

Effect of brachytherapy on NF- κ B and VEGF in gastric carcinoma xenografts

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Abstract. Iodine-125 (¹²⁵I) seed irradiation can be used as an important supplementary treatment for unresectable advanced gastric cancer. However, the radiobiological mechanism underlying brachytherapy remains unclear. Therefore, we investigated the influence of continuous and low-energy ¹²⁵I irradiation on the cell cycle distribution, apoptosis, expression of NF- κ B and VEGF and tumor growth in a human gastric cancer xenograft model. To create an animal model of gastric cancer, SGC-7901 cells were surgically implanted into mice. The 60 mice bearing SGC-7901 gastric cancer xenografts were randomly separated into 2 groups. Sham seeds (0 mCi) were implanted into the control group (n=30); ¹²⁵I seeds (0.6 mCi) were implanted into the treatment group (n=30). At 28 days after irradiation, apoptosis was detected by flow cytometry. Fluorescence micrograph detected intense VEGF and NF- κ B immunofluorescence in the tumor samples, and changes in NF- κ B and VEGF mRNA and protein expression were assessed by real-time PCR and western blot analysis, respectively. The tumor volume and weight were measured 0-28 days after ¹²⁵I seed implantation. ¹²⁵I seed irradiation induced significant apoptosis and G2/M phase arrest. Reduction in the intensities of VEGF and NF- κ B immunofluorescence in tumor vessels was observed after treatment. NF- κ B and VEGF mRNA and protein expression levels were substantially lower in the implantation treatment group than in the control group. Consequently, ¹²⁵I seed implantation inhibited cancer growth and reduced cancer volume. The present study revealed that ¹²⁵I seed irradiation significantly induced apoptosis and cell cycle arrest in the human gastric cancer xenografts. ¹²⁵I-induced changes in NF- κ B and VEGF expression are suggested as potential mechanisms underlying effective brachytherapy.

Introduction

Gastric cancer is one of the most common types of cancer worldwide, and almost 50% of gastric cancer-related deaths occur in China (1-3). Surgery offers the only realistic chance of cure; however, many of the patients present with unresectable tumors at the time of diagnosis. Even with resection, more than 50% of patients relapse and eventually die of their disease (4,5). Therefore, non-surgical methods have attracted increasing attention. In recent years, ¹²⁵I implantation has been widely used to treat prostate cancer (6,7) and other tumor types (8,9) because of its ability to offer high precision, little trauma, strong lethality and few complications (10-12). Wang and colleagues (13) applied ¹²⁵I implantation to treat advanced gastric cancer and found significant improvement in clinical symptoms and the quality of life of the patients. Although ¹²⁵I seed implantations have been successfully applied in the clinic, its biological effects and underlying molecular mechanisms are far from fully understood. Takabayashi and colleagues (14) demonstrated that a continuous low-dose rate of irradiation influenced the proliferation of cells and the apoptosis rate which possibly was the main mechanism of the cell-killing effects in CL187 cells. Ma and colleagues (15) demonstrated that ¹²⁵I irradiation at 4 Gy significantly induced cell apoptosis and cell cycle arrest in gastric cancer cells.

The best known and the most efficient growth factors involved in tumor angiogenesis are vascular endothelial growth factor (VEGF) and nuclear factor- κ B (NF- κ B). Their activation has been connected with multiple aspects of oncogenesis, such as apoptotic resistance, transformation, growth, metastasis and angiogenesis. Li *et al* (16) found that inhibition of the NF- κ B p65 signaling pathway may be considered as a potential strategy for treating gastric cancer. Various studies examined the relationship between VEGF expression and the clinical outcome of patients with gastric cancer and found that VEGF expression in gastric cancer tissue is associated with poor survival (17). Thus, irradiation-induced apoptosis, inhibition of cell proliferation and VEGF and NF- κ B signal transduction may be key mechanisms underlying the therapeutic effect of low energy ¹²⁵I seed implantation. In the present study, we investigated the role of VEGF and NF- κ B in

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the process of 125 I brachytherapy and 125 I-induced cell apoptosis and cell cycle changes in a xenograft model.

Materials and methods

Animal model. Human SGC-7901 cells (3×10^6 /mouse) were subcutaneously injected into the right dorsal flank of BALB/c-nu/nu nude mice. After 1-2 weeks of implantation with tumor cells, when tumors reached ~ 20 - 30 mm³, the animals were randomized into control and treatment groups (30 animals per group). The 125 I seeds (0.6 mCi) were injected into the mice in the treatment group through an 18-gauge needle, while ghost seed were injected into the mice in the control group. The tumor size was measured using calipers, and the tumor volume (V) was estimated by the following formula: $(V) (\text{mm}^3) = (L \times W^2) \times 1/2$, where L is the length and W is the width of the tumor.

Tumor volumes and body weights were monitored every 3 days over the course of treatment. The tumor weight was measured when the mice were sacrificed. Mice were sacrificed after 28 days of treatments, and tumors were removed and fixed in 10% neutral buffered formalin for histologic and immunohistochemical analyses. All animal procedures were carried out with the approval of the Animal Ethics Committee of Kunming Medical College.

Histological analysis of tumors and immunofluorescence examination of VEGF and NF- κ B. Tumors were embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA). For the immunofluorescence staining of NF- κ B and VEGF, the frozen sections were maintained at room temperature for 30 min, incubated in distilled water for 5 min and in PBS for 5 min and permeabilized in 1 g/l Triton X-100 for 10 min. The sections were subsequently washed with PBS (5 min \times 3), blocked with 100 ml sheep serum (Sigma) at 37°C for 20 min and incubated in the primary antibodies: rat anti-mouse NF- κ B (BioLegend, San Diego, CA, USA) and rabbit anti-human VEGF polyclonal antibody (LabVision Corp., Fremont, CA, USA) at 4°C overnight and washed with PBS (5 min \times 3). Incubation in the secondary antibody (goat anti-rat IgG-conjugated TRITC or sheep anti-rabbit IgG-conjugated FITC; Sigma) was carried out for 1 h at 37°C. Sections were washed with PBS (10 min \times 3) and then examined under a TCS SP2 laser confocal microscope. For each group, several field images of VEGF and NF- κ B in each tumor tissue section were captured under a confocal microscope. The fluorescence intensity of each section in the confocal fluorescence images was measured using the Leica confocal analysis system. The mean fluorescence intensity in each section was then calculated.

Cell cycle distribution analysis. Cells in the mono-dispersed suspension were fixed with ethanol, followed by propidium iodide staining (PI; Sigma) and analyzed using the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Percentages of cells resting in the G1, S and G2/M phases were determined with CellQuest software (BD Biosciences) and ModFit LT software (Verity Software House, Topsham, ME, USA).

Annexin V/PI assay of 125 I-induced apoptosis. The cells were stained with Annexin V-FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA). Both early (Annexin V-positive, PI-negative) and late (Annexin V-positive, PI-positive) apoptotic cells were counted as apoptotic cells.

Total RNA preparation. The samples were ground by using liquid nitrogen. Total RNA was extracted from lung tissues by using an animal tissue RNA purification kit (Norgen Biotek Corp., Thorold, ON, Canada) as recommended by the manufacturer. RNA samples were measured by using a bioanalyzer to determine RNA integrity number.

Quantitative real-time PCR. The approximate length of miRNA at 21-23 nt resulted in difficulties in conventional PCR test. We used TaqMan[®] MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) to examine miRNA differential expression profiling in PTC as recommended by the manufacturer. Sample RNA (10 ng) was reversely transcribed into cDNA by using specific stem-loop primers and TaqMan[®] MicroRNA reverse transcription kit. With cDNA as the template, TaqMan MicroRNA Assay and the TaqMan[®] Universal PCR Master Mix were used for the serial real-time PCR. RNU48 was used as an internal control to minimize the variation among reverse transcription, PCR and samples. The data were collected, analyzed, and normalized by using the Applied Biosystems analysis software to determine the differential expression profiles of miRNAs. All of the experiments were performed in triplicate. Expression levels were calculated by using the relative quantification method ($\Delta\Delta\text{CT}$) in the ABI PRISM 7500 Sequence Detection system (Applied Biosystems), according to the manufacturer's protocol.

Western blot analysis. Proteins were resolved on 12% polyacrylamide gels, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with 5% non-fat dairy milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.4) with 0.1% Tween-20.

Statistical analysis. The results of the animal experiments and real-time PCR were analyzed using SPSS 13.0 software. (SPSS Inc., Chicago, IL, USA). All data were plotted as mean \pm standard deviation. Student's t-test was used to compare values between two independent groups. Differences were considered to be significant at $P < 0.05$.

Results

Inhibitory effect of 125 I seed irradiation on the growth of gastric cancer. The effectiveness of 125 I seed irradiation to inhibit the growth of implanted SGC-7901 tumors was examined in a nude mouse model. There were no significant changes in the tumor volumes for the first 10 days of the 125 I seed treatment. However, after 13 days, the 125 I-irradiated tumors were much smaller, and a significant difference in tumor volume was observed over time between the control and 125 I treatment group (Fig. 1A). On day 28, the mice were sacrificed and tumor weights were measured. Statistical difference in the tumor weight was observed between the control

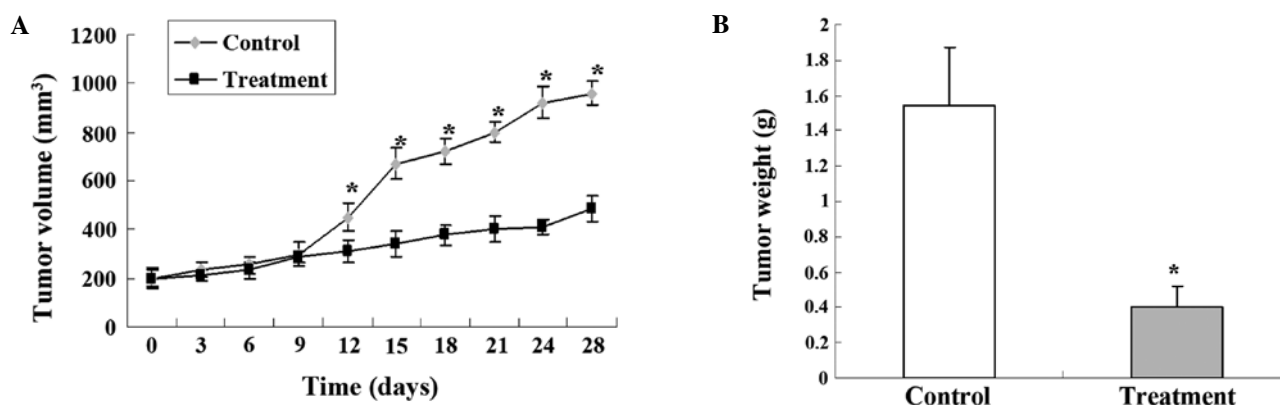


Figure 1. Effect of ^{125}I on the gastric cancer xenograft model. Data represent means \pm SD (n=30/group). (A) The treatment group exhibited a decreased tumor weight compared to the control group ($P<0.05$). (B) The treatment group had a smaller tumor volume compared to the control group from day 13 ($P<0.05$).

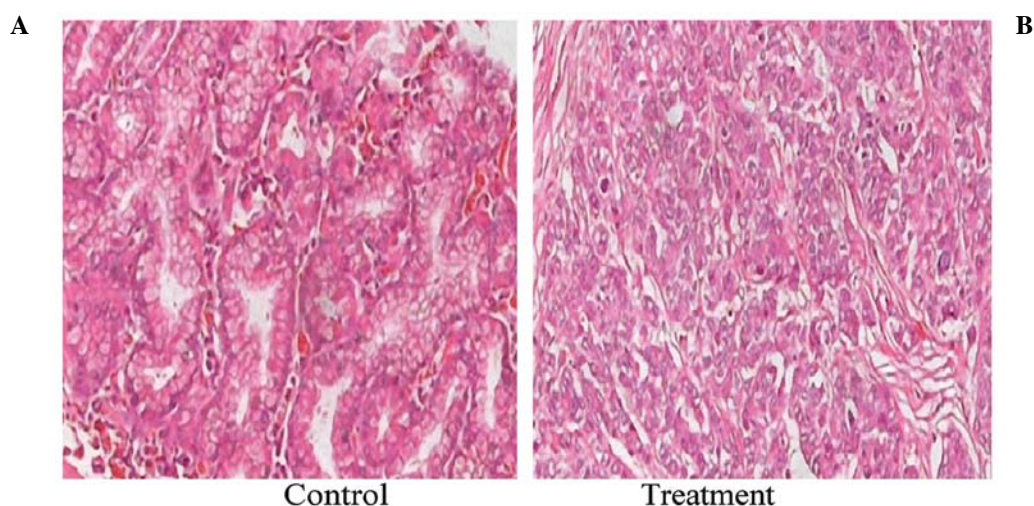


Figure 2. Evaluation of the antitumor efficacy of ^{125}I on xenograft tumors. Images of representative H&E staining of (A) control and (B) treated tumor tissue sections.

and treatment group (Fig. 1B). All these data clearly indicated that ^{125}I seed implantation effectively inhibited tumor growth. In addition, the body weights of the mice were not affected by the ^{125}I irradiation and no obvious radiation-induced damage was observed in the vital organs of the mice (data not shown), indicating the safety of the ^{125}I seed treatment.

Effect of ^{125}I seed irradiation on morphology of gastric cancer tumors. To investigate the effect of the ^{125}I irradiation on the histology of the SGC-7901 xenografts, tumor sections were obtained from mice in the control and the ^{125}I treatment group and were stained using H&E. As shown in Fig. 2, the histological appearance of the tumors in the control group was quite different from that in the ^{125}I treatment group. In the control group, the cancer cells were densely arranged with large darkly stained nuclei and obvious karyokinesis. In the treatment group, large necrotic regions were observed around the ^{125}I seed. The cancer cells adjacent to the necrotic region were loosely arranged with condensed nuclei and reduced eosinophilic cytoplasm. These results indicated that ^{125}I seed implantation caused growth inhibition of cancer cells in the SGC-7901 xenografts.

Effect of ^{125}I seed irradiation on cell apoptosis and cell cycle distribution in the gastric cancer tumors. To quantitatively compare the cell cycle and apoptotic index of tumors treated with ^{125}I seed irradiation, FACS was performed. The cell cycle was blocked in the G2/M phase in the tumors in the ^{125}I treatment group when compared to the tumors in the control group (Fig. 3A). The percentage of apoptotic cells was significantly increased in the ^{125}I treatment group when compared to the percentage of apoptotic cells in the control group (Fig. 3B).

Effect of ^{125}I on VEGF and NF- κB immunofluorescence staining. Expression of VEGF and NF- κB was confirmed by the presence of fluorescence-stained cytoplasm in the cells. Strong immunoreactivity to VEGF and NF- κB was found in the SGC-7901 tumor xenografts of the control group (Fig. 4A-a and -b). Weaker fluorescence intensity expression was observed in the SGC-7901 tumors of the group treated with ^{125}I (Fig. 4A-c and -d). The fluorescence intensity levels of VEGF and NF- κB in the tumor cells were significantly lower in the radiation-treated group than levels in the control group (Fig. 4B; $P<0.01$).

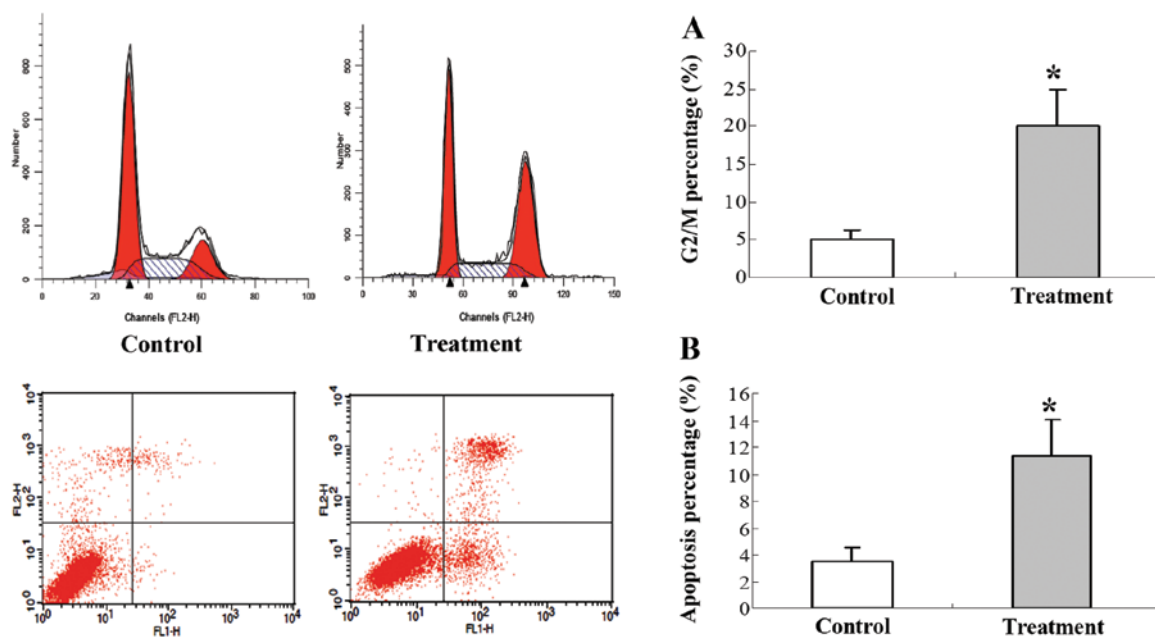


Figure 3. Summary of G2/M phase distribution and percentage of apoptosis. The treatment group exhibited significant increases in (A) G2/M cell cycle distribution and (B) the percentage of cell apoptosis. * $P < 0.05$ for the comparison between control and 125 I treatment.

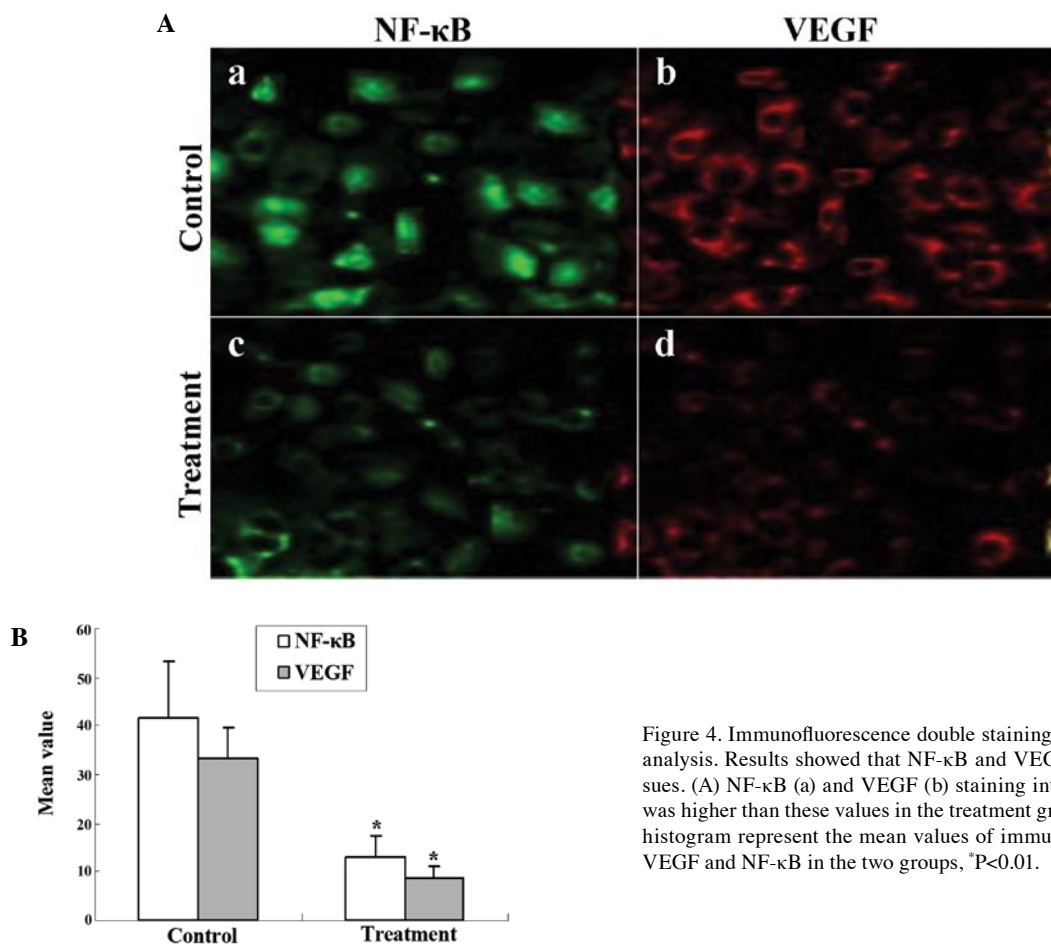


Figure 4. Immunofluorescence double staining and confocal laser scanning analysis. Results showed that NF- κ B and VEGF were expressed in the tissues. (A) NF- κ B (a) and VEGF (b) staining intensities in the control group was higher than these values in the treatment group (c and d). (B) Bars in the histogram represent the mean values of immunofluorescence intensities of VEGF and NF- κ B in the two groups, * $P < 0.01$.

Effect of 125 I on the expression of VEGF and NF- κ B mRNA and protein in the tumor tissues. To further analyze the effect of 125 I radiation on VEGF and NF- κ B expression in tumors, we assessed VEGF and NF- κ B expression in SGC-7901 cells

in vitro using western blotting and fluorescent quantitative RT-PCR. Western blot analysis revealed that the expression of VEGF and NF- κ B protein was decreased in the treatment group (Fig. 5B). Furthermore, VEGF and NF- κ B mRNA

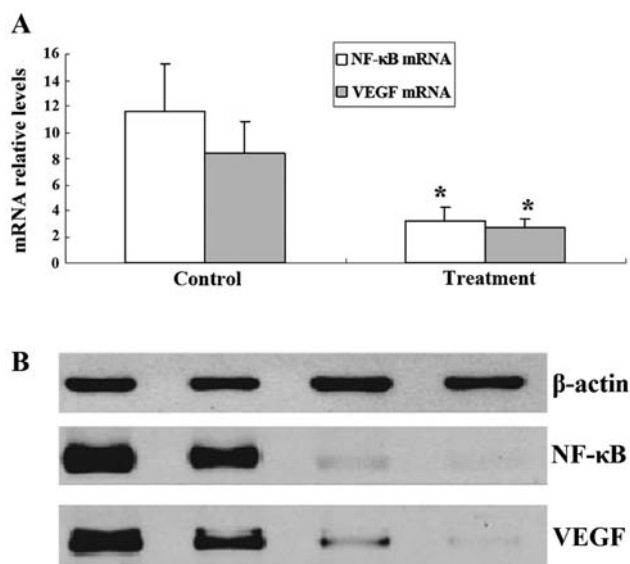


Figure 5. ^{125}I inhibits VEGF and NF- κB expression. (A) VEGF and NF- κB mRNA and (B) protein levels were significantly decreased in the ^{125}I seed implantation xenograft tissue samples.

expression was in agreement with the protein expression results as mRNA data also revealed a significant difference between the control and the treatment group (Fig. 5A).

Discussion

Several recent studies suggest that apoptosis and cell cycle arrest may have important roles in the therapeutic effects of continuous low-energy ^{125}I irradiation (14,15,18). In addition, the VEGF and NF- κB signaling pathway may be involved in the gastric cancer oncogenic signaling pathway (19). However, comprehensive knowledge on this topic, particularly at the molecular level, is still lacking. In the present study, analysis of cell apoptosis and the cell cycle, as well as VEGF and NF- κB expression analysis of human gastric cancer xenografts exposed to ^{125}I seed irradiation were performed to gain insight into the mechanisms underlying the biological effects of ^{125}I irradiation.

SGC-7901 gastric cancer cells were implanted into nude mice to create a xenograft animal model. The growth curves of tumors indicated that irradiation induced significant tumor growth inhibition. By observing H&E-stained slides, a large number of apoptotic cells was observed in the gastric cancer tumors receiving ^{125}I seed implantation. These results showed that ^{125}I suppressed the growth of the gastric cancer xenografts in the nude mice, while inhibiting cell proliferation and inducing apoptosis. Our results further demonstrated that continuous low-dose-rate irradiation by ^{125}I seeds reduced cell viability and induced cell apoptosis, and led to the accumulation of cells in the G2/M phase. These data suggest that the cell cycle was blocked in the G2 phase after radiation. Cells in the G2 phase are more sensitive to radiation (20), and therefore, more tumor cells were eliminated.

Our VEGF and NF- κB analyses in the tumor cells indicated decreased fluorescence intensity implying reduced mRNA and protein levels in the treatment cells as compared with the control cells. A previous study demonstrated a correlative expression relationship between VEGF and NF- κB in 80 ACC

clinical samples (21). Evidence also indicates that overexpression of NF- κB is the key component of the angiogenic cascade, which contributes to VEGF-induced angiogenesis through upregulation of VEGF mRNA expression in many tumor types (22,23).

NF- κB is a family of homodimeric or heterodimeric transcription factors formed by proteins of the Rel family. Recently, it has been suggested that NF- κB plays an important role in carcinogenesis (24). There is evidence that NF- κB is constitutively activated in gastric cancer tissues, with higher levels in gastric carcinoma cells in comparison to normal adjacent epithelial cells (25). In gastric cancer, abnormal NF- κB activation has been shown to lead to enhanced proliferation, evasion of apoptosis, genomic instability, increased rate of glycolysis and drug resistance (26-28).

VEGF, a dimeric 42-kDa protein, is a multifunctional cytokine that plays a key role in both physiological and pathological angiogenesis. It was identified in tumor cells of gastric cancer more than 10 years ago (29). Several groups of investigators have reported a correlation between VEGF expression and microvessel density in human gastric cancer (30). Our data together with previous research evidence suggest that NF- κB and VEGF play an important role in the therapeutic effects of continuous low-energy ^{125}I irradiation and are involved in the mechanism of the ^{125}I seed implantation therapy process.

In conclusion, the present study demonstrated that human gastric tumor cells following ^{125}I brachytherapy showed induced cell apoptosis and cell cycle arrest in the G2/M phase in a xenograft model. Furthermore, suppression of NF- κB activity through significantly decreased VEGF expression facilitates the ^{125}I clinical effect on gastric tumors. The results indicate that brachytherapy is a useful strategy in gastric tumor therapy. Further study by us will investigate the function of NF- κB and VEGF signaling in angiogenesis and metastasis of gastric tumor cells.

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