laser group; T: sorafenib-treated laser group.

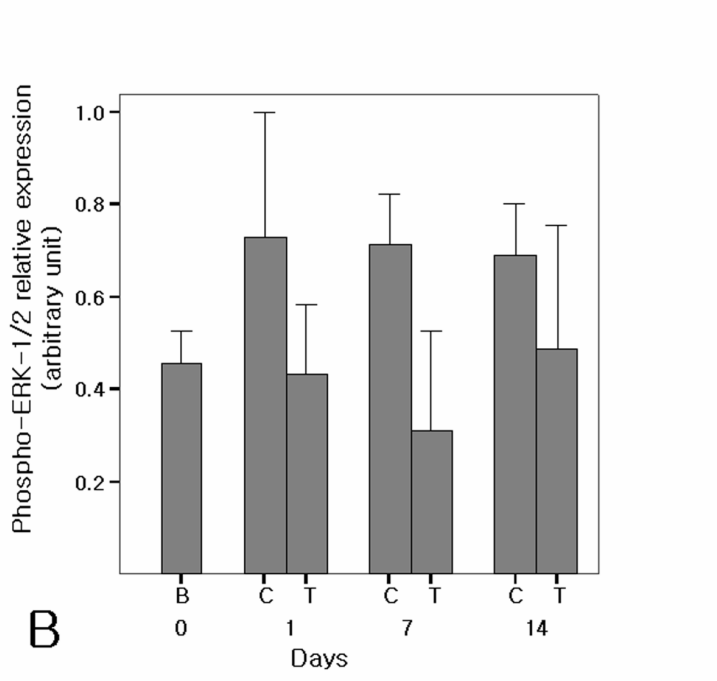

Figure 4B. Densitometric analysis of three independent experiments showed an increase in relative p-ERK expression (values normalized to total ERK expression) after laser photocoagulation compared with the non-treated group, and this increase was suppressed by sorafenib treatment. B: baseline without laser treatment; C: vehicle-treated laser group; T: sorafenib-treated laser group.

IV. DISCUSSION

The laser-induced CNV model is widely used for the study but does not completely mimic naturally occurring CNV in age-related macular degeneration.[24] Nonetheless, the essential process such as the break-up of the basement membrane, the migration and proliferation of vascular endothelial cells, and tubular formation is similar, which validates the use of the laser-photocoagulation-induced model for evaluating the effect of a drug on CNV.[24]

In this study, we showed that oral administration of sorafenib markedly inhibited CNV in a dose-dependent manner. Sorafenib treatment that began prior to laser-induced coagulation suppressed the development of CNV, and when administered after the establishment of neovascularization (7 days after Bruch's membrane rupture) caused CNV regression. Choroidal flat mounts demonstrated a significant reduction in CNV areas after sorafenib treatment. Western-blot analyses of RPE/choroid layer revealed increased expression of

p-ERK in RPE/choroid tissues, which was evident 1 day after Bruch's membrane rupture and was maintained through 2 weeks. The expression of p-ERK was suppressed by orally administered sorafenib at every time point.

Sorafenib is an oral multikinase inhibitor that inhibits VEGFR2, PDGFR-β and the serine threonine kinase Raf, which acts through the Raf/MEK/ERK kinase signaling pathway. [25] [26] In addition to direct antitumor activity, sorafenib has been shown to possess anti-angiogenic properties. Recent reports have demonstrated that the anti-angiogenic effect of sorafenib might be a primary consequence of therapy and not a secondary effect owing to tumor cell loss and reduced production of angiogenic factors.[27] [28] Recent case reports have also suggested possible therapeutic benefits of sorafenib in the treatment of exudative age-related macular degeneration. However, in these reports, sorafenib was administered either after or in conjunction with intravitreal injection of anti-VEGF drugs; thus, the direct therapeutic effect of

sorafenib in CNV could not be proven.[29 30]

Although targeting VEGF has recently been validated in clinical trials as an effective therapy for diseases associated with pathologic angiogenesis[31-33], several other growth factor pathways have been shown to be involved in the process of pathologic angiogenesis,[34 35] and there is evidence that anti-VEGF therapy alone may not be sufficient to cause vessel regression in advanced stages of aberrant angiogenesis. An RTK inhibitor targeting VEGFR2 and PDGFR-β was shown to potently promote tumor-vessel regression[18] and a recent report has also shown that inhibition of PDGF-β signaling rendered growing vessels more sensitive to anti-VEGF blockade.[19] Sorafenib has inhibitory activities against a number of kinases implicated in angiogenesis, including VEGFR2 and PDGFR-β, indicates that inhibition of neovascularization might be especially effective. Further experiments should reveal whether this theoretical advantage of sorafenib holds true when

comparing it to the anti-VEGF therapy alone.

Increased phosphorylation of ERK 1/2 has been reported in oxygen-exposed rats and in other models of retinal ischemia, and has been suggested to have a critical role in ocular angiogenesis.[36-38] Our observation that ERK phosphorylation was increased after Bruch's membrane rupture and was suppressed by sorafenib treatment in association with a significant reduction in CNV areas suggests that activation of the MAP kinase pathway (and/or VEGF/PDGF signaling) is also involved in the development of CNV in this mouse model. However, inhibition of any one of the VEGF, PDGF or MAP kinase pathways can inhibit angiogenesis. [39-41] Future investigations using drugs with different, but overlapping, *in vitro* activities should allow identification of the specific kinase targeted by sorafenib in a CNV model and provide insight into the molecular signals involved in the development of CNV.

V. CONCLUSION

The current study showed that oral administration of the multikinase inhibitor sorafenib significantly suppressed the development of laser-induced CNV and caused regression of established CNV in mice. Sorafenib interferes with multiple pro-angiogenic receptor tyrosine kinases, including VEGFR 2, PDGFR-β and Raf kinase, and thus holds promise for the treatment of CNV in clinical settings.

REFERENCES

1. Green WR. Histopathology of age-related macular degeneration. Mol Vis 1999;5:27.

2. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669-76.

3. Grossniklaus HE, Ling JX, Wallace TM, Dithmar S, Lawson DH, Cohen C, et al. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. Mol Vis 2002;8:119-26.

4. Ishibashi T, Hata Y, Yoshikawa H, Nakagawa K, Sueishi K, Inomata H. Expression of vascular endothelial growth factor in experimental choroidal neovascularization. Graefes Arch Clin Exp Ophthalmol 1997;235:159-67.

5. 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.

6. Shen WY, Yu MJ, Barry CJ, Constable IJ, Rakoczy PE. Expression of cell adhesion molecules and vascular endothelial growth factor in experimental choroidal neovascularisation in the rat. Br J Ophthalmol 1998;82:1063-71.

7. Wells JA, Murthy R, Chibber R, Nunn A, Molinatti PA, Kohner EM, et al. Levels of vascular endothelial growth factor are elevated in the vitreous of patients with subretinal neovascularisation. Br J Ophthalmol 1996;80:363-6.

8. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem

1994;269:26988-95.

9. D'Angelo G, Lee H, Weiner RI. cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. J Cell Biochem 1997;67:353-66.

10. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 2001;280:C1358-66.

11. Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 1998;273:30336-43.

12. Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by

vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene 1997;15:2169-77.

13. Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 1999;18:2221-30.

14. Bikfalvi A, Bicknell R. Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. Trends Pharmacol Sci 2002;23:576-82.

15. Cristofanilli M, Charnsangavej C, Hortobagyi GN. Angiogenesis modulation in cancer research: novel clinical approaches. Nat Rev Drug Discov 2002;1:415-26.

16 Jain RK. Molecular regulation of vessel maturation. Nat Med 2003;9:685-93.

17. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003;111:1287-95.

18. Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, et al. Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J 2004;18:338-40.

19. Jo N, Mailhos C, Ju M, Cheung E, Bradley J, Nishijima K, et al. Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. Am J Pathol 2006;168:2036-53.

20. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases

involved in tumor progression and angiogenesis. Cancer Res 2004;64:7099-109.

21. Tobe T, Ortega S, Luna JD, Ozaki H, Okamoto N, Derevjanik NL, et al. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am J Pathol 1998;153:1641-6.

22. Edelman JL, Castro MR. Quantitative image analysis of laser-induced choroidal neovascularization in rat. Exp Eye Res 2000;71:523-33.

23. Tobe T, Okamoto N, Vinores MA, Derevjanik NL, Vinores SA, Zack DJ, et al. Evolution of neovascularization in mice with overexpression of vascular endothelial growth factor in photoreceptors. Invest Ophthalmol Vis Sci 1998;39:180-8.

24. Yanagi Y, Tamaki Y, Obata R, Muranaka K, Homma N, Matsuoka H, et al. Subconjunctival administration of bucillamine suppresses choroidal neovascularization in rat. Invest Ophthalmol Vis Sci 2002;43:3495-9.

25. Hood JD, Cheresh DA. Targeted delivery of mutant Raf kinase to neovessels causes tumor regression. Cold Spring Harb Symp Quant Biol 2002;67:285-91.

26. Rini BI. Vascular endothelial growth factor-targeted therapy in renal cell carcinoma: current status and future directions. Clin Cancer Res 2007;13:1098-106.

27. Kim S, Yazici YD, Calzada G, Wang ZY, Younes MN, Jasser SA, et al. Sorafenib inhibits the angiogenesis and growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice. Mol Cancer Ther 2007;6:1785-92.

28. Murphy DA, Makonnen S, Lassoued W, Feldman MD, Carter C, Lee WM. Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). Am J Pathol 2006;169:1875-85.

29. Diago T, Pulido JS, Molina JR, Collett LC, Link TP, Ryan EH, Jr. Ranibizumab combined with low-dose sorafenib for exudative age-related macular degeneration. Mayo Clin Proc 2008;83:231-4.

30. Kernt M, Staehler M, Stief C, Kampik A, Neubauer AS. Resolution of macular oedema in occult choroidal neovascularization under oral Sorafenib treatment. Acta Ophthalmol 2008;86:456-8.

31. Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR. Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004;351:2805-16.

32. 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.

33. Zondor SD, Medina PJ. Bevacizumab: an angiogenesis inhibitor with efficacy in colorectal and other malignancies. Ann Pharmacother

2004;38:1258-64.

34. 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.

35. Kwak N, Okamoto N, Wood JM, Campochiaro PA. VEGF is major stimulator in model of choroidal neovascularization. Invest Ophthalmol Vis Sci 2000;41:3158-64.

36. Bullard LE, Qi X, Penn JS. Role for extracellular signal-responsive kinase-1 and -2 in retinal angiogenesis. Invest Ophthalmol Vis Sci 2003;44:1722-31.

37. Hayashi A, Imai K, Kim HC, de Juan E, Jr. Activation of protein tyrosine phosphorylation after retinal branch vein occlusion in cats. Invest Ophthalmol Vis Sci 1997;38:372-80.

38. Hayashi A, Koroma BM, Imai K, de Juan E, Jr. Increase of protein tyrosine phosphorylation in rat retina after ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 1996;37:2146-56.

39. Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ, et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res 1999;59:99-106.

40. Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 2004;10:145-7.

41. Zhu WH, MacIntyre A, Nicosia RF. Regulation of angiogenesis by vascular endothelial growth factor and angiopoietin-1 in the rat aorta

model: distinct temporal patterns of intracellular signaling correlate with induction of angiogenic sprouting. Am J Pathol 2002;161:823-30.

ABSTRACT (in Korean)

레이저로 유도된 실험적 맥락막 신생혈관 마우스 모델에서 경구로 투여된 소라페닙의 효과

<지도교수 고 형 준>

연세대학교 대학원 의학과
정 은 지

연령 관련 황반 변성에서 동반되는 맥락막 신생혈관은 노인 인구에서 중요한 실명의 원인으로 알려져 있다. Vascular endothelial growth factor (VEGF)는 최근 여러 보고에 의하면 안구 내 병적인 신생혈관의 형성에서 가장 중요한 역할을 할 것으로 알려진 인자이다. 또한 VEGF이외에도 platelet-derived growth factor (PDGF)나 fibroblast growth factor(FGF)등 여러 다른 성장인자들도 병적인 혈관 신생의 과정에서 중요한 역할을 할 것으로 생각되고 있으며 이를 뒷받침하는 연구 결과들이 보고된 바 있다.

소라페닙은 새로운 multikinase inhibitor로서 최근 신장세포암의

치료제로서 미국 Food and Drug Administration의 승인을 받은 경구용 투여 약제이다. 기존의 작용기전으로 알려진 Raf kinase의 억제이외에도 VEGFR2, VEGFR3, PDGFR-β과 같은 여러 성장인자들의 수용체의 활성화를 억제시키는 multikinase inhibitor로 알려져 있다.

본 연구는 실험적으로 유도된 마우스의 맥락막 신생혈관에서 경구로 투여된 소라페닙의 신생혈관 억제 효과를 보고자 하였다.

레이저를 시행하여 맥락막 신생혈관을 유도한 마우스에서 레이저 치료 1일 전부터 경구로 소라페닙 또는 vehicle을 투여하였다. 레이저 치료 후 14일째 마우스에서 choroidal flat mount를 통하여 맥락막 신생혈관 병변의 크기를 측정하여 비교 분석하였다. 또 다른 실험군에서는 이미 형성된 맥락막 신생혈관에서의 소라페닙의 효과를 확인하기 위하여 레이저 시행 후 7일째부터 경구로 소라페닙 또는 vehicle을 투여하여 레이저 후 14일에 병변의 크기를 측정하였다.

경구로 투여된 소라페닙은 용량과 비례하여 맥락막 신생혈관의 형성을 억제하는 것으로 나타났다. 맥락막 신생혈관의 크기는 30-mg·kg^{-1}·day^{-1} 그룹에서 대조군과 비교하여 43% 감소하였으며

60-mg·kg^{-1}·day^{-1} 그룹에서는 61% 감소하여 통계적으로 유의한 차이를 나타내었다 (P < 0.0001). 또한 이미 형성된 맥락막 신생혈관의 퇴행을 효과적으로 유도하는 것으로 나타났다. 맥락막 신생혈관 병변의 크기는 30-mg·kg^{-1}·day^{-1} 그룹에서 대조군과 비교하여 59% 감소하였으며 60-mg·kg^{-1}·day^{-1} 그룹에서는 66% 감소하여 통계적으로 유의한 차이를 나타내었다 (P < 0.0001).

이번 실험을 통하여 multikinase inhibitor인 소라페닙은 레이저로 유도된 실험적 맥락막 신생혈관의 생성을 억제하고 형성된 신생혈관의 퇴행을 유도하는 것으로 나타났다. 소라페닙은 신생혈관의 형성에 관여하는 여러 pro-angiogenic receptor tyrosine kinase인 VEGFR 2, PDGFR-β, Raf kinase등을 동시에 억제하여 신생혈관 형성을 억제하므로 임상적인 맥락막 신생혈관에서도 그 효과를 기대할 수 있을 것으로 사료된다.

핵심되는 말: 맥락막 신생혈관, extracellular signal-regulated kinase, antiangiogenesis, 소라페닙