EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 2, 173-179, April 2006

Inhibition of corneal neovascularization by rapamycin

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Accepted 31 March 2006

Abbreviations: bFGF, basic fibroblast growth factor; HDMEC, human dermal microvascular endothelial cell; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor

Abstract

The purpose of this study was to determine whether rapamycin could inhibit corneal angiogenesis induced by basic fibroblast growth factor (bFGF). Using human dermal microvascular endothelial cells (HDMECs), we examined the effect of rapamycin on cell proliferation and migration, and the expression of vascular endothelial growth factor (VEGF). The rabbit's eye was implanted intrastromally into the superior cornea with pellet containing bFGF for the control group and pellet containing bFGF and rapamycin for the rapamycin group. Biomicrographically, corneal angiogenesis was evaluated for 10 days after pellet implantation. The neovascularized cornea also was examined histologically. bFGF induced corneal neovascularization was significantly reduced by treatment with rapamycin. Using *in vitro* model, rapamycin strongly inhibited bFGF induced proliferation, migration, and VEGF secretion of HDMECs. We could observe that the bFGF induced corneal angiogenesis was inhibited by rapamycin in a micropocket rabbit model. The score of neovascularization was significantly decreased in the rapamycin group than in the control group at 10 days after pellet implantation. Histologically, the cornea of rapamycin group also showed much less new vessels than that of control group. Collectively, rapamycin appears to inhibit bFGF induced angiogenesis in a rabbit corneal micropocket assay and may have therapeutic potential as an antiangiogenic agent.

Keywords: cornea; neovascularization; rabbit; rapamycin; vascular endothelial growth factor

Introduction

Angiogenesis is essential in tissue reproduction, development and wound repair. However, angiogenesis is also associated with pathogenesis of numerous eye diseases such as loss of visual acuity, neovascular glaucoma, diabetic retinopathy, chemical burns and viral infections of the cornea (Epstein *et al*., 1987). Corneal neovascularization is also thought to predispose to the rejection of corneal allograft by facilitating the exposure of antigens to the host immune system (Hill and Maske, 1988). The mechanisms of corneal neovascularization have been investigated and various mediators are involved in this process, such as basic fibroblast growth factor (bFGF) (Adamis *et al*., 1991; Kim *et al.,* 1999), leptin (Park *et al.,* 2001), vascular endothelial growth factor (VEGF) (Amano *et al*., 1998), angiogenin (Shin *et al*., 2000), prostaglandins (Ziche *et al*., 1982), interleukin-2 and -8 (Koch *et al*., 1992) and platelet-derived growth factor (PDGF) (Risau., 1990). Particularly, bFGF has been demonstrated to be a major factor in corneal neovascularization, which is released from the cell surface and extracellular matrix by various stresses, including mechanical cell injury and inflammation, and plays an important role in the migration and proliferation of vascular endothelial cells, resulting in formation of a capillary network. Rapamycin is produced by *Streptomyces hygroscopicus*, and it was first isolated in 1975 from a soil sample taken from Easter Island (Vezina *et al*., 1975). Although rapamycin was originally found to be an effective antifungal agent, especially against *Candida albicans* (Baker *et al*., 1978), it was later found to have potent immunosuppressive qualities (Martel *et al*., 1977), similar to tacrolimus, which was identified in the late 1980s. Olsen *et al*. (1994) have reported that rapamycin inhibited corneal allograft rejection and neovascularization in a rat model. Furthermore, it has been shown that rapamycin is not cytotoxic *in vivo* and *in vitro* and that it has pharmacologic potential, not only as an antiangiogenic agent, but also as a clinical immunosuppressant. In this study, antiangiogenic activity of rapamycin was examined using *in vitro* and *in vivo* assay systems for neovascularization. The results suggest the applicability of rapamycin for treatment of corneal disorders with angiogenesis.

Materials and Methods

Cell culture

Human dermal microvascular endothelial cells (HDMECs) were obtained from Clonetics (Walkersville, MD) and cultured in growth medium (EGM-2; Clonetics) supplemented with 2% fetal calf serum, 10 ng/ml human epidermal growth factor (EGF), 5 ng/ml human bFGF, 1 μg/ml hydrocortisone, 50 ng/ml gentamicin, 50 ng/ml amphotericin B, and 10 μg/ml heparin at 37 μl in a humidified, 5% CO_2 and 95% air atmosphere.

Proliferation assay

Cell proliferation was determined by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) assay (Roche, Mannheim, Germany), according to the manufacturer's protocol. HDMECs were seeded in 96 well microplates (2×10^4 cells/well), and treated with bFGF (100 ng/ml) and rapamycin (0-1,000 ng/ml) for durations of 48 h. Cell cultures of the control group were treated with only bFGF. Ten microliters of MTT labeling reagent was added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader at a test wavelength of 570 nm. The optical density was calculated as the difference between the absorbance at the reference wavelength and the absorbance at the test wavelength.

Migration assay

When the cells were at a confluent state, the medium was replaced with a growth factor-free starvation medium (EBM-2 containing 2% FCS), and the cells were cultivated for another 36 h. Endothelial cell invasion was assessed by using membrane inserts with 8.0 μm-sized pores (Transwell; Falcon, Franklin Lakes, NJ). Three hundred microliters of HDMEC suspension in starvation medium (2×10^4 cells) was plated on the upper insert. Seven hundred microliters of medium containing 100 ng/ml bFGF and various amounts (0.1-1,000 ng/ml) of rapamycin was prepared in a 24-well culture plate. The HDMEC insert was set into each well of the 24-well plate, and the cells were incubated for 6 h at 37°C. The cells that had migrated to the distal side of the filter membrane were stained with crystal violet, and the number of migrating cells was counted in five randomly chosen fields at a magnification of × 400. The results are expressed as the mean ± SD of four independent experiments.

ELISA for VEGF

ELISA was performed to quantitate VEGF protein released from the cultured cells. When HDMECs were at 70% confluent state, bFGF (100 ng/ml) and/or rapamycin (1,000 ng/ml) were treated for 2 days in serum free condition. The culture medium was taken for VEGF assay using a commercially available kit (R&D system, Minneapolis, MN) according to the manufacturer's protocol. In brief, 50 μl of the assay buffer and 50 μl of the standard dilutions of human VEGF, and samples were dispensed into a 96-well microtiter plate coated with anti- VEGF polyclonal antibody. The plate was sealed and incubated at RT for 2 h. After this, the plates were washed four times, and 200 μl of rabbit anti-VEGF conjugated with horseradish peroxidase was added into each well and this was allowed to incubated at RT for 2 h. 200 μl aliquots of the color reagent 3,3', 5,5'-tetramethylbenzidine (TMB) were then applied for 20 to 30 min to develop a blue color, and the reaction was stopped by adding 50 μl of 1 M H_2SO_4. The absorbance was measured at a 450 nm wavelength by an automatic plate reader with a reference wavelength of 570 nm.

Corneal neovascularization assay

The animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In this study, 12 female New Zealand White rabbits (3-3.5 kg) were divided into 2 groups and anesthetized systemically with an intramuscular injection of ketamine hydrochloride (5 mg/kg) and xylazine (2 mg/kg), locally with one drop of 0.5% proparacaine hydrochloride (Alcon Laboratories, Fort Worth, TX) on the corneal surface. For assessment of neovascularization *in vivo*, we used a corneal micropocket assay. In brief, an aliquot of bFGF (Sigma-Aldrich, St. Louis, MO) solution was added 2 μl of 12% polyhydroxyethylmethacrylate (Sigma-Aldrich, St. Louis, MO) in ethanol. Four microliter aliquouts of the mixture were placed on Parafilm (American National Can, Greenwich, CT) and allowed to dry to produce pellets, each containing 1 μg of bFGF. The pellet containing either 2 μg of rapamycin or PBS alone was prepared as described earlier. A partial incision was made 3 mm from the limbus to dissect the corneal stroma and the pellet containing bFGF was placed in this pocket. After that, the pellet containing PBS and rapamycin was inserted in the corneal pocket for the control group and the rapamycin group, respectively. The rabbit's eyes were examined under slit-lamp biomicroscopy by two masked observers. After general anesthesia, the pictures of corneal neovascularization were taken with a zoom photographic

slit lamp (SM-50F; Takagi, Nakano, Japan). To evaluate the corneal neovascularization, measurements were performed directly from the slides by using an image analyzer system consisting of a charge-coupled device (CCD) camera (CCD TR-900; Sony, Tokyo, Japan) coupled with a digital analyzer system (Optimas 5 Image Analysis Software; Optimas, Bothell, WA) on an IBM-compatible computer. Angiogenic activity was scored by the number and length of newly developed vessels, and the scores were calculated as the number of vessels multiplied by the length from the limbus on each of the 10 days after the pellet implantation. A score of 0 was assigned to the length of $<$ 0.3 mm, 1 for lengths from 0.3 to 0.6 mm, 2 for lengths from 0.7 to 1.0 mm, and 3 for lengths $>$ 1.0 mm. When several vessels had extensive branching, we took the longest vessel as a standard score. The scores by two masked observers were added, and the final score was the average of the two. For histologic examination, animals were euthanized after general anesthesia on post operation day 10. The tissue sample were enucleated and fixed in 4% paraformaldehyde solution. The 4 μm sections were stained by hematoxylin and eosin, and observed with a light microscope.

Statistical analysis

All data are expressed as the mean ± SD. The data were statistically evaluated by paired Student's *t*-test. $P < 0.05$ was considered significant.

Results

Effect of rapamycin on the HDMECs growth and migration

To investigate the effect of rapamycin on bFGF induced cell growth at the concentration of 0, 0.1, 1, 10, 100, or 1000 ng/ml for a treatment period of 2 days, an MTT assay was performed. The cell growth of HDMECs was suppressed by the treatment of rapamycin in a dose dependent manner. When the HDMECs were exposed by concentrations of rapamycin more than 100 ng/ml, the growth was significantly inhibited ($P < 0.05$; Figure 1). By using a double-chamber assay, we next examined the effect of rapamycin on the migration of HDMECs, which was promoted by bFGF as a chemoattractant, at concentrations of 0, 0.1, 1, 10, 100, or 1000 ng/ml for a treatment period of up to 6 h, and it was observed that rapamycin at concentrations of 10, 100, and 1000 ng/ml significantly inhibited the migration rate of HDMECs ($P < 0.05$; Figure 2). Therefore, rapamycin successfully inhibited HDMECs migration at the concentration of 10 ng/ml, which is a drug concentration that only minimally affected cell growth of the HDMECs.

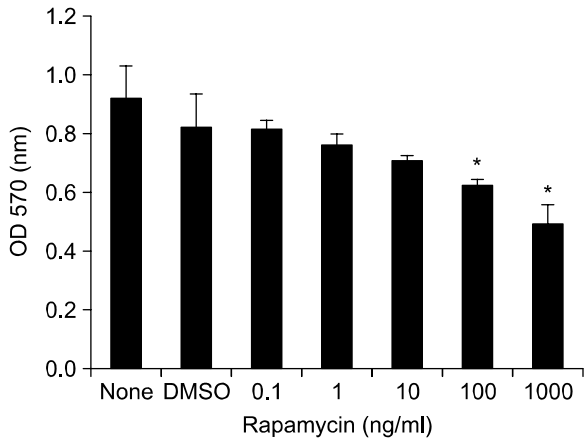

Figure 1. The effect of rapamycin on HDMEC growth. HDMECs were treated with 100 ng/ml bFGF and 0 to 1000 ng/ml rapamycin for 48 h. Cell growth was determined by MTT assay. *$P < 0.05$ compared with the control.

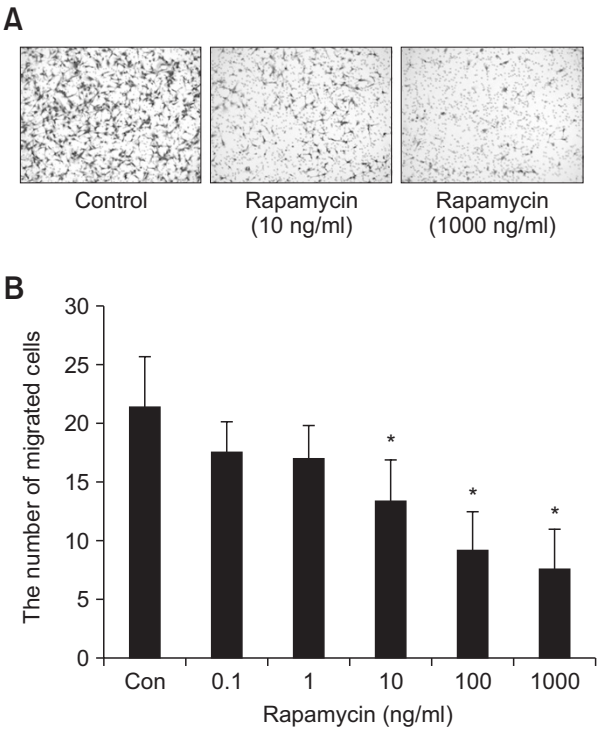

Figure 2. The effect of rapamycin on the migration of HDMECs. (A) The cells that had migrated to the distal side of the filter were stained with crystal violet. × 200. (B) The number of migrated cells was counted in five randomly chosen fields at the magnification of 400-folds. *$P < 0.05$ compared with the control.

Effect of rapamycin on HDMECs' VEGF production

VEGF is one of the central regulators of angiogenesis. To investigate the ability of treatment with rapamycin to reduce the VEGF secretion of HDMECs, VEGF levels of culture supernatant were measured by an ELISA. Two days after rapamycin treatment, VEGF secretion was significantly decreased by concentrations of rapamycin higher than 100 ng/ml ($P < 0.05$; Figure 3).

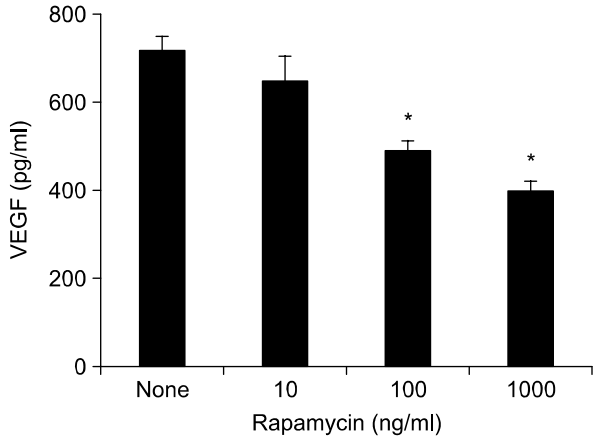

Figure 3. Effect of Rapamycin on HDMECs' VEGF production. Rapamycin significantly caused a decrease in VEGF secretion. HDMECs were cultured with and without rapamycin. *$P < 0.05$ compared with the control.

Effect of rapamycin on corneal neovascularization

The antiangiogenic activity of rapamycin was examined using a corneal micropocket assay. The corneal angiogenesis gradually increased in both groups. However, there was less angiogensis observed in the rapamycin group than in the control group (Figure 4) The scores for corneal neovascularization were 17.59 $\pm$ 5.29 and 5.48 $\pm$ 2.59 in the control and rapamycin groups at day 3, respectively; 22.46 $\pm$ 6.18 and 9.87 $\pm$ 2.48 at day 7, respectively; and 27.61 $\pm$ 6.89 and 9.88 $\pm$ 2.56 at day 10, respectively (Figure 5). Thus, corneal neovascularization was significantly inhibited by the treatment with rapamycin ($P < 0.05$). Each eye was examined for histology. As shown in Figure 6, a number of typical capillaries as well as edema were observed in the area between the limbus and the pellet in the control group. By contrast, very little change was observed in the cornea treated with rapamycin.

Discussion

Corneal neovascularization is a major sight-threatening complication of corneal infections, chemical injury, and keratoplasty. It is characterized by a corneal ingrowth of new vessels originating from the limbus, often accompanied by an inflammatory response. Generally, angiogenesis is a complex process that is especially related to vascular endothelial cells (Auerbach *et al*., 1994; Cockerill *et al*., 1995). To form new capillary tubes, the proliferation and

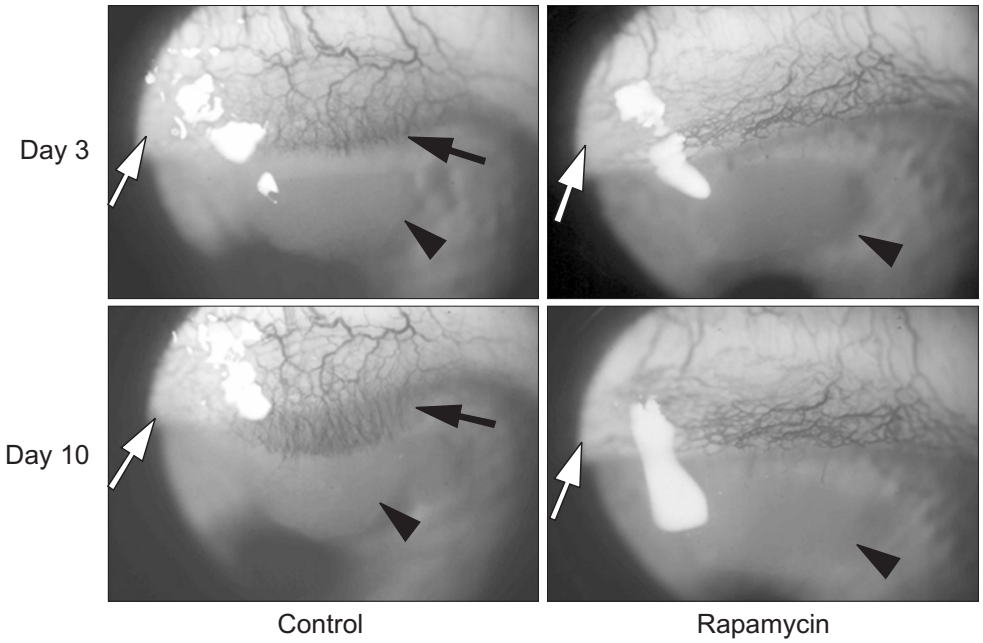

Figure 4. Slit lamp photographs of rabbit corneas at 3 and 10 days after pellet implantation. The corneal neovascularization of control group was observed at the limbus after bFGF implantation. The angiogenesis was remarkably inhibited in the rapamycin group at day 10. White arrow, limbus; Arrow head, bFGF pellet; Black arrow, grown capillary $\times$ 30.

migration of endothelial cells are a prerequisite. Therefore, previous studies have demonstrated whether the proliferation and migration of vascular endothelial cells could be inhibited by anti-angiogenic agents (Jimi *et al*., 1995; Vermeulen *et al*., 1996; Kobayashi *et al*., 2002). Recently, it was suggested that the rapamycin had antiangiogenic activities in tumor and cornea models (Guba *et al*., 2002; Kwon *et al*., 2005) In this study, it was observed that rapamycin, an antibiotic macrolide and immunosuppressant, inhibited endothelial cell proliferation and migration, reduced VEGF protein level, and thus inhibited angiogenesis in a rabbit model of corneal neovascularization.

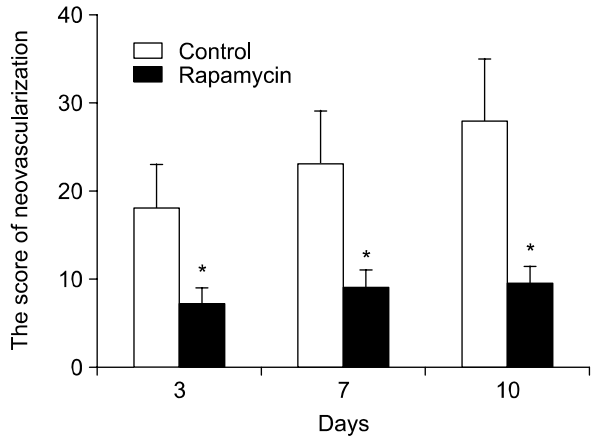

Figure 5. The score of neovascularization was calculated by number of newly developed vessels multiplied by length of vessels from the limbus 3, 7 and 10 days after pellet implantation. The score of rapamycin group was significantly lower than that of the control group during the experiment period. *$P < 0.05$ compared with the control group.

Endothelial proliferation and migration are key steps in angiogenesis, so we examined the effects of rapamycin using an *in vitro* and *in vivo* model for these processes. We found that the HDMECs proliferation was suppressed by the treatment of rapamycin in a dose dependent manner. And it was observed that rapamycin significantly inhibited the migration of HDMECs at concentration of more than 10 ng/ml. Previous studies suggested that Rapamycin specifically inhibits mammalian target of rapamycin (mTOR) (Sabatini *et al*., 1994; Sabers *et al*., 1995; Hosoi *et al*., 1999; Humar *et al*., 2002), which is the initially identified member of a novel family of PI kinase-related kinases that function in surveillance pathways and plays a central role in controlling cellular growth (Barbet *et al*., 1996; Thomas and Hall, 1997; Schmelzle and Hall, 2000). It was also reported that rapamycin suppressed cell migration via mTOR signaling (Gomez-Cambronero, 2003). Our previous study showed that the expression of mTOR of endothelial cell was suppressed with rapamycin treatment (Kwon *et al*., 2005). Among the various angiogenic factors identified to date, VEGF specifically acts on endothelial cells (Connolly, 1991) and its cell surface receptors are exclusively expressed in endothelial cells (plate *et al*., 1993). Because VEGF plays an important role in vessel development, we tested whether rapamycin could affect its secretion by endothelial cell's activation by bFGF. Consequently, rapamycin significantly inhibited the secretion of VEGF. Interestingly, HDMEC migration was significantly inhibited at the doses of rapamycin (10 ng/ml) that did not cause the change of VEGF production in our *in vitro* model. These our *in vitro* results partially support that rapamycin had a strong anti-chemotactic effect

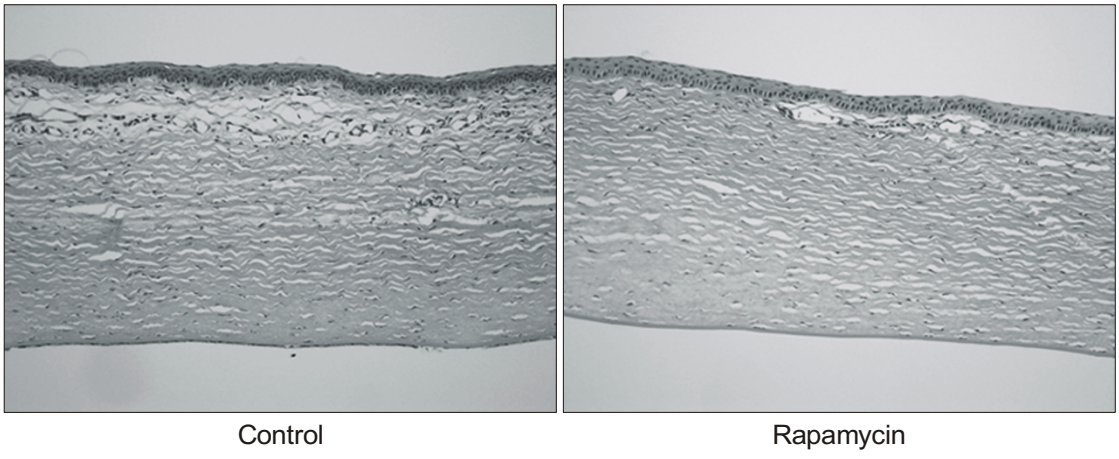

Figure 6. Histological photographs of rabbit cornea on day 10 after pellet implantation. Angiogenesis was observed in the stromal layer between the limbus and the pellet. The number of new vessels was decreased in the rapamycin group. × 200.

against vascular endothelial cell. Based upon these results, we can suppose that rapamycin regulate the HDMEC migration via other pathway such as mTOR or FKBP. The mechanism between rapamycin and its anti-migration effect has to be further elucidated in future studies. Accordingly, we could propose that rapamycin have an antiangiogenic effect by inhibition of endothelial proliferation and migration and suppression of VEGF secretion.

Furthermore, we confirmed that rapamycin dramatically suppressed bFGF induced angiogenesis in a rabbit corneal model by macroscopic and histologic examination. Although rapamycin has been investigated for multiple purposes, its anti-angiogenic effect using corneal micropocket assay has not yet been reported. Therefore, these findings provide a direct evidence that corneal neovascularization induced by bFGF is inhibited by rapamycin *in vivo*.

Collectively, this study suggests that rapamycin have a strong inhibitory effect on bFGF induced neovascularization. The effect is explained in part by inhibition of proliferation, migration, and VEGF secretion of vascular endothelial cells. Therefore, rapamycin may be used useful for the treatment of corneal diseases with angiogenesis.

Acknowledgement

This work was supported by the Postdoctoral Research Program of Chung-Ang University 2004 year.

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