J Vet Clin 18(4) : 418-423(2001)

The Role of Nerve Growth Factor on Corneal Wound Healing in Dogs

Heung-myong Woo[1]

Department of Surgical Sciences, School of Veterinary Medicine, University of Wisconsin Madison

Abstract : To investigate the modulation of nerve growth factor (NGF) during corneal epithelial wound healing and the effect of topical NGF on corneal epithelial wound healing in dogs. An axial epithelial defect was created in the right eye using 6mm axial corneal mechanical debridement while the left served as an unwounded control. The tears were collected from both eyes during 1 week and the corneal epithelium was processed for the measurement of NGF at day 0 and 7. The NGF content of tears and corneal epithelium was determined by enzyme-linked immunosorbent assay. In another experiment, the animals were divided into 3 groups. The right eyes in each group were treated every six hours with 200 ug/ml of recombinant human (rh) NGF, murine NGF, or 600 ug/ml of anti-NGF blocking antibody. The left eye of each animal was treated with bovine serum albumin (BSA) to serve as controls. Wound healing was analyzed using NIH image software. Tear NGF was markedly increased in the wounded eyes, relative to tears from control eyes during the early healing period. The NGF content of the corneal epithelium was elevated in the wounded eye ($p = 0.024$). Time to wound closure and rate of epithelial migration were not significantly different between the NGF treated or the NGF antibody treated, and the control BSA treated eyes. Corneal epithelial wounding increased NGF content only on the wounded side during the early healing period. Neither topical recombinant human or murine NGF affected corneal epithelial wound healing in the normal dog.

Key words **:** corneal wound, nerve growth factor, dog

Introduction

The survival of primary sensory neurons depends on neurotrophins, exerting their biological function by signaling through specific receptors of the *trk* family of tyrosine kinase receptors[11,26]. Nerve growth factor (NGF) represents the first isolated and best characterized neuropeptide of a growing family of neurotrophins that is best known for their profound effects on cells of the nervous system[1]. In its active form, NGF isolated and purified from male mouse salivary gland (2.5S) is a dimer weighing approximately 26 kDa[4]. In recent years, numerous studies have provided evidence that the effects of NGF are not limited to the peripheral and central nervous system but also act on non-neural cells such as the endocrine, immune system and other tissue cell types [7,20,29]. They have been shown to play important roles in diverse biological activities including modulation of proliferation, migration and differentiation in various cells.

The cornea is one of the most densely innervated structures in the body and contains numerous cytoactive compounds[10,19]. In the recent years a multitude of cytokines have also been identified in the corneas of humans and other vertebrates[6,10,13,14,15,19,21]. It has been shown *in vitro* that corneal primary epithelial cells produce NGF[14]. In preliminary studies, we have also documented that NGF not only is synthesized by SV-40 human corneal epithelial cells (SV-40 HCEC)[6], but also play an important role in migration, proliferation of the cells[21]. In clinical studies, it has been demonstrated that topical exogenous NGF can restore corneal integrity in human patients with immune or neurotropic corneal ulcers[13,15]. NGF has recently gained attention for the treatment of chronic corneal epithelial defects in human. These observations support that tear and corneal epithelial NGF may play an important functional role in corneal wound healing.

However, in corneal wound healing, the functional significance of the large amounts of endogenous NGF observed in tears and corneas of the various species is not fully understood. Recent studies by several laboratories have demonstrated that the NGF produced in the cornea may not only be released into the tears from normal cornea, but may also be increased after corneal wounding and stimulation[14,28]. Because NGF exists in various forms and has been isolated from a variety of sources[5,9,25,31]. It has also been reported that exogenous 7S NGF and high molecular weight (HMW)-NGF obtained from mouse salivary gland didn't enhance wound healing in the hamster skin[17], although exogenous murine 2.5S NGF accelerated corneal wound healing[14]. These findings also lead to the hypothesis that the effect of exogenous NGF may differ depending on cell types and the species being investigated.

Dogs are the most commonly seen patients in veterinary practice to develop spontaneous chronic corneal epithelial defects (SCCED). Recently this spontaneous dog model has been used to evaluate the efficacy of Substance P (SP) ± insulin-like growth factor (IGF) in the treatment of chronic epithelial defects[2,22]. However, the role of NGF on corneal wound healing in the dog has not yet been studied.

When studying homeostasis and wound healing in the cornea, it is important to consider potential interactions between the cornea and the tears that bathe and protect the anterior

[1]Corresponding author.
E-mail : woo@surgery.wisc.edu

surface of the eye. Therefore, identifying the change of NGF content in the tears and the cornea is important in improving our understanding of their relation associated with corneal wound healing.

To investigate the role of NGF in the dog cornea, we determined the expression of NGF in dog tears and corneal epithelium.

Materials and Methods

In all experimental procedures, the animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Effect of corneal wounding on the NGF content of the tissues associated with healing.

Six normal Beagle dogs were used in the experiment. Tears were collected from both eyes for two days prior to wounding using sterile, soft, fine diameter silicone tubing attached to a 3 ml syringe. Six mm axial epithelial defects were created in the right eye using mechanical debridement (n=6) while the left served as an unwounded control. Debrided epithelium was collected and stored in 50 ul of PBS immediately after surgery and placed on dry ice. Tears were collected once daily from both eyes for 1 week following debridement. After 1 week the dogs were euthanized with an IV injection of pentobarbital (100-200 mg/kg) and then the corneas of each dog were rapidly collected and stored at -70°C until processing for the measurement of NGF.

NGF immunoassay procedure

Tissues were rapidly dissected, weighed, and NGF was extracted according to the method of Zettler et al[32] with minor modifications. Briefly, the tissues were homogenized in 0.5 ml ice cold extraction medium consisting of Tris-HCl (100 mM), NaCl (0.4 M), BSA (1%), sodium azide (0.05%), Triton X-100 (1%), phenylmethylsulphonyl fluoride (1mM) EDTA (4 mM) and aprotinin (0.09 TIU/ml) pH 7.0. After homogenization, samples were spun at 3000 RPM for 15 min. at 4°C and supernatants were collected for NGF immunoassay. NGF concentrations were determined using a commercially available ELISA (NGF EmaxTM immunoassay system from Promega™), which has detection limit of 5 pg/ml, and expressed as ng/ml of tears or ng/g of tissue. All results were expressed as the mean ± SD. Differences were assessed using a paired t-test.

Effect of the NGFs or anti-NGF blocking antibody on corneal epithelial wound healing.

Young adult female beagle dogs with clinically normal eyes weighing 8-10 kg were used in the experiment. All dogs were sedated with 1 mg/kg IM morphine and 0.1 mg/kg IM acepromazine. Six-mm axial corneal epithelial defects were created using mechanical debridement in both eyes of 16 dogs. The animals were divided into 3 groups. The right eyes in each group were treated every six hours with 200 ug/ml of recombinant human NGF (provided by Genentech; n = 4), murine NGF (provided by Dr. Rama, Hospital of Venice "SS. Giovanni e Paolo," Italy; n = 6), or 600 ug/ml anti-NGF blocking antibody (provided by Genentech; n = 6). The left eye of each animal was treated with an equimolar concentration of bovine serum albumin (BSA) in phosphate buffered saline to account for non-specific protein effects. The size of the defect was measured every six hours for the first 72 hours after surgery by instillation of fluorescein and subsequent image capture and analysis using NIH Image software. All images were taken at a fixed focal length under a cobalt blue filter. The healing rates were expressed as a linear decrease of the wound radius per hour[30].

Results

Effect of corneal wounding on NGF content.

Debrided corneas were almost entirely re-epithelialized by 72 hours after surgery. Fig 1 shows that tear NGF was markedly increased for 3 days after epithelial wounding bilaterally. Tear NGF concentration in tears from wounded eyes was significantly greater than tears from unwounded eyes.

NGF content of epithelial cells from wounded eyes was increased at 1 week following debridement (Fig 2).

Effect of the exogenous NGFs or anti-NGF blocking antibody on epithelial wound healing.

As Fig 3 shows, The spherical radiuses of debrided cornea after corneal wounding were reduced with the similar

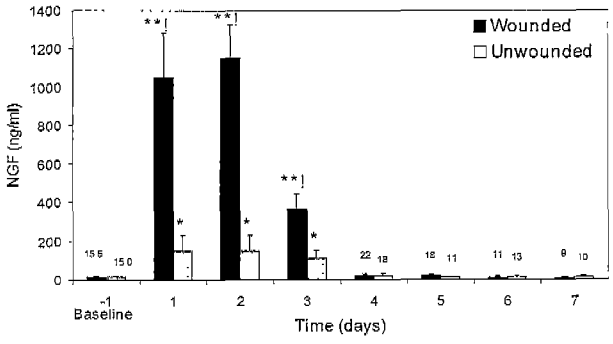

Fig 1. Tear NGF content after corneal epithelial debridement. *p<0.001 comparing the value to baseline; **p<0.0001 comparing the value to baseline; !p<0.001 comparing the value to the unwounded

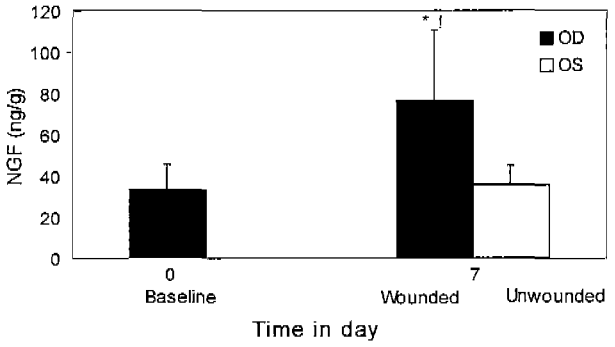

Fig 2. Corneal epithelium NGF content 1 week after corneal epithelial debridement. *P = 0.024 comparing the value to baseline: P = 0.029 comparing the value to the unwounded group.

slopes between the treated eyes and the control BSA treated eyes (Fig 3 A, B, C). Time to wound closure and rate of epithelial migration excluded latent phase were also not significantly different between the NGFs treated or the anti-NGF antibody treated, and the control BSA treated eyes (Table 1).

Discussion

The cornea is one of the most densely innervated structures in the body containing numerous cytoactive compounds[19,10]. NGF is known to play a significant role in the wound healing process including: the formation of adhesion complexes, the acceleration of corneal epithelial cell migration, the induction of mitogenesis, and the promotion of cellular differentiation[12,13,15].

These experimental studies demonstrated that NGF is stored and produced in the corneal epithelium, and that tear NGF is present at high concentrations in the normal dog.

In the dog NGF mRNA has also been found in elements of the adult peripheral nervous system-the sciatic nerve and the sympathetic and sensory ganglia[24]. It has been reported that wounding of normal tissue leads to an increase in levels of endogenous NGF in mouse skin as well as in the rat and human cornea[20,28]. In this study, we demonstrated that NGF content of the tear film increased to a greater degree on the wounded side for 3 days until debrided corneas were

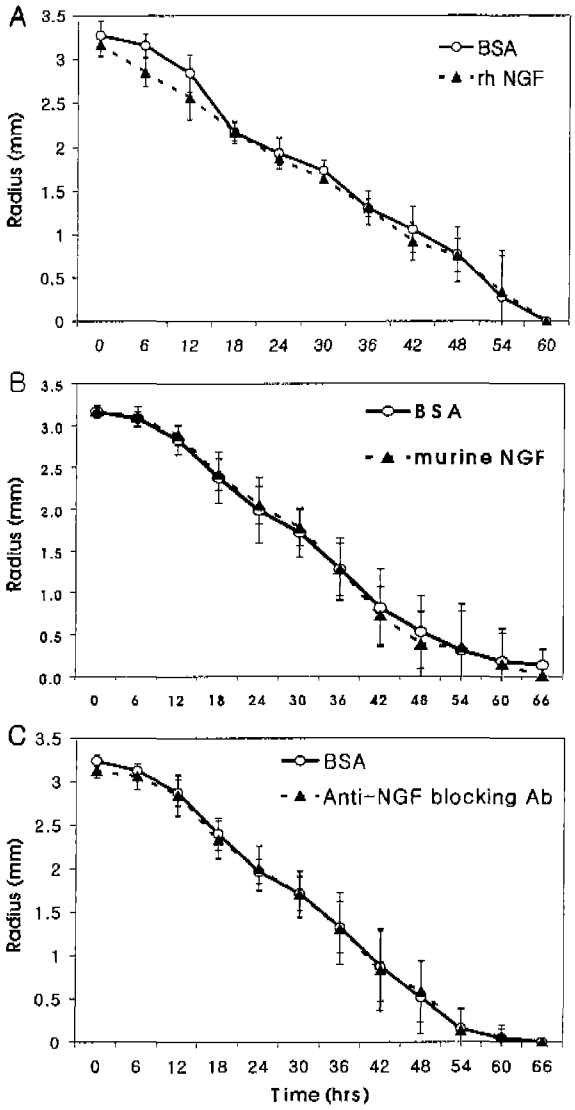

Fig 3. Change in corneal epithelial wound radius: topical NGFs and anti-NGF blocking antibody. Topical NGF does not accelerate corneal epithelial wound healing.

entirely re-epithelialized. Interestingly, tear NGF content also showed a significant increase in the tears contralateral to the wounded cornea during the early healing period, though the degree of the increase was less than on the

Table 1. Mean (SD) epithelial healing rates and time to healing after corneal epithelial debridement

Treatments	Healing rate* (um/hour)			Time to heal (hour)		
	OD (treated)	OS (BSA**)	P value	OD (treated)	OS (BSA)	P value
Murine NGF (n = 6)	66.7 (11.8)	62.1 (11.9)	0.051	53.8 (7.2)	59.5 (14.5)	0.18
Rh NGF[†] (n = 4)	62.3 (17.9)	64.8 (13.5)	0.46	58.0 (9.4)	54.5 (8.3)	0.17
Anti-NGF Ab.(n = 6)	49.1 (6.1)	51.0 (8.9)	0.60	64.7 (8.9)	61.0 (8.2)	0.12

*The rates were calculated by the decrease in wound radius; mean healing rate excluded a latent phase.
**Bovine serum albumin
[†]Recombinant human NGF

wounded side.

Despite these findings, neither topical NGF's nor anti-NGF blocking antibody affected corneal epithelial wound healing in the normal dogs in this study. However, in recent years, several studies reported by other laboratories have demonstrated that topical NGFs altered biological activity in corneal epithelium. Topical NGF has been shown to increase migration, proliferation and differentiation of cultured rabbit corneal epithelial cell and SV-40 HCEC cell[21,12]. A clinical study has demonstrated that NGF treatment accelerates the healing of corneal neurotropic ulcers that had developed subsequent to dysfunction of corneal sensitivity innervation[15]. Lambiase et al have also reported that topical application of murine NGF accelerates corneal epithelial wound healing, conversely anti-NGF antibody attenuated healing compared with nonspecific IgG after chemical burn in the rabbit cornea[14].

There are several ways of interpreting these data.

First, comparing to the model of heptanol-induced epithelial wounding Lambiase et al[14] used, we used a limited mechanical debridemented wound model of the central cornea without damage to the basal epithelial membrane complexes and anterior stroma[3]. In our model the corneal stroma is disrupted to much lesser extent than in the chemical burn wound model, which is associated with damage to corneal nerve terminals of mainly unmyelinated polymodal nociceptor fibers[28]. It is possible that an effect of NGF on canine corneal wound healing would have been observed if a more severe model had been utilized, such as complete debridement of the epithelium with limbal compromise[27].

Second, It is likely that endogenous NGF concentrations after corneal wounding would be optimal in the normal dog and thus supplementation through topical application would not improve the wound healing response.

Finally, the results may indicate a possibility that murine 2.5S and rh NGF that we used in this study didnt bind to the receptors that we observed in dog cornea or differences of intracellular pathways activated subsequent to binding were different, or that the relevant concentrations of NGF were not physiologically optimal. NGF with different forms has been isolated from various sources such as 2.5S NGF with 26 kDa, 7S NGF with 140 kDa or 116kDa from mouse salivary, submaxillary, or submandibular gland, respectively [18,19,25,31]. The topical NGFs used in this study been shown to act on other species such as mice, rats, rabbits and humans [12,14,18]. Species variability in response to NGF from different sources has also been reported. Exogenous 7S NGF and HMW-NGF obtained from mouse salivary gland affected skin wound healing differently when tested using the Syrian hamster and the mouse[18]. Exogenous murine 2.5S NGF accelerated corneal wound healing in rabbit[14]. Collectively, these data suggest that the wound healing properties of topical NGFs may be more species-specific than its neurotrophic effects.

The effect of NGF has also been documented to stimulate and enhance the synthesis and release of neurotrotransmitters and neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP) in peripheral neurons[8,23]. Additionally, a recent study reported by our laboratory has demonstrated that NGF modulates migration, proliferation of SV-40 HCEC by interaction with SP, IGF and CGRP[16].

Thus, clearly to distinguish between these possibilities, further studies on the interaction with the neurotrotransmitters and neuropeptides are required.

References

1. Barde YA. The nerve growth factor family. Prog Growth Factor Res 1990; 2: 237-248.
2. Bentley E, Abram G, Reid TW, Cook C, Fisher C, Covitz D, Stur C, Hacker D, Murphy CJ. Morphology and Immunohistochemistry of chronoc, non-septic, corneal epithelial defects in dogs. Invest Ophthalmol Vis Sci:inpress.
3. Bentley E, Campbell SF, Woo HM, Murphy CJ. The effect of repeated corneal epithelial debridement on epithelial and stromal morphology in the dog. Invest Ophthalmol Vis Sci 2001;42:S792. Abstract nr 4773.
4. Bocchini V, Angeletti PU. The nerve growth factor: purification as a 30,000-molecular-weight protein. Proc Natl Acad Sci U S A 1969; 64: 787-794.
5. Bradshaw RA. Nerve growth factor. Annu Rev Biochem 1978; 47: 191-216.
6. Campbell SF, Reid TW, Murphy CJ. The effect of growth factors, neuropeptides and neurotransmitters on NGF content in SV-40 transformed human corneal epithelial cells. Invest Ophthalmol Vis Sci 2000; 41: S905. Abstract nr 4806.
7. Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 2001; 142: 2078-2086.
8. Donnerer J, Schuligoi R, Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. Neuroscience 1992; 49: 693-698.
9. Fedorushchenko AN, Koval TI, Khamidov DK. [The effect of in-situ nerve growth factor from different biological sources on the reinitiation of mouse oocyte meiotic maturation in culture and on parthenogenetic activation]. Ontogenez 1999; 30: 453-455.
10. Jones MA, Marfurt CF. Peptidergic innervation of the rat cornea. Exp Eye Res 1998; 66: 421-435.
11. Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 1991; 65: 189-197.
12. Kruse FE, Tseng SC. Growth factors modulate clonal growth and differentiation of cultured rabbit limbal and corneal epithelium. Invest Ophthalmol Vis Sci 1993; 34: 1963-1976.
13. Lambiase A, Bonini S, Aloe L, Rama P. Anti-inflammatory and healing properties of nerve growth factor in immune corneal ulcers with stromal melting. Arch Ophthalmol 2000;

118: 1446-1449.
14. Lambiase A, Manni L, Bonini S, Rama P, Micera A, 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.
15. Lambiase A, Rama P, Bonini S, Caprioglio G, Aloe L. Topical treatment with nerve growth factor for corneal neurotrophic ulcers. N Engl J Med 1998; 338: 1174-1180.
16. Lee CH, Nelson M, Barney NP, Brightbill FS, Araki-Sasaki K, Reid TW, Murphy CJ. Migration and proliferation characteristics of SV-40 transformed human corneal epithelial cells in response to neuropeptides (ARVO abstract). Invest Ophthalmol Vis Sci 1997; 37: S861. Abstract nr 3934.
17. Leitzel K, Cano C, Marks J, Jr., Lipton A. Failure of nerve growth factor to enhance wound healing in the hamster. J Neurosci Res 1982; 8: 413-417.
18. Li AK, Koroly MJ, Schattenkerk ME, Malt RA, Young M. Nerve growth factor: acceleration of the rate of wound healing in mice. Proc Natl Acad Sci U S A 1980; 77: 4379-4381.
19. Marfurt CF, Ellis LC. Immunohistochemical localization of tyrosine hydroxylase in corneal nerves. J Comp Neurol 1993; 336: 517-531.
20. Matsuda H, Koyama H, Sato H, Sawada J, Itakura A, Tanaka A, Matsumoto M, Konno K, Ushio H, Matsuda K. Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. J Exp Med 1998; 187: 297-306.
21. Murphy CJ, Campbell SF, Reid TW. Modulation of SV-40 transformed human corneal epithelial cell proliferation, migration, and adhesion by nerve growth factor (ARVO abstract). Invest Ophthalmol Vis Sci 2000; 41: S903. Abstract nr 4803.
22. Murphy CJ, Marfurt CF, MaDermott C, Bentley E, Abram G, Reid TW, Campbell S. Chronic non-septiccorneal epithelial defects in dogs: Clinical fetures, innervation and effect of topical SP and IGF-1. Invest Ophthalmol Vis Sci:in press.
23. Schicho R, Donnerer J. Nerve growth factor stimulates synthesis of calcitonin gene-related peptide in dorsal root ganglion cells during sensory regeneration in capsaicin-treated rats. Neurosci Res 1999; 35: 183-187.
24. Shelton DL, Reichardt LF. Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc Natl Acad Sci U S A 1984; 81: 7951-7955.
25. Shooter EM. Early days of the nerve growth factor proteins. Annu Rev Neurosci 2001; 24: 601-629.
26. Snider WD. Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 1994; 77: 627-638.
27. Tseng SC. Regulation and clinical implications of corneal epithelial stem cells. Mol Biol Rep 1996; 23: 47-58.
28. Vesaluoma M, Muller L, Gallar J, Lambiase A, Moilanen J, Hack T, Belmonte C, Tervo T. Effects of oleoresin capsicum pepper spray on human corneal morphology and sensitivity. Invest Ophthalmol Vis Sci 2000; 41: 2138-2147.
29. Villoslada P, Hauser SL, Bartke I, Unger J, Heald N, Rosenberg D, Cheung SW, Mobley WC, Fisher S, Genain CP. Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. J Exp Med 2000; 191: 1799-1806.
30. Woo HM, Kim MS, Kweon OK, Kim DY, Nam TC, Kim JH. Effects of amniotic membrane on epithelial wound healing and stromal remodelling after excimer laser keratectomy in rabbit cornea. Br J Ophthalmol 2001; 85: 345-349.
31. Young M, Saide JD, Murphy RA, Blanchard. Nerve growth factor: multiple dissociation products in homogenates of the mouse submandibular gland. Purification and molecular properties of the intact undissociated form of the protein. Biochemistry 1978; 17: 1490-1498.
32. Zettler C, Bridges DC, Zhou XF, Rush RA. Detection of increased tissue concentrations of nerve growth factor with an improved extraction procedure. J Neurosci Res 1996; 46: 581-594.

Department of Surgical Sciences, School of Veterinary Medicine, University of Wisconsin Madison

우 흥 명
위스콘신대학교 수의과대학 외과학교실

요 약 : 각막상피의 창상치유에 중요한 역할을 하는 것으로 알려진 Nerve Growth Factor(NGF)의 내인성 변화와 국소적 투여효과를 알아보았다. 6마리의 Beagle 을 사용하여 비교군인 오른쪽 눈에만 6 mm의 기계적인 각막 창상을 만들었고 왼쪽 눈은 대조군으로 사용하였다. 눈물은 수술후 1주일동안 매일, 각막 상피는 수술중 그리고 수술후 7일에 각각 채취하여 NGF 농도를 enzyme-linked immunosorbent assay (ELISA)를 사용하여 측정하였다. 2차 실험으로, 16마리의 Beagle을 사용하여 오른쪽 눈에 위와 동일한 창상을 만든 후, 창상 부위가 회복될 때까지 recombinant human (rh) NGF (n = 4), murine NGF (n = 6) 그리고 anti-NGF blocking antibody (n = 6)를 6시간마다 점안하였으며, 왼쪽 눈은 bovine serum albumin (BSA)를 점안하여 대조군으로 사용하였다. 창상 면적은 NIH image software를 이용하여 분석하였다. 대조군에 비하여, 비교군의 눈물내 NGF는 치유초기에 현저하게 증가하였으며, 각막 상피의 NGF도 유의적인 증가를 보였다. 그러나 rh NGF, murine NGF, anti-NGF blocking antibody처치군과 BSA처치군 간에는 각막창상 치유에 유의적인 차이가 인정되지 않았다. 각막창상후, rh NGF 혹은 murine NGF의 추가적인 국소점안은 정상개의 각막상피 치유에 효과가 없었으나, 창상후 초기 치유기동안 눈물과 손상받은 각막상피에서 내인성 NGF의 급격한 보상성 증가로 미루어 NGF는 각막 상피의 창상치유에 중요한 역할을 하는것으로 사료된다.