

Angiogenesis effects of nerve growth factor (NGF) on rat corneas

Kangmoon Seo*, Jongil Choi, Myungjin Park¹ and Changhun Rhee¹

Department of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea

¹Lab. of Cell Biology, Korea Cancer Hospital, Seoul 139-706, Korea

This study was performed to evaluate the effects of nerve growth factor (NGF) upon angiogenesis in the rat cornea, to examine its possible application as an alternative angiogenic inducer and to provide basic data for further studies. Angiogenesis was induced by cornea micropocket assay, as previously described. Eight of thirty two eyes of Sprague-Dawley rats were randomly assigned to one of four groups, namely, a non-NGF group (Group 0), a 0.5 ng of NGF group (Group 0.5), a 1.0 ng of NGF group (Group 1.0) and a 5.0 ng of NGF group (Group 5.0). Pellets made of poly-2-hydroxyethylmethacrylate and sucralfate were implanted into the corneal stroma no closer than 1 mm from the limbus. After the implantation, the number of new vessels, vessel length and circumferential neovascularization were examined daily under the surgical microscope over a period of 7 days. The area of neovascularization was determined using a mathematical formula. Although new vessels in Group 0 and Group 0.5 were first observed at day 5, those of Groups 1.0 and 5.0 were first noted on days 4 and 3, respectively. However, the growth rates of new vessels in Groups 1.0 and 5.0 were higher than those of Groups 0 and 0.5 with the passage of time. The number, length, circumferential neovascularization and areas covered by the vessels in Groups 1.0 and 5.0 were significantly more than in Group 0 and Group 0.5 ($p < 0.05$). This study showed that NGF had a dose-dependent angiogenic effects on the rat cornea and that the minimal effective dose of NGF was 1.0 ng per cornea. Also, it showed that NGF would be useful in angiogenic studies as an alternative angiogenic inducer.

Key words: Nerve growth factor (NGF), angiogenesis, cornea micropocket assay, rat

Introduction

Angiogenesis is known to be essential for wound healing, female reproduction, embryogenic development, organ formation, tissue regeneration, and wound remodeling [13,15,27]. It is a complex multistep process that includes proliferative migration and the differentiation of endothelial cells, the degradation of extracellular matrix, microtubule formation, and the sprouting of new capillary branches [12,15,27].

Overgrowth of blood vessels may lead to the development and progression of diseases such as tumor growth and diabetic retinopathy. Many lines of evidence support the original hypothesis that tumor growth and metastasis are angiogenically dependent [3,4,17]. Thus, the study of angiogenesis is required to elucidate the mechanism of tumor growth and other neovascular diseases or to determine antitumor and wound healing efficacy.

In the field of neovascular research, the testing of angiogenic and antiangiogenic substances relies substantially on the sensitivity and specificity of *in vivo* and *in vitro* bioassays. Various bioassay methods have been used in order to identify and elucidate the action mechanisms of various positive and negative angiogenic regulators. These methods include the hamster cheek pouch assay [5], dorsal air sac assay [14], rabbit ear chamber assay [19], chick chorioallantoic membrane assay (CAM) [6], dorsal mouse skin assays [9], monkey iris neovascularization model [23], cornea micropocket assay [16,26], and the disc angiogenesis assay [11]. All of these methods allow the neovascularized area to be directly inspect and rely upon a vascular pattern which can be clearly distinguished from newly formed vessels. Nowadays, the CAM and the cornea micropocket assay are widely used in neovascular research. However, in the CAM assay is difficult to distinguish new vessels from the previous vascular network because it contains previously developed vascular network. On the other hand, in the case of the cornea micropocket assay is easy to observe new vessels because the cornea has high visibility, accessibility,

*Corresponding author
Phone: +82-33-250-8651; Fax: +82-33-244-2367
E-mail: kmseo@kangwon.ac.kr

and avascularity. Therefore, the cornea micropocket assay can avoid inherent problems of interpretation.

Angiogenic factors of basic fibroblast growth factor (bFGF) [5,8,9,20], vessel endothelial growth factor (VEGF) [9,10,16,24] and epidermal growth factor (EGF) [24] have been used as an angiogenic inducers. Nerve growth factor (NGF) is known to promote the neural differentiation and survival of several peripheral and central neurons [1,2,7,18,25,29,30]. NGF is also known to enhance the survival of cholinergic neurons [21] and to have neuroprotective effects on adult rat hippocampal neurons [22]. In addition, some studies have reported that NGF has angiogenic effects associated with nerve growth effects in several nerve ganglions [24,28]. However, there have been no reports to the effect that NGF may be used as an angiogenic inducer. Therefore, this study was performed using a cornea micropocket assay to evaluate the dose dependent angiogenic effects of NGF, to elucidate the effective minimal dose of NGF, and to provide an alternative choice as an angiogenic inducer for the study of angiogenesis.

Materials and Methods

Experimental animals

Female and male Sprague-Dawley rats, weighing 250 to 300 g, were used in this study. The animals were allowed unrestricted access to pelleted food and tap water, and were confirmed to have no vessels on their corneas before NGF-impregnated pellets were implanted.

Pellet preparation

Pellets were prepared according to the method previously described [26]. Sterile casting solution was prepared by dissolving the poly-2-hydroxyethylmethacrylate (Hydron, Sigma Co. USA) powder in absolute ethanol (12% w/v) at 37°C with continuous stirring for 24 hours. An equal volume of Hydron and sucralfate (12% w/v, Sigma Co, USA) were combined. Also each concentration of nerve growth factor (NGF), such as 0.5 ng, 1.0 ng, and 5.0 ng, was mixed with 2 µl of Hydron and sucralfate solution. This solution was pipetted onto the surface of sterile teflon rods glued to the surface of a petri dish to make a pellet of 2 mm diameter. After drying at room temperature for 1 to 2 hours in a sterile environment the pellets were stored at 4°C. Using this techniques, each pellet contained 0 ng, 0.5 ng, 1.0 ng, or 5.0 ng of NGF.

Pellet implantation

Pellets were implanted into rat corneas according to the previously described method [26]. Rats were anesthetized with a combination of xylazine (6 mg/kg, IM) and ketamine (20 mg/kg, IM). The eyes were topically

anesthetized with 0.5% proparacaine (Alcaine®, Alcon, USA), and gently proptosed and secured by clamping the upper eyelid with a non-traumatic hemostat. Under a surgical microscope, a 1.5-mm incision was made at the center of the cornea but not through it (Fig. 1, A). A curved microdissector, approximately 1.5 mm in width, was then inserted under the lip of the incision and gently blunt-dissected through the stroma toward the limbus of the eye. Slight finger pressure against the globe of the eye helped steady it during dissection. Once the corneal pocket was made, the microdissector was removed, and the distance between the limbus and base of the pocket was measured to make sure it was no closer than 1 mm (Fig. 1, B). Just before implantation, the pellet was rehydrated with saline, and positioned down to the base of the pocket, which then sealed spontaneously (Fig. 1, C). No more than half of the pocket was filled with implant material (Fig. 1, D).

Corneas were examined daily with the aid of a surgical microscope to monitor angiogenic responses to NGF, and then antibiotic ointment (Terramycin®, Pfizer, Korea) not containing corticosteroids, was applied to the eyes once per day.

Biomicroscopic examination

Eyes were examined under a surgical microscope daily for 7 days after pellet implantation. The number of vessels, vessel length, and the area of the neovascularization were determined using a computer program (Image Tools, ver. 2.0, University of Texas health science center in San Antonio, USA). Photographs of the rat cornea were obtained with a digital camera. Each photograph was analyzed at the same magnification with a computer program. If needed, digitized images were optimized for analysis by erasing nonvascular structures and completing vascular profiles. The contiguous circumferential zone of neovascularization was measured as clock hours with a 360° reticule (where 30° of arc equalled 1 clock hour). The area of corneal neovascularization was determined with a reticule by measuring the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. Only the uniform contiguous band of neovascularization adjacent to the pellet was measured. A formula was used to determine the area of the circular band segment, as previously described [8]: $C/12 \times 3.1416[r^2 - (r-L)^2]$, where $r = 2.5$ mm, the measured radius of the rat cornea.

Experimental design

Eight out of thirty-two eyes were randomly assigned to each of four groups, namely, the non-NGF group (Group 0), 0.5 ng of NGF group (Group 0.5), 1.0 ng of NGF group (Group 1.0), and the 5.0 ng of NGF group (Group 5.0).

Data analysis

The significant differences between groups were

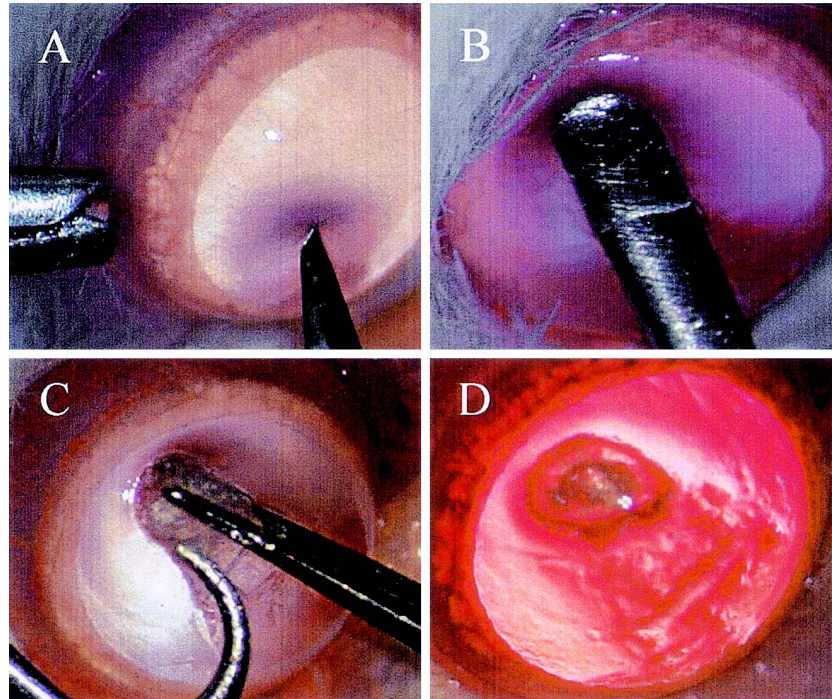


Fig. 1. Surgical procedure for NGF pellet implantation into the rat corneal stroma. A. An 1.5 mm incision was made at the center of the cornea. B. A curved microdissector was inserted under the lip of the incision and gently blunt-dissected through the stroma. C. Pellet was positioned at the base of the pocket. D. Completed pellet implantation.

analyzed by one-way ANOVA with ranked data. The number of vessels, length of vessels, clock hour of neovascularization, and area of vessels were determined (mean \pm S.E.) and statistically analyzed with one-way ANOVA. The level of significance was set at $p<0.05$.

Results

To evaluate the angiogenesis effects of NGF, non-NGF pellets (Group 0) and pellets containing 0.5 ng of NGF (Group 0.5), 1.0 ng of NGF (Group 1.0), and 5.0 ng of NGF (Group 5.0) were implanted into the rat corneas as described. After NGF pellet implantation, the number of vessels, vessels length, clock hour, and vessels area were measured from day 1 to day 7, and statistically analyzed.

The number of vessels

Pellets containing less than 0.5 ng NGF (Groups 0 and 0.5) did not induce neovascularization until day 4. In eyes containing 1.0 ng (Group 1.0) and 5.0 ng of NGF (Group 5.0), limbal vessels began sprouting into the cornea on postoperative days 4 and 3, respectively. The number of vessels increased in all groups with time. The number of vessels in high dose groups (Groups 1.0 and 5.0) was significantly greater than in the low dose groups (Groups 0 and 0.5) ($p<0.05$). However, there was no significant difference between Groups 1.0 and 5.0 (Fig. 2).

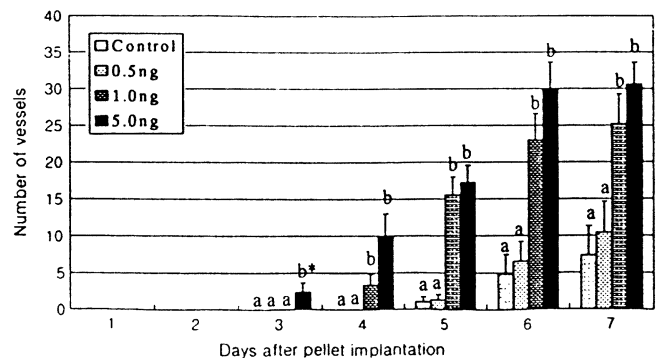


Fig. 2. Changes of the number of vessels after NGF pellet implantation in rat corneas. Different superscripts on the same day show significant differences at $p<0.05$. * mean \pm S.E.

The length of vessels

Vessel length changes in each group showed a pattern that was similar to the number of vessels. The vessel length in Groups 1.0 and 5.0 was increased significantly faster than those of Groups 0 and 0.5 ($p<0.05$).

However, the vessel length changes in Groups 1.0 and 5.0 were not statistically different (Fig. 3).

The clock hours of neovascularization

Clock hour changes of neovascularization in each group showed a growth pattern that was similar to that of the

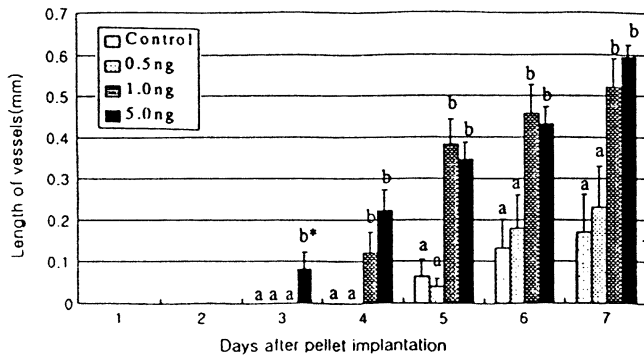


Fig. 3. Changes of the vessel length after NGF pellet implantation in the rat cornea. Different superscripts on the same day indicate significant difference at $p < 0.05$. * mean \pm S.E.

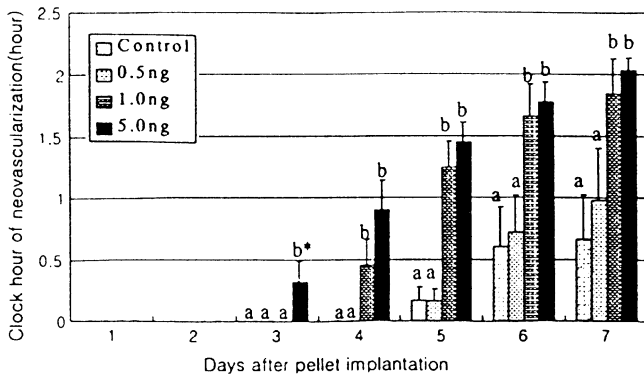


Fig. 4. Changes of clock hour of vessels after NGF pellet implantation in the rat cornea. Different superscripts on the same day indicate significant difference at $p < 0.05$. * mean \pm S.E.

other criteria. As the vessels increased in number and length over the experimental period, the extent of circumferential neovascularization also increased. However, there was no difference in clock hours of neovascularization between Groups 1.0 and 5.0. The clock hours of neovascularization in Groups 1.0 and 5.0 were significantly wider than in Groups 0 and 0.5 ($p < 0.05$) (Fig. 4).

The areas of vessels

The number, length and clock hours of new vessels resulted in a similar pattern of changes in the vessel area. The vessel area in the high dose group (Groups 1.0 and 5.0) was significantly greater than in the low dose groups (Groups 0 and 0.5) ($p < 0.05$). However, there was no significant difference in vessel areas of Groups 1.0 and 5.0 (Fig. 5).

Discussion

This study showed that nerve growth factor (NGF) has the potential to be used in angiogenic studies, as an angiogenic inducer. In addition, the angiogenic effect of

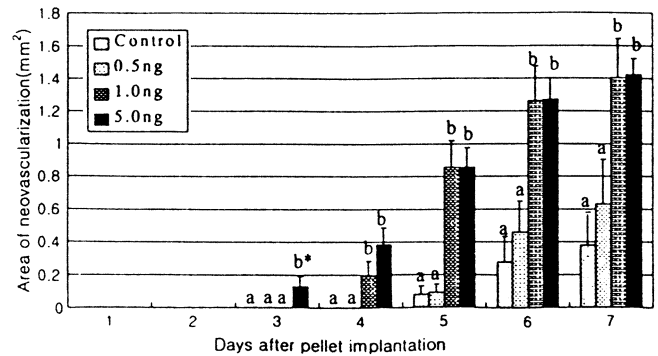


Fig. 5. Changes of vessel area after NGF pellet implantation in the rat cornea. Different superscripts on the same day indicate significant difference at $p < 0.05$. * mean \pm S.E.

NGF was dose-dependent on the rat cornea and its minimal effective dose was 1.0 ng per cornea.

Nerve growth factor (NGF) is known as a protein that promotes the survival, during development growth, and neurite differentiation of neurons, and NGF has also been used to regenerate nerves. However, a number of studies have reported that NGF is more effective at promoting angiogenesis rather than nervous regeneration [24,28]. Nevertheless, no reports have been issued concerning the angiogenic effects of NGF by previous established bioassay techniques.

To identify angiogenesis induced by NGF in this study, a cornea micropocket assay was performed. The cornea micropocket assay has been generally performed in the study of angiogenesis of potent angiogenic growth factors, such as, bFGF, EGF, and VEGF. CAM has also been used to identify the angiogenic or antiangiogenic effects of growth factors in the study of angiogenesis. CAM is the method that involves observation of the growth of vessels in the chick embryo. Because CAM is performed during the embryogenic period, it is difficult to distinguish between new vessels and previously established vascular networks. On the other hand, the cornea micropocket assay avoids any confusion between new vessels and previously existing vessels, and any vessels penetrating into the corneal stroma can be readily identified as newly formed, as the cornea is avascular.

To determine the dose of NGF per pellet, a preliminary study was performed (data not shown). Pellets containing 10 ng and 100 ng of NGF also stimulated increased vessel length and area of neovascularization but also induced intraocular hemorrhage and corneal edema, and therefore, the dose was reduced to less than 10 ng in this study.

Changes in the vessels after NGF pellet implantation were measured in items of the number of vessels, the vessel length, the clock hours of vessels, and the area of neovascularization for quantitative assay and statistically analyzed from postoperative day 1 to day 7. Vessels were first noted on postoperative day 3. As progressed, the

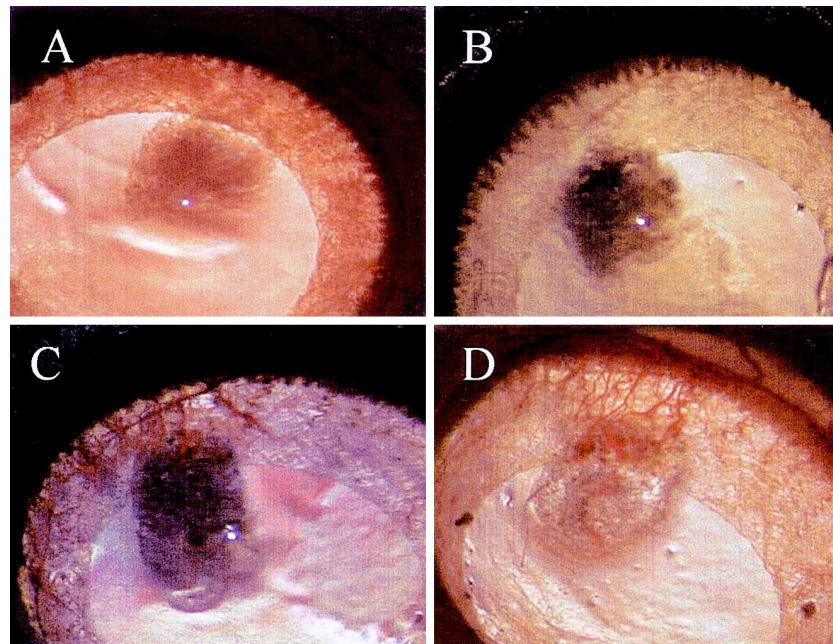


Fig. 6. Appearance of angiogenesis on day 7 after NGF implantation into the rat corneal stroma. A. Non-NGF pellet implanted (Group 0). B. 0.5 ng NGF pellet implanted (Group 0.5). C. 1.0 ng NGF pellet implanted (Group 1.0). D. 5.0 ng NGF pellet implanted (Group 5.0). The higher the NGF dose, the greater was the number of vessels in the rat cornea.

number, length, clock hours and areas of the vessels gradually increased. This is in agreement with the observation of Kenyon *et al.* [16], that neovascularization induced by bFGF began on day 3 and was sustained through to day 8. It was also reported that pellets containing sucralfate alone did not induce neovascularization and that pellets containing a lower dose of bFGF, caused a decrease in the linear and circumferential neovascular response. In this study, all observed criteria in Groups 0 and 0.5 were slightly increased after day 5.

In the high dose groups, Groups 1.0 and 5.0, the length, number, clock hours and areas of vessels were significantly greater than in the low dose groups, and there were no side-effects, such as corneal edema and intraocular hemorrhage, which were evident in the preliminary study using 10 ng and 100 ng of NGF. Kenyon *et al.* [16] demonstrated that high doses (145 ng and 180 ng) of bFGF induced stromal edema and hemorrhage in mice.

More than 1.0 ng of NGF had no further influence on the vessel length or the extent of circumferential neovascularization in this study, which was similar to that previously observed for more than 180 ng of bFGF [16].

Therefore, the dose-dependent relationships of bFGF and NGF show similar patterns, even though their effective doses are somewhat different. It is likely that the dose differences between bFGF and NGF are related to the experimental animal species and the characteristics of the growth factors chosen. It is probable that NGF has more

potent angiogenic effects than bFGF, as determined from results in the mouse cornea. Further studies will be needed to elucidate this point.

References

1. Ahmed, Z., Brown, R., Ljungberg, C., Wiberg, M. and Terenghi, G. Nerve growth factor enhances peripheral nerve regeneration in non-human primates. *Scand. J. Plast. Reconstr. Surg. Han. Surg.* 1999, **33**, 393-401.
2. Amico-Roxas, M., Caruso, A., Leone, M., Scifo, R., Vanella, A. and Scapagnini, U. Nerve growth factor inhibits some acute experimental inflammations. *Arch. Int. Pharmacodyn. Ther.* 1989, **299**, 269-285.
3. Boehim, T., Folkman, J., Browder, T. and O'Reilly, M. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 1997, **390**, 404-407.
4. Brooks, P., Montgomery, A., Rosenfeld, R., Hu, T., Klier, G. and Cheres, D. Intergrin $\alpha_v\beta_3$ antagonist promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994, **79**, 1157-1164.
5. Brown, W., Hudlicka, O., Damon, D. and Duling, B. Vasoactive effects of basic and acidic fibroblast growth factors in hamster cheek pouch arterioles. *Int. J. Microcirc. Clin. Exp.*, 1996, **16**, 308-312.
6. Cao, R., Farnebo, J., Kurimoto, M. and Cao, Y. Interleukin-18 acts as an angiogenesis and tumor suppressor. *FASEB J.* 1999, **13**, 2195-2202.
7. Chen, Z. and Wang, M. Effects of nerve growth factor on crushed sciatic nerve regeneration in rats. *Microsurgery* 1995, **16**, 547-551.

8. **D'Amato, R., Loughnan, M., Flynn, E. and Folkman, J.** Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci.* 1994, **91**, 4082-4085.
9. **Dellian, M., Witwer, B., Salehi, H., Yuan, F. and Jain, R.** Quantitation and physiological characterization of angiogenic vessels in mice: effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. *Am. J. Pathol.* 1996, **149**, 59-71.
10. **Edelman, J., Castro, M. and Wen, Y.** Correlation of VEGF expression by leukocytes with the growth and regression of blood vessels in the rat cornea. *Invest. Ophthalmol. Vis. Sci.* 1999, **40**, 1112-1123.
11. **Fajardo, L., Kowalski, J., Kwan, H., Prionas, S. and Allison, A.** The disc angiogenesis system. *Lab. Invest.* 1988, **58**, 718-724.
12. **Folkman, J.** Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1995, **1**, 27-31.
13. **Folkman, J. and D'Amore, P.** Blood vessel formation : what is its molecular basis. *Cell* 1996, **87**, 1153-1154.
14. **Funahashi, Y., Wakabayashi, T., Semba, T., Sonoda, J., Kitoh, K. and Yoshimatsu, K.** Establishment of a quantitative mouse dorsal air sac model and its application to evaluate a new angiogenesis inhibitor. *Oncol. Res.* 1999, **11**, 319-329.
15. **Hanahan, D. and Folkman, J.** Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, **86**, 353-364.
16. **Kenyon, B., Voest, E., Chen, C., Flynn, E., Folkman, J. and D'Amato, R.** A model of angiogenesis in the mouse cornea. *Invest. Ophthalmol. Vis. Sci.* 1996, **37**, 1625-1632.
17. **Kim, J., Li, B., Winer, J., Armanini, M., Gillet, N., Phillips, H. and Ferrara, N.** Inhibition of vascular endothelial growth factor-induced angiogenesis suppress tumor growth in vivo. *Nature* 1993, **362**, 841-844.
18. **Lambiase, A., Manni, L., Bonini, S., Rama, P., Micera, A. and Aloe, L.** Nerve growth factor promotes corneal healing: structural, biochemical, and molecular analyses of rat and human corneas. *Invest. Ophthalmol. Vis. Sci.* 2000, **41**, 1063-1069.
19. **Lebel, L. and Gerdin, B.** Sodium hyaluronate increases vascular ingrowth in the rabbit ear chamber. *Int. J. Exp. Pathol.* 1991, **72**, 111-118.
20. **Loughman, M., Chatzistefanou, K., Gonzalez, E., Flynn, E., Adamis A. P., Shing Y., D'Amato, R. and Folkman, J.** Experimental corneal neovascularization using sucralfate and basic fibroblast growth factor. *Aust. N. Z. J. Ophthalmol.* 1996, **24**, 289-295.
21. **Mahoney, M. and Saltzman, M.** Millimeter-scale positioning of a nerve growth factor source and biological activity in the brain. *Proc. Natl. Acad. Sci.* 1999, **96**, 4536-4539.
22. **Martinez, A. and Bjarklund, A.** Protection of the Neostriatum against Excitotoxic Damage by Neurotrophic Producing, Genetically Modified Neural Stem Cells. *J. Neuro. Sci.* 1996, **16**, 4604-4616.
23. **Miller, J., Stinson, W., Gregory, W., Koumy, H. and Puliafito, C.A.** Phthalocyanine photodynamic therapy of experinemtal iris neovascularization. *Ophthalmology* 1991, **98**, 1711-1719.
24. **Ohta, H., Ishiyama, J., Saito, H. and Nishiyama, N.** Effects of pretreatment with basic fibroblast growth factor, epidermal growth factor and nerve growth factor on neuron survival and neovascularization of superior cervical ganglion transplanted into the third ventricle in rats. *Jpn. J. Pharmacol.* 1991, **55**, 255-262.
25. **Pang, Q., Luo, Y., Wu, U., Li, Z., Fang, H., Yuan, T., Zhang, W. and Chen, Q.** Experimental studies on peripheral nerve regeneration enhanced by nerve growth factor. *J. Tongji Med. Univ.* 1993, **13**, 34-39.
26. **Polverini, P., Bouck, N. and Razinejad, F.** Assay and purification of naturally occurring inhibitor of angiogenesis. *Methods Enzymol.* 1991, **198**, 440-450.
27. **Risau, W.** Mechanisms of angiogenesis. *Nature* 1997, **386**, 671-674.
28. **Santos, P., Winterowd, J., Allen, G., Bothwell, M. and Rubel, E.** Nerve growth factor: increased angiogenesis without improved nerve regeneration. *Otolaryngol. Head Neck Surg.* 1991, **105**, 12-25.
29. **Santos, X., Rodrigo, J., Hontanilla, B. and Bilbao, G.** Evaluation of periphral nerve regeneration by nerve growth factor locally administered with a novel system. *J. Neurosci. Methods* 1998, **85**, 119-127.
30. **Varon, S. and Conner J.** Nerve growth factor in CNS repair. *J. Neurotrauma* 1994, **11**, 473-486.