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Full Length Research Paper

Effects of recombinant human nerve growth factor on cervical cancer

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Nerve growth factor (NGF) plays a crucial role in the life of the sympathetic and sensory nervous systems. However, the roles of NGF to cervical cancer remain deeply unknown. This study investigated the effect of recombinant human nerve growth factor (rhNGF) on cervical cancer. It was found that the proliferation of hela cells was inhibited by rhNGF and there were some apoptotic-like cell deaths. After tumor modeling of mice implanted U14 cells, it was shown that the growth of tumor tissues was inhibited in response to rhNGF and the concentration of virus been rhNGF was not more than 1.0×10^7 pfu/ml. In addition, the immune abilities of thymus and spleen were improved by rhNGF. Finally, it was shown that SOD level of tumor tissues was improved under the right concentration of rhNGF. Therefore, the mentioned result showed that cervical cancer is probably treated by rhNGF, which is depended on the concentration of rhNGF.

Key words: rhNGF, cervical cancer, tumor inhibition.

INTRODUCTION

Nerve growth factor (NGF), an evolutionarily conserved polypeptide neurotrophin, plays a crucial role in the life of the sympathetic and sensory nervous systems (Sofroniev et al., 2001). The biologic effects of NGF on neural cells are mediated by 2 different receptor classes; the tropomyosin-related kinase A (TrkA) of tyrosine kinase receptor and the neurotrophin receptor p75, a member of the tumor necrosis factor receptor family (Frade and Barde, 1998; Frade et al., 1996; Rabizadeh et al., 1993; Wang et al., 2001). The binding of NGF to TrkA receptor promotes cell survival. When NGF binds to TrkA, it phosphorylates TrkA, which leads to the activation of PI 3 Kinase, ras and PLC signaling pathways. However, NGF binding to p75 receptor triggers cell apoptosis (Park et al., 2007). P75NTR induces apoptosis through its death domain in a NGF-independent manner and Caspases 9, 6, and 3 are activated by p75NTR (Wang et al., 2001). When p75NTR is activated by NGF, the activation of p75NTR kills cells in the central nervous system during normal development (Frade and Barde, 1998). The simultaneous expression of both receptors promotes cell proliferation, as the survival-enhancing effects of TrkA receptor negate the apoptosis-inducing signals of p75 receptor. The precise ratio of TrkA and p75 receptors is thought to be an important determinant of cell survival and death (Arrighi et al., 2010).

At present, there are many reports on NGF in neoplasms unrelated to the nervous system (Fanburg-Smith and Miettinen, 2001), such as myeloma, acute myeloid leukemia, fibrosarcoma, hepatocellular carcinoma, pancreatic cancer, lung cancer and thyroid papillary carcincoma (Eguchi et al., 1999; Knezevich et al., 1998; McGregor et al., 1999; Okada et al., 2004; Pearse et al., 2005; Ricci et al., 2001; Yang et al., 2005). Bothwell (1991) reported that TrKA was the producer of oncogene. Dollé et al. (2003) proved that, NGF was the promoter of breast cancer. Adriaenssens et al. (2008) showed that NGF was the new target for breast cancer treatment. However, other reports showed that cell death was induced by NGF through its p75 receptor (Frade et al., 1996; Rabizadeh et al., 1993). Missale et al. (1993) reported that NGF suppressed the transforming phenol-

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type of human prolactinomas and NGF could cause differentiation of rat C6 glioma cells and strongly inhibit their proliferation in vitro (Kimura et al., 2002). Although, the effect of NGF on the cancer possibly depends on the tumor types (Krüttgen et al., 2006), the detailed role of NGF to tumor cells is still not clear and controversial. This study was aimed to detect the effect of NGF on cervical cancer. It was shown that the proliferation of cervical cancer cells was inhibited by rhNGF in vitro and in vivo; whereas the action was related to the concentration of rhNGF. In addition, it was found that the immunity of cervical cancer mice was improved.

MATERIALS AND METHODS

Reagents

MTT and Trypanblau were from Sigma. Hoechst was purchased from Biyuntian biotechnology research center. 5-fluorouracil was purchased from Jinyao amino acid Inc. (Tianjin, China) and 50 μg/ml with normal sodium was made when needed. CTX (Cyclophosphamide, Batch no. H14020373) was purchased from Taisheng drugs manufacture (Shanxi, China) and 100 mg/L was made with normal sodium when needed for use. Unless otherwise specified, all the chemicals were purchased from China.

RhNGF, 293T cells, hela cells, U14 cells and animals

RhNGF was a gift from the Biotechnology Laboratory of Yanshan University, Qinhuangdao, China. 293T cells, hela cells and mouse UCC U14 cell lines were from the Institute of Materia Medica of Chinese Academy of Medical Sciences. Kunming cleaning female mice (Lic No. SCXK (capital) 2004-0001) were from the Institute of Zooscopy of Chinese Academy of Medical Sciences. The test temperature of the mice was 20 to 25°C and the relative humidity was 40 to 60%.

Cell culture and the effect of rhNGF on hela cells

293T cells infected GFP-rhNGF virus were grown at 37°C in Dulbecco's modified eagle's medium (DMEM) containing 10% FBS and maintained in a 5% CO_2 atmosphere. When the rate of cytopathic effect (CPE) was up to 90%, the cells were collected, lysated and centrifugated. After that, the supernatant was collected to detect the concentration of GFP-rhNGF virus.

The inhibition rate of hela cells was detected by MTT. Six experimental groups were prepared; negative control group, positive control group and 4 rhNGF-treated groups. Each group was inoculated with about 1.0×10^4 pfu hela cells. After the hela cells were cultured for 24 h, GFP-rhNGF virus was added and the concentrations of 4 rhNGF groups were 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 pfu/ml, respectively. The medium was added into the cells of the negative control group and 5-fluorouracil was added into the cells of the positive control group. The hela cells were incubated for 24 h again, 0.1 mg MTT was added to each group and then, the cells were cultured for 4 h. After that, the old medium was removed and 150 μl DMSO was added. Finally, the OD value was determined at less than 492 nm through ELISA reader, to count the cell inhibition rate.

The cell apoptosis was checked by Hoechst. Hela cells of the rhNGF-treated groups (1.0×10^7 pfu/ml) were incubated for 24 and48 h. Then, the old medium was removed, the hela cells were washed with PBS and 100 μ and Hoechst was added. After staining for 20 min, the cells were observed and photographs were taken.

Mouse U_{14} cells modeling and analyzing

The fasted (one-day) and weighted mice were implanted with about 1.5×10^6 pfu U14 cells and were left forefoot oxter. After that, the mice were randomly divided into 6 groups which included a negative and a positive control group and 4 rhNGF-treated groups. Each group had 8 mice. The following day, all the mice in the negative control group were given 0.2 ml distilled water and all mice of the positive control group were given 1 mg of CTX per 10 g wt. The dosages of GFP-rhNGF virus in 4 rhNGF groups were 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 pfu, respectively. The treatment to the mice was given once per day for 15 days. After the discontinuation, mice were weighted and then, sacrificed to investigate the changes in the tumor, spleen, thymus, SOD and so on.

Statistics

All experiments were repeated at least two times. Results were expressed as mean ± S.E. The data were analyzed with the software package SPSS 16.0. P- values < 0.05 were considered significant.

RESULTS

Effect of rhNGF on hela cells

The four rhNGF-treated groups (1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 pfu/ml) were firstly in turn named rhNGF-1, rhNGF-2, rhNGF-3 and rhNGF-4 respectively for convenient records. According to Figure 1, the proliferation of hela cells was inhibited by rhNGF. The inhibition rate gradually went up with the increase of GFP-rhNGF virus concentration. When the concentration of GFP-rhNGF virus reached 1.0×10^7 pfu/ml, the inhibitory action of rhNGF to hela cells was increased greatly and the inhibition rate was 72.63%. However, the inhibitory action on the basis of rhNGF-3 group was not improved when the concentration of rhNGF virus surpassed 1.0×10^7 pfu/ml. Therefore, it was concluded that the proliferation of hela cells was inhibited in response to rhNGF and the inhibitory action was best when the concentration of rhNGF virus was 1.0×10^7 pfu/ml.

RhNGF induces the apoptosis of hela cells

The apoptosis of hela cells was detected through Hoechst. Cell nucleus was stained blue. Apoptotic body was not found when hela cells of the negative control group were cultured for 48 h. However, some apoptotic bodies were observed when rhNGF-treated cells (1.0×10^7 pfu/ml) were cultured for 24 h and more apoptotic bodies were seen after 48 h (Figure 2b,c). Therefore, it was speculated that cell apoptosis was developed in response to rhNGF and the changes were increasingly obvious

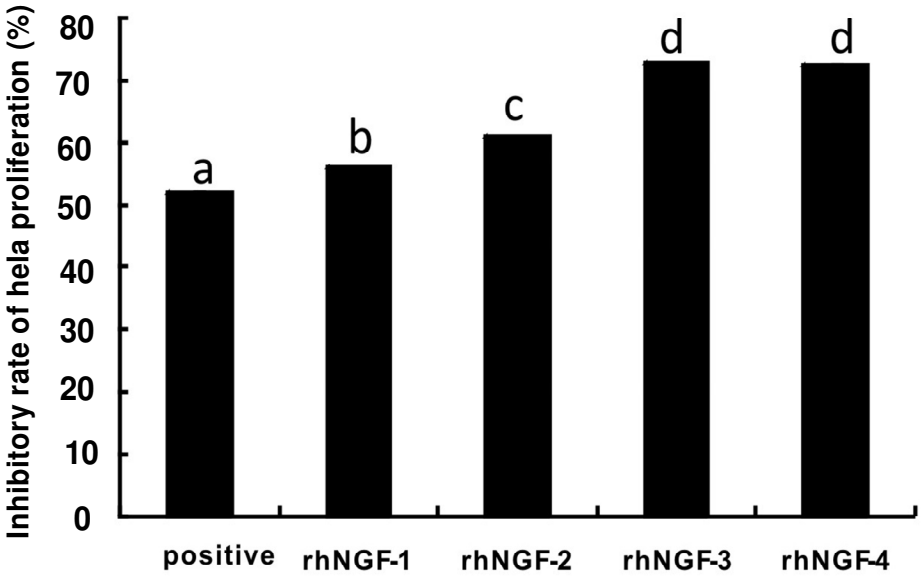

Figure 1. The proliferation of hela cells blocked by rhNGF. The cytostasis rate was the percentage of the OD value of the negative control group which minus the OD value of the drug-treated group divided by the OD value of the negative control group (×100). Through Elisa reader, OD values of all the experimental groups were obtained and the cytostasis rate of hela cells was counted. The cytostasis rate in the positive, rhNGF-1, rhNGF-2, rhNGF-3 and rhNGF-4 groups were 52.01, 56.06, 60.83, 72.63 and 72.52%, respectively. Values with different letter showed significant discrepancy ($P < 0.05$) and same letter showed $P > 0.05$.

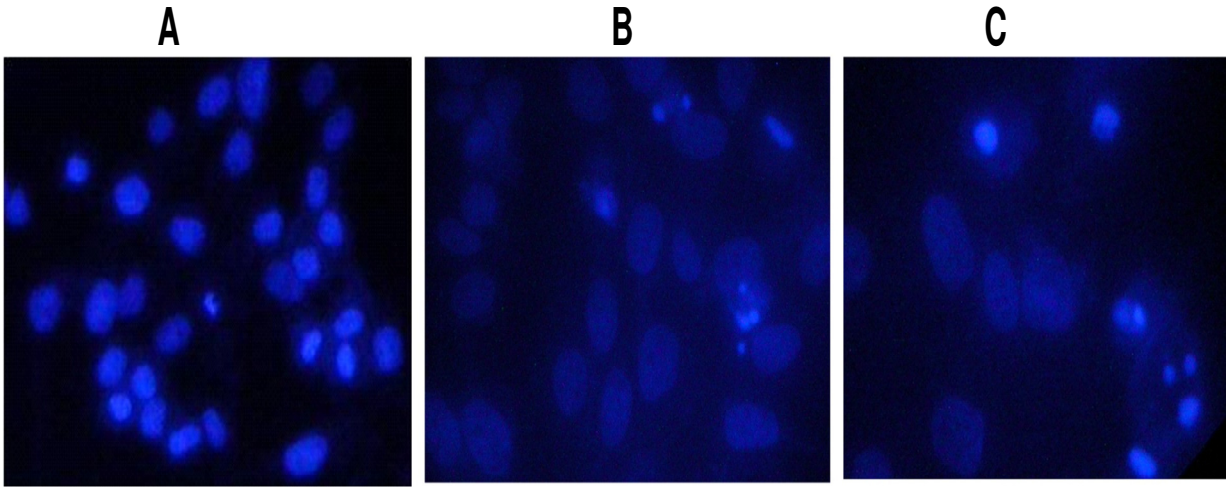

Figure 2. Cell apoptosis detected through Hoechst. Hela cell nucleus was stained blue. In the negative control group, there were no apoptotic bodies (A). However, there were some apoptotic bodies in rhNGF-3 group when hela cells were incubated for 24 h (B) and more apoptotic bodies were observed at 48 h (C).

with the time extending.

Inhibition of rhNGF to mouse tumors

In this experiment, tumor inhibitory rate of rhNGF-3 group was 50.89% and almost reached the result of the positive group ($P > 0.05$). When the concentration of rhNGF virus was less than 1×10^7 pfu/ml, the tumor inhibitory rate went up with the increase of rhNGF concentration, which showed that the tumor was significantly inhibited. However, when the concentration of rhNGF virus reached 1.0×10^8 pfu/ml, the tumor inhibitory rate dropped greatly and was under zero, which showed that the tumor was

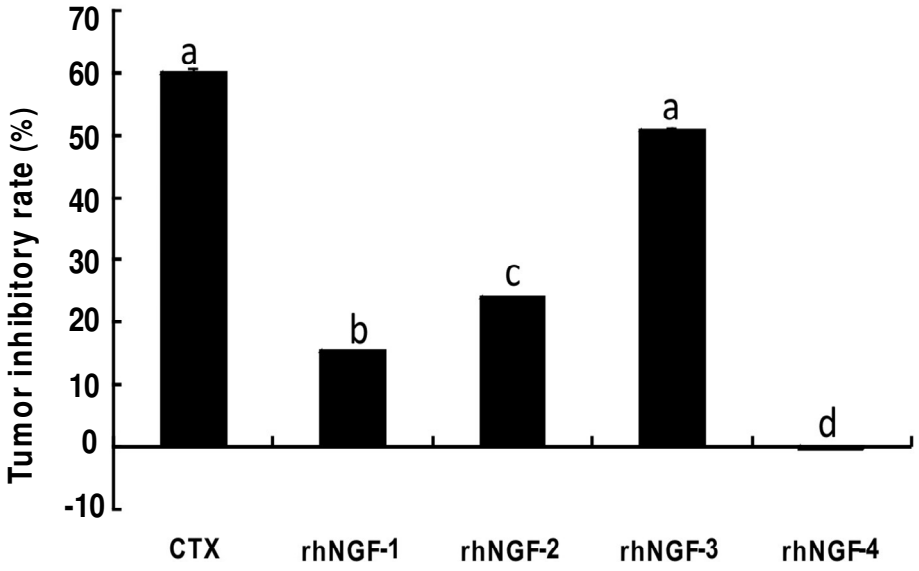

Figure 3. Effect of rhNGF on tumors of mice. Tumor inhibitory rate for all the test groups was counted and they were 59.95% for CTX group, 15.32% for rhNGF-1 group, 23.86% for rhNGF-2 group, 50.89% for rhNGF-3 group and -0.13% for rhNGF-4 group, respectively. Values with different letter showed significant discrepancy ($P < 0.05$) and same letter showed $P > 0.05$.

not inhibited (Figure 3). Therefore, it was shown that the inhibition of tumor was best when the concentration of rhNGF virus was 1.0×10^7pfu/ml.

Changes of spleen and thymus in response to rhNGF

To know the effect of rhNGF on animal immune function, the thymus and spleen were investigated. It was found that the qualities of thymus and spleen in cervical cancer mice were normal. The indexes of thymus and spleen greatly went up in response to rhNGF when compared with the negative control group (Figure 4, $P < 0.05$). The stated results showed that the immunity of mice was imported by rhNGF.

SOD level in response to rhNGF

SOD is a defensive enzyme of organisms and it catalyses the conversion of O_2 to H_2O_2 and the latter again forms nontoxic H_2O under the action of peroxydase so that free radicals which result in tumorigenesis are completely cleared (Breimer, 1990; Dizdaroglu et al., 2002; Vaca et al., 1988; Yang and Fu, 2001). In this experiment, except for rhNGF-4 group, the SOD levels of tumor organs in the other rhNGF groups were greatly improved when compared with the negative group, but they were less than that of the positive group ($P < 0.05$). Significantly, SOD level of tumor organs greatly went down instead when the concentration of GFP-rhNGF virus was increased from 1.0×10^7 to 1.0×10^8 pfu/ml (Figure 5).

Effect of rhNGF on cervical cancer

The direct action of rhNGF to cancer was determined through the inhibitory rates of cancer cells *in vitro* and *in vivo*. The effect of rhNGF on mouse immunity was showed through the indexes of spleen and thymus and free radicals were cleared, which indicated the treatment of SOD to cancer. From Figure 6, it can be seen that, the anti-cancer action of rhNGF was depended on its concentration. When the concentration of GFP-rhNGF virus was less than 1.0×10^7 pfu/ml, tumor inhibition, mouse immunity and SOD level were improved. Nevertheless, the anti-cancer action went down when the concentration of GFP-rhNGF virus surpassed 1.0×10^7 pfu/ml. Therefore, it was concluded that rhNGF had positive correlation with the inhibition of cervical cancer when the concentration of rhNGF virus was less than 1.0×10^7 pfu/ml.

DISCUSSION

In this study, it was shown that the cells of cervical cancer were inhibited in response to rhNGF and the immunity of mice and SOD of tumor organs were improved. Nevertheless, the anticancer roles were depended on the concentration of the GFP-rhNGF virus.

Previous finding indicated that, the growth of hela cells

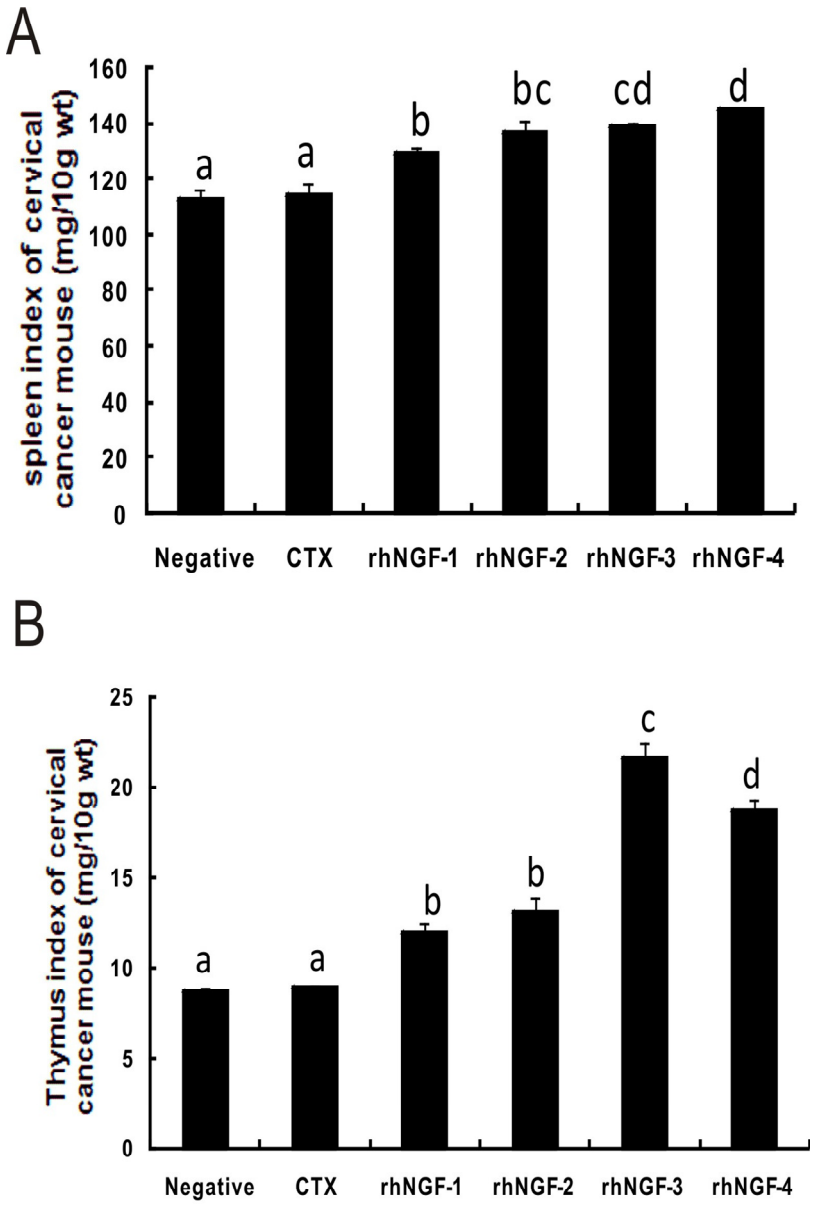

Figure 4. Spleen and thymus of cervical cancer mice affected by rhNGF. Mouse spleen indexes were 112.5 mg for the negative control group, 114.1 mg for positive control group, 129.1 mg for rhNGF-1 group, 136.4 mg for rhNGF-2 group, 139.1 mg for rhNGF-3 group, 144.9 mg for rhNGF-4 group per 10 g wt, respectively (A) and mouse thymus indexes were 8.8 mg for the negative control group, 9.0 mg for the positive control group, 12.0 mg for rhNGF-1 group, 13.1 mg for rhNGF-2 group, 21.7 mg for rhNGF-3 group and 18.7 mg for rhNGF-4 group per 10 g wt, respectively (B). Values with different letter showed significant discrepancy ($P < 0.05$) and same letter showed $P > 0.05$.

Was affected by rhNGF (Zhang et al., 2003). In this experiment, rhNGF was used to treat hela cells and the tumors resulted from U14 cells. It was found that the cell proliferation was inhibited in response to rhNGF and the inhibitory action gradually went up with the increase of rhNGF when the concentration of GFP-rhNGF virus was not more than 1.0×10^7 pfu/ml. This showed that, rhNGF greatly bound p75NTR because p75NTR induces cell death (Park et al., 2007). Nevertheless, when the concentration of GFP-rhNGF virus was more than 1.0×10^7 pfu/ml, the anti-cancer action of rhNGF greatly went down instead. Since the binding of NGF to the TrkA receptor promotes cell survival (Bothwell, 1991), it was considered that rhNGF could have greatly bound TrkA on the high dosage of rhNGF which resulted in the cell proliferation and the anti-cancer action was lost. Certainly,

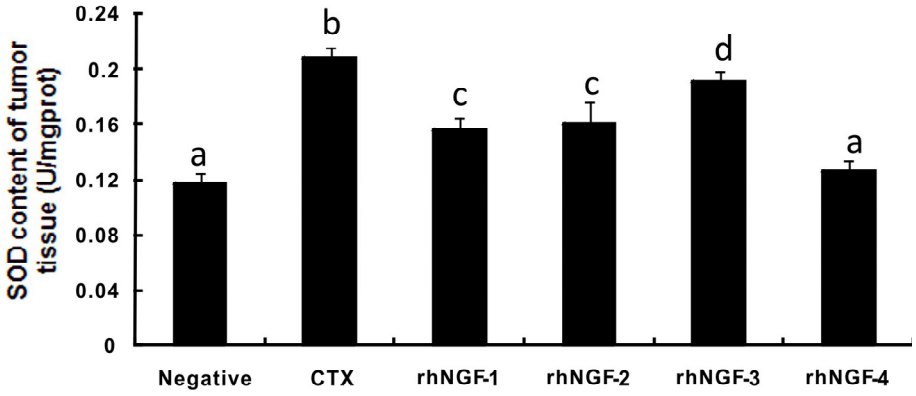

Figure 5. SOD level in tumor-bearing mice was investigated through biochemical experiments. SOD levels of tumor organs in cervical cancer mice were 0.1170 U/mgprot of negative control group, 0.2083 U/mgprot of CTX group, 0.1557 U/mgprot of rhNGF-1 group, 0.1602 U/mgprot of rhNGF-2 group, 0.1909 U/mgprot of rhNGF-3 group and 0.1259 U/mgprot of rhNGF-4 group, respectively. Values with different letter showed significant discrepancy ($P < 0.05$) and same letter showed quiet ($P > 0.05$).

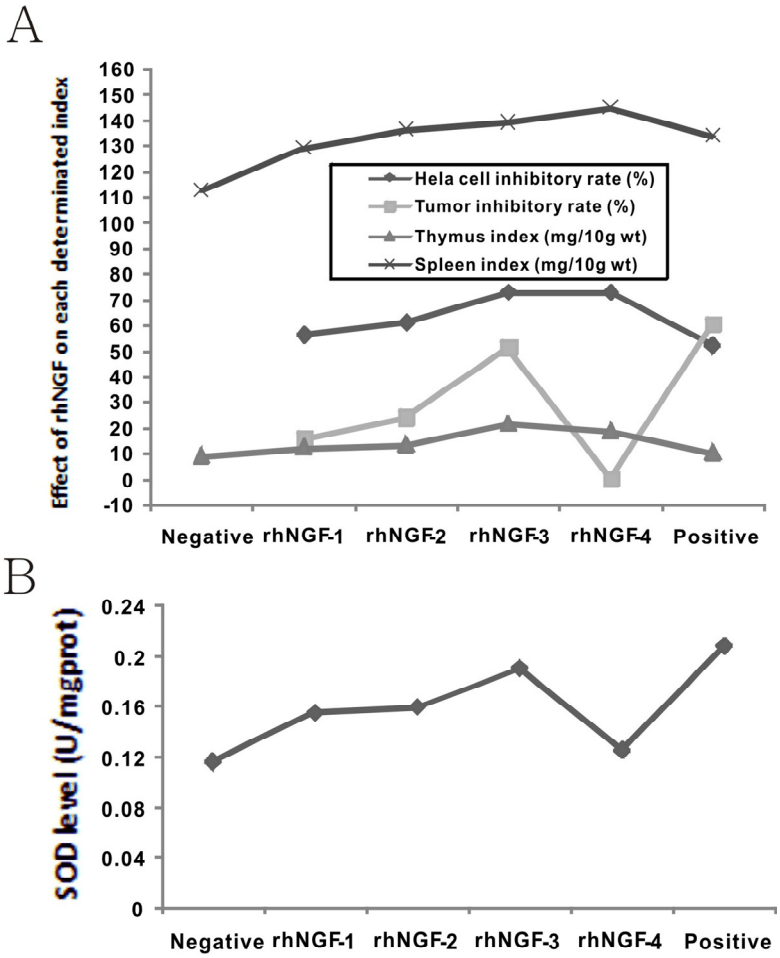

Figure 6. Cell inhibitory rate, tumor inhibitory rate, spleen index, thymus index and SOD level of tumor tissue in response to rhNGF. When the concentration of rhNGF virus was less than 1.0×10^{7} pfu/ml, rhNGF had positive correlation with inhibitory rate of cell proliferation, tumor inhibition, spleen index, thymus index and SOD content.

the detailed mechanism should be studied further.

Report showed that NGF has a significant role in the nerve, endocrine and immune system (Miao et al., 2006). Since the mentioned experiments showed that the proliferation of cancer cells was inhibited by rhNGF, the immune organs were possibly involved in the process because they were related with the tumor inhibition. Therefore, thymus and spleen were investigated to check the reaction of mouse immune function in response to rhNGF. It was shown that, thymus and spleen weights were added and rhNGF groups had obvious discrepancy in comparison with the negative control group ($P < 0.05$). Spleen contains many T cells which possibly kill tumor cells. Because the spleen weight of rhNGF groups was enhanced, it was suggested that the immunity of the tumor-bearing mice was improved. The above results were consistent with that of Klaus et al. (Knezevich et al., 1998). Next, it was found that the content of SOD got to the highest point when the concentration of rhNGF virus reached 1.0×10^7 pfu/ml. However, when the concentration of rhNGF virus was more than 1.0×10^7 pfu/ml, the content of SOD went down which maybe the reason why the tumor cells of rhNGF-4 group were not inhibited. As such, the content of SOD was improved under the right concentration of rhNGF, which helped in the treatment of cervical cancer.

In summary, rhNGF had inhibitory action on cervical cancer in which cell apoptosis was developed and mouse immunity was improved. However, there are still some questions that can be studied further.

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