*Short Communication***Nerve Growth Factor Suppresses Prostate Tumor Growth**

Mitsuhiro Goda¹, Saori Atagi¹, Keisuke Amitani¹, Narumi Hobara², Yoshihisa Kitamura³, and Hiromu Kawasaki^{1,*}

¹Department of Clinical Pharmaceutical Science, ³Department of Pharmaceutical Care and Health Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan

²Department of Life Science, Okayama University of Science, 1-1 Ridai-cho, Okayama 700-0005, Japan

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Abstract. Nerve growth factor (NGF) facilitates reinnervation of perivascular nerves that regulate vascular tone and blood flow. This study investigated whether NGF prevents tumor growth by promoting neuronal regulation of tumor blood flow. The growth rate of DU145 prostate carcinoma cells subcutaneously implanted into nude mice was significantly inhibited by subcutaneous NGF administration. Significant suppression of tumor growth continued after withdrawing NGF. NGF increased vascular smooth muscle cells in tumor tissues, but had no cytotoxic action on tumor cells *in vitro*. These results suggest that NGF prevents tumor growth via an indirect effect, probably innervation or maturation of the tumor neovasculature.

Keywords: nerve growth factor, prostate tumor growth, angiogenesis

Angiogenesis is fundamental to physiological processes such as tissue development and regeneration. It also contributes to the development and progression of various pathological conditions, including tumor growth and metastasis (1, 2). Therefore, it appears that tumor proliferative activity is dependent on blood flow to tumor tissues (3). Perivascular nerves and endothelial cells play an important role in maintenance of vascular tone and regulate local blood flow (4). In normal tissues, the distribution of perivascular innervation in the microcirculation, such as terminal arterioles and precapillary arterioles, has been anatomically confirmed (5, 6). However, in tumor tissues, several immunohistochemical studies showed no innervation in tumor blood vessels and progressive loss of perivascular innervation from the tumor edge as the tumor grade advanced (7). Therefore, it is hypothesized that the neuronal regulation of tumor blood flow may induce tumor growth suppression. Recently, we demonstrated that nerve growth factor (NGF), which is known to promote innervation of central and peripheral nerves (8), facilitated reinnervation of perivascular sympathetic nerves and nonadrenergic noncholinergic nerves

in small mesenteric arteries, which were lesioned by topical phenol application of the superior mesenteric artery (9). NGF may have a suppressive effect on tumor growth by facilitating innervation on the neovasculature in tumor tissues. Therefore, the aim of this study was to investigate whether NGF has effects on tumor growth.

Five-week-old BALB/c Slc nu/nu mice (Shimizu Experimental Animals, Shizuoka) were used in this study. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C with 50 ± 10% relative humidity. This study was done in accordance with the Guidelines for the Animal Experiments at Okayama University Advanced Science Research Center.

Human prostate cancer DU145 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). DU145 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich Japan, Tokyo), 100 unit/mL penicillin, and 100 µg/mL streptomycin. DU145 cells (1×10^6 cell/50 µL) mixed with 50 µL of Matrigel were injected into the flanks of nude mice (100 µL/site). On Day 21 after the injections of tumor cells, a micro-osmotic pump (model 1002, Alzet; Alza, Palo Alto, CA, USA) containing NGF (TOYOBO Co., Ltd., Osaka) or sterile saline was subcutaneously implanted in

*Corresponding author. kawasaki@pheasant.pharm.okayama-u.ac.jp
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the dorsal area. NGF was administered at a rate of 20 or 40 ng/h for 14 days. Then, after 2 weeks of administration, the osmotic pump was surgically removed under ether anesthesia. Tumor growth was determined by measuring the tumor size, starting at Day 14 and ending at Day 42. Tumor volumes were calculated according to the formula $(\text{width}^2 \times \text{length}) / 2$.

To examine the direct effect of NGF on DU145 cells, DU145 cells were cultured in RPMI 1640 medium supplemented with saline or 50 ng/mL of NGF for 10 days (10) and the medium with NGF or saline was changed every day for 10 days. The cells (1×10^6 cell/100 μL) were injected into the flanks of nude mice and the tumor growth rate was measured as described above.

On Day 35, after the saline or NGF treatment for 14 days, tumor tissues were surgically removed and immersion-fixed in Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) for 48 h and prepared for paraffin section staining with hematoxylin and eosin. We immunohistochemically quantified

the density of microvessels by first scanning the tumor at a low power and identifying five areas at the tumor periphery that contained the maximum number of discrete microvessels and then counting individual microvessels. The density of vessels in each field was determined and expressed as vessels per field.

To examine proliferative effects of NGF on smooth muscle cells, tumor tissues dehydrated and paraffined were sliced in 7- μm -thick sections, which were incubated in Cy3-labeled anti- α -smooth muscle actin (α -SMA) mouse IgG (Sigma Aldrich Japan, Tokyo) at a dilution of 1:100 for 1 h at room temperature and observed under a confocal laser scanning microscope (CLSM510; Carl Zeiss, Tokyo) in the Okayama University Medical School Central Research Laboratory.

To examine the effect of NGF on DU145 cell viability, cell proliferation was determined using a WST-8 reduction method. Briefly, DU145 cells (100 μL , 1×10^4 cells) in the logarithmic growth phase were cultured onto 96-well plates for 24 h and incubated in NGF solution (50 or 500 ng/mL) or saline for an additional 24 or 48 h. After adding 10 μL of the working solution WST-8 kit (Kishida Chemical Ltd., Osaka), the absorbance of each well was

Fig. 1. Effect of nerve growth factor (NGF) on tumor growth (A) (NGF: 20 or 40 ng/h) and the survival rate (B) (NGF: 40 ng/h) in nude mice implanted with DU145 prostate cancer cells. NGF or saline was administered on Day 21 until Day 35 with an osmotic pump. Tumor volumes are expressed as the percentage relative to the values on Day 14 in each group. Note that NGF caused significant inhibition of DU145 tumor growth in tumor-bearing mice and the inhibitory effect lasted after NGF administration was discontinued. Each bar indicates a mean $\pm$ S.E.M. * $P < 0.05$ vs. Saline (Tukey's test) (A). The survival rate was expressed as the percentage of mice with adequate tumor control ($<1000 \text{ mm}^3$) in each group (B).

Fig. 2. Changes in tumor growth (A) and the survival rate (B) after implantation of DU145 cells (1×10^6 cell/100 μL) incubated with nerve growth factor (NGF: 50 ng/mL for 10 days) in nude mice. Tumor volumes are expressed as the percentage relative to the values on Day 14 in each group. Each bar indicates a mean $\pm$ S.E.M. (A). The survival rate was expressed as the percentage of mice with adequate tumor control ($<1000 \text{ mm}^3$) in each group (B).

measured at 450 nm with a reference wavelength at 650 nm with a microplate reader Model 550 (Bio-Rad Laboratories, Inc., Tokyo).

All data are expressed as the mean $\pm$ S.E.M. Statistical analysis was performed using unpaired Student's *t*-tests or analysis of variance (ANOVA) followed by Tukey's test. *P* values <0.05 were considered statistically significant.

In all mice implanted with DU145 cells (1×10^6 cells/site), measurable tumors developed within 14 days after implantation. These mice were divided into three groups: one group ($n = 10$) was treated with saline and the other groups were treated with NGF at 20 ($n = 8$) or 40 ng/h ($n = 6$) via a micro-osmotic pump for 14 days. As shown in Fig. 1A, tumor sizes in saline- and NGF-administered mice gradually increased during administration and after discontinuation. There was no significant difference between the saline and NGF (20 ng/h) groups. However, NGF administration at 40 ng/h for 14 days markedly inhibited tumor size as shown in Fig. 1A. There was a significant difference in tumor size between the NGF- and saline-treated groups. Furthermore, the tumor growth inhibition was maintained even after the discontinuation of NGF administration (Fig. 1A). The survival rate,

which was expressed as the percentage of mice with an adequate tumor volume less than 1000 mm^3 , was prolonged by NGF administration (40 ng/h) for 2 weeks (Fig. 1B).

To examine the direct effect of NGF on DU145 cells, we evaluated the effect on cell viability using the WST-8 assay. Neither treatment with NGF (50 or 500 ng/mL) for 24 nor 48 h had any effect on the viability of DU145 cells (data not shown). Furthermore, as shown in Fig. 2A, in nude mice implanted with DU145 cells (1×10^6 cell/100 μL) previously cultured with saline or 50 ng/mL of NGF for 10 days, the tumor size in saline ($n = 7$)– and NGF ($n = 8$)–treated mice gradually increased and there was no significant difference between saline and NGF treatment groups. Also, pretreatment with NGF had no effect on the survival rate of tumor-bearing mice (Fig. 2B), indicating that NGF exerts no effect on the viability of DU145 cells.

As shown in Fig. 3, A and B, tumors removed from animals administered saline and NGF had high vessel density, indicating the presence of extensive angiogenesis. However, tumors from NGF-treated animals showed similar microvessel density to that from saline-treated animals (Fig. 3C). Figure 3, D and E showed α -SMA–

Fig. 3. Effects of nerve growth factor (NGF: 40 ng/h) on tumor angiogenesis in nude mice implanted with DU145 cells. In the left panel, representative light micrographs show microvessels (open triangles) in tumors from animals that received saline (A) or NGF (B). A bar graph (C) shows quantitative analysis of microvessel density by counting the positively stained cells in five different fields ($\times 200$). Each bar indicates a mean $\pm$ S.E.M. In the right panel, representative fluorescence micrographs show α -smooth muscle actin (α -SMA) immunopositive microvessels in tumors from animals that received saline (D) or NGF (E). A bar graph (F) shows quantitative analysis of vascular smooth muscle density by computer-assisted image processing in five different fields ($\times 200$). Each bar indicates a mean $\pm$ S.E.M. **P* < 0.05 vs. Saline (Student's *t*-test).

immunopositive stainings in tumor tissues, indicating the presence of vessel mural cells in the tumors. As shown in Fig. 3F, quantitative analysis demonstrated a significant increase in the density of α -SMA-immunopositive cells in tumors from NGF-treated animals compared with saline-treated animals.

The present study demonstrated that NGF inhibited tumor growth of DU145 prostate cancer cells, which have NGF receptors, Trk A and p75 (10) and was implanted into nude mice, and prolonged the survival rate. Furthermore, the inhibitory effect of NGF was maintained even after its discontinuation, suggesting that it causes tumor growth inhibition by an indirect effect, probably by changing the features of tumor areas. This notion is supported by the findings that NGF had no effect on DU145 cell viability and NGF-pretreated DU145 cells caused tumor growth similar to non-treated tumor cells. NGF has been reported to promote neovessel formation in mouse hindlimb ischemia (11), which is a model of physiological angiogenesis. However, the present study showed that NGF had no effect on the density of neovessels in tumor tissues. Therefore, it seems unlikely that NGF promotes angiogenesis under pathologic conditions. Several reports showed enhanced migration of human lung fibroblasts, as well as human vascular smooth muscle cells, after NGF treatment (12). Here, NGF-treated tumor tissues had increased α -SMA immunopositive cells, suggesting that NGF promotes the migration of vascular smooth muscle cells to tumor neovessels, directly or via perivascular nerves. Human and rat vascular smooth muscle cells have been shown to express NGF receptor, Trk A (13). In addition, the smooth muscle cell migration induced by NGF is comparable to the migratory effects obtained in vitro with platelet-derived growth factor-BB (PDGF-BB), indicating that the neurotrophin NGF is a potent chemoattractant for cultured vascular smooth muscle cells (13). Furthermore, peripheral nerves have been reported to provide a template that determines the organotypic pattern of blood vessel branching and arterial differentiation in the skin (14). Additionally, enhancement of blood vessel maturation has been shown to inhibit tumor growth without suppression of angiogenesis (15). Taken together, it is inferred that NGF facilitates maturation of tumor neovessels by migrating smooth muscle cells, regulating tumor tissue blood flow, and resulting in suppression of tumor growth. However, further study is needed to clarify whether NGF facilitates innervation of neovessels in tumor tissues.

In conclusion, the present findings suggest that NGF suppresses the growth of prostate tumors in nude mice by accelerating the maturation of neovasculatures in tumor tissues; therefore NGF could be a novel anticancer drug

and approach for cancer therapy.

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