# Effects of Basic Fibroblast Growth Factor on Radiation-Induced Proliferation Inhibition and Apoptosis in Thymocytes and Splenocytes

**Xiaokun Li,*[a, b] Yanli Xu,[b] Yan Hong,[b] Wei Li,[c] and Guanjun Wang[c]**

*[a]School of Pharmaceutical Sciences, Wenzhou Medical College, Chashang College Park, Wenzhou, Zhejiang 325035, P.R. China, [b]School of Pharmacy and Medicine, Jinan University, Guangzhou, P.R. China, and [c]Department of Hematology and Oncology, First Clinical College, Jilin University, Changchun 130021, P.R. China*

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Fibroblast growth factors (FGFs) stimulate the proliferation of a variety of cells and protect against stress-induced apoptosis. We have demonstrated that basic FGF (bFGF) significantly stimulates intestinal cell proliferation and protects against dexamethasone-induced apoptosis. In this study, we further investigate the effects of bFGF on radiation-induced inhibition of thymocyte and splenocyte proliferation and apoptotic cell death. Mice were subjected to whole-body X-rays irradiation by at a dose of 0.5 Gy or 1.0 Gy and 14 hr later thymocytes and splenocytes were collected for *in vitro* culture with or without bFGF. Radiation induced a significant decrease in the cell proliferation of both thymocytes and splenocytes, which was not significantly inhibited by incubation with bFGF. Radiation with 0.5 and 1.0 Gy also significantly increased apoptotic cell death in both thymocytes and splenocytes, and incubation with bFGF did not significantly affect the apoptotic effects of radiation. These results suggest that incubation of bFGF *in vitro* with thymocytes and splenocytes from irradiated mice did not significantly prevent radiation-induced inhibition of cell proliferation and apoptotic effects.

**Key words** —— radiation protection, fibroblast growth factor, cell proliferation, apoptotic cell death, thymocytes, splenocytes

## INTRODUCTION

Fibroblast growth factors (FGFs) are a family of at least nine heparin-binding polypeptides that have mitogenic activity toward various types of cells of mesenchymal, neuronal, and epithelial origin.[1)] Both acidic (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) are expressed in normal adult tissues and may regulate cellular events involved in wound repair.[1–3)] We have shown that bFGF improves of wound recovery of burned skin in animals and humans.[4)] We also demonstrated the protective effects of both aFGF and bFGF against ischemia-induced skin and intestinal injury.[5)]

Studies[6–10)] have shown that bFGF enhances the survival of a variety of endothelial, epithelial, and hematopoietic cell lines subjected to ionizing radiation. However, little is known about the effects of bFGF on organs involved in immune regulation such as the thymus and spleen in response to ionizing radiation. In the present study therefore, we investigated the effects of bFGF on radiation-induced splenocyte and thymocyte apoptosis and cell proliferation inhibition.

*To whom correspondence should be addressed: School of Pharmaceutical Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325035, P.R. China. Tel.: +11-86-13676701796; Fax: +11-86-577-86689983; E-mail: xiaokunli@163.net

## MATERIALS AND METHODS

**Animals** —— Kunming adult male mice, 7–8 weeks age old (weighing 20 ± 2 g), were used in compliance with the Principles of Laboratory Animal Care formulated by the Wenzhou Medical College Guide for the Care and Use of Laboratory Animals prepared based on the guidelines of the Institute of Laboratory Animal Resources. Mice were subjected to whole-body at 0.5 or 1.0 Gy with a Phillips therapeutic X-ray machine operated at 200 kVp and 10 mA in the presence of 1.0 mm Al and 0.5-mm Cu filter plates. The dose rate was 0.287 Gy/min.

**Cell Cultures** —— Fourteen hours after irradiation, the mice were killed under sterile conditions to collect the spleen and thymus. Splenocyte and thy-

mocyte suspensions were prepared as described in previous studies.[11–13] Both types of cells were incubated in 24-well plates at $5 \times 10^6$ cells/ml in the presence or absence of bFGF 1.0 or 2.0 $\mu$g/ml. Since it is well known that radiation-induced cell death has two phases, the immediate effect of predominantly necrotic cell death and the delayed mainly apoptotic cell death, the antiapoptotic effects of bFGF were observed by adding it to the cultured cells when they were collected from animals 14 hr after irradiation.[11] In addition, since there are several types of cells in the spleen in addition to splenocytes, the incubation of splenocytes for apoptotic study was longer than that for thymocytes, based on our previous results.[11–13]

**Cell Proliferation Assay** —— Both splenocytes and thymocytes at a final concentration of $2.5 \times 10^5$ were incubated in 96-well plates with and without bFGF at final concentration of 0.25 or 0.50 $\mu$g/ml. For splenocytes the cultures also included Con-A 20 $\mu$g/ml for 72 hr. Before harvesting, [$^3$H]-TdR 20 $\mu$l (18.5 kBq) was added to all cultures for 6 hr and then the cells were collected on type 49 membrane to measure the incorporated [$^3$H] using a gamma counter for calculating radioactivity as an index of cell proliferation.

**Detection of Apoptotic Cell Death** —— Apoptotic cell death was confirmed using the DNA fragmentation assay and then quantitatively analyzed with flow cytometry using PI staining. The DNA fragmentation assay was performed based on the methods used in our previous study.[14] Procedures for analyzing apoptotic cell death using flow cytometry were also described in a previous study.[11]

**Statistical Analysis** —— The results, expressed as mean ± S.D., were obtained from at least three separate experiments with 3 mice in each group and triple samples of cultures for each animal. These data were subjected to one-way analysis of variance (ANOVA) analysis, followed by Student's *t*-test. The differences were considered to be significant at $p < 0.05$.

## RESULTS

### Radiation-Induced Inhibition of Cell Proliferation and Effects of bFGF

Radiation with 0.5 or 1.0 Gy significantly inhibited the proliferation rate of splenocytes in response to Con-A stimulation (Fig. 1A) and thymocytes (Fig. 1B) in a dose-dependent manner. Incubation with bFGF 0.25 or 0.5 $\mu$g/ml for 72 hr of thymocytes and splenocytes irradiated *in vivo* showed no significant protection against radiation-induced inhibitory effects on both splenocyte and thymocyte proliferation.

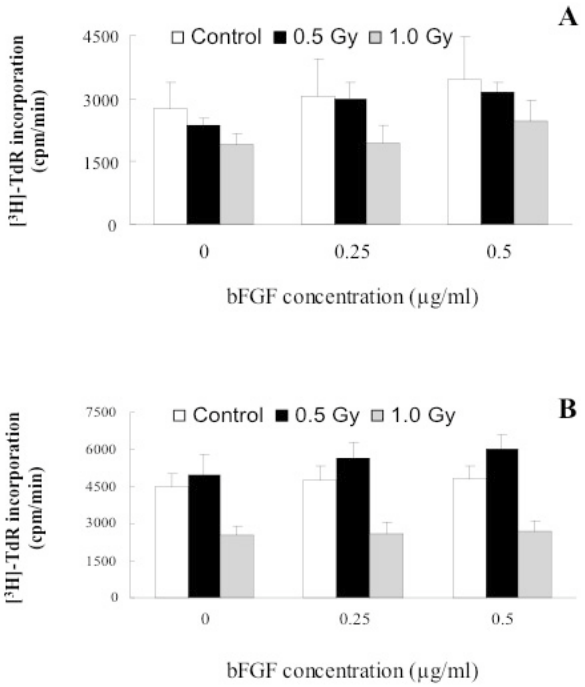


**Fig. 1.** Effects of bFGF on Radiation-Induced Cell Proliferation Inhibition

Mice were whole-body irradiated with 0.5 or 1.0 Gy X-rays and 14 hr later, the spleen and thymus were harvested to prepare for *in vitro* cultures of splenocytes (A) and thymocytes (B). Both splenocytes and thymocytes were incubated *in vitro* in the presence of the bFGF 0.25 or 0.5 $\mu$g/ml for 72 hr. Cell proliferation was evaluated based on incorporation of $^3$H-TdR, as described in the MATERIALS AND METHODS. Data obtained from three separate experiments with 3 mice and three samples for one mouse in each experiment are presented as mean ± S.D.

### Radiation-Induced Apoptotic Cell Death and Effects of bFGF

Radiation-induced cytotoxic effects in thymocytes were examined firstly based on DNA fragmentation (Fig. 2), which showed a dose-dependent induction of DNA ladders. After identification of apoptotic death, we used flow cytometry to analyze quantitatively the apoptotic effects of 0.5 and 1.0 Gy X-rays and bFGF effects on radiation-induced apoptosis. Figure 3 shows that both thymocytes and splenocytes from irradiated mice did not exhibit any increase in apoptotic cell death if they were not incubated *in vitro* with bFGF (*i.e.*, group 0 in Fig. 3). Apoptotic cell death increased with longer incubation times, particular in thymocytes (Fig. 3C and 3D).

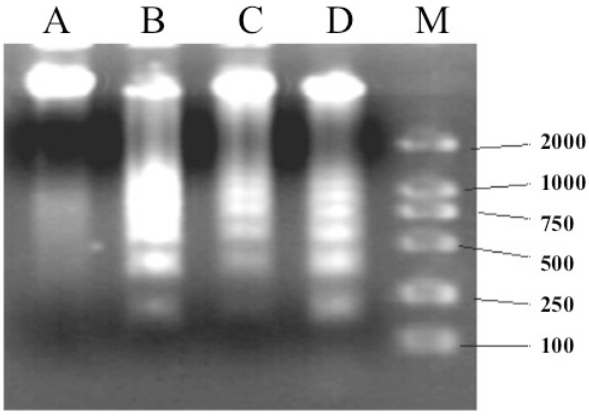


**Fig. 2.** Representative Results of DNA Fragmentation Analyses of Apoptotic Cell Death

Mice were whole-body irradiated with 0.5 or 1.0 Gy X-rays and 14 hr later the thymus was harvested to prepare for *in vitro* cultures of thymocytes. After thymocytes from irradiated mice were incubated *in vitro* for 8 hr, cells were collected and DNA fragmentation was analyzed. A, Control; B, 0.5 Gy; C, 0.5 Gy + bFGF 2.0 $\mu$g/ml; D, 1.0 Gy; M, DNA marker.

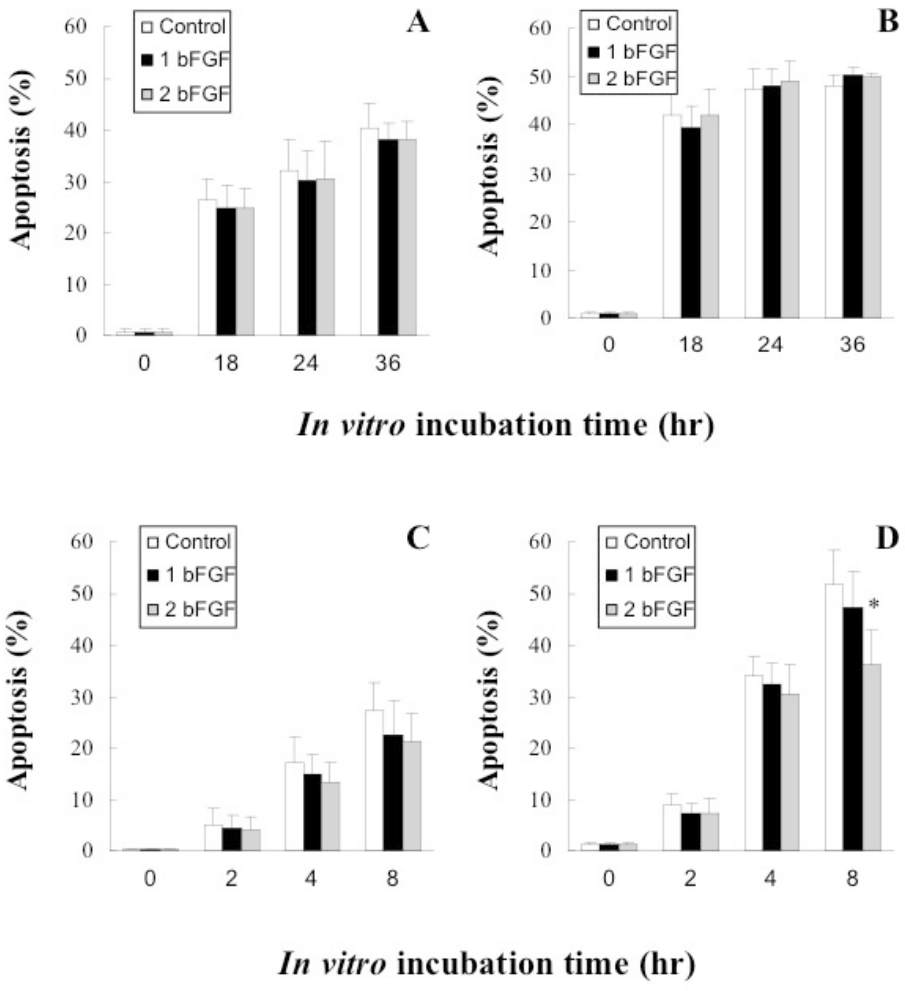


**Fig. 3.** Effects of bFGF on Radiation-Induced Apoptotic Cell death

Mice were whole-body irradiated with 0.5 (A, C) or 1.0 Gy (B, D) X-rays and 14 hr later, the spleen and thymus were harvested to prepare *in vitro* cultures of splenocytes (A, B) and thymocytes (C, D). Both splenocytes and thymocytes were incubated *in vitro* in the presence of bFGF 1.0 (black column, 1 bFGF) or 2.0 $\mu$g/ml with (gray column, 2 bFGF) for the indicated times. The apoptotic cell death was measured with flow cytometry as described in MATERIALS AND METHODS. Data obtained from three separate experiments with 3 mice and three samples for each mouse in each experiment are presented as mean ± S.D. $^*p < 0.05$ *vs.* corresponding control group.

Radiation induced a significant increase in apoptotic cell death in a dose-dependent manner. Compared to cells exposed to 0.5 Gy of X-rays (Fig. 3A and 3C), cells exposed to 1.0 Gy showed a significantly higher incidence of apoptosis.

Since we did not found a protective effect of bFGF 0.2–0.5 $\mu$g/ml (Fig. 1), we increased the dose of bFGF to investigate its effects on radiation-induced apoptosis. The addition of bFGF 1.0 or 2.0 $\mu$g/ml to splenocytes did not affect radiation-induced apoptotic effects (Fig. 3A and 3B). In contrast, the addition of bFGF to thymocytes seemed to provide slight protection against radiation-induced apoptosis, especially in the high-dose group receiving bFGF 2 $\mu$g/ml for 8 hr (Fig. 3D).

## DISCUSSION

The protective effects of FGF against tissue injury in response to various stresses including ischemia and exposure to dexamethasone (Dex) have been documented in the literature and our own studies.[1–5] The radioprotective effects of FGF, especially bFGF, on skin and bone marrow *in vivo* were also documented.[6–10] The present study was the first time to explore the protection by bFGF against radiation-induced proliferation inhibition and apoptotic effects in the splenocytes and thymocytes of mice whole-body X-ray irradiated with 0.5 or 1.0 Gy (clinically relevant doses). We found that bFGF did not provide significant protection when splenocytes and thymocytes were irradiated *in vivo* and incubated with bFGF *in vitro* at the current concentrations. To understand this negative finding, a few issues should be discussed.

First, the experimental approaches should not be a concern in regard to the finding of no significant protection of bFGF against radiation-induced apoptosis and cell proliferation inhibition. Flow cytometry analysis of apoptotic cell death in cultured thymocytes and splenocytes has been extensively used in our previous studies.[11–13] In addition, apoptotic cell death has also been confirmed by another standard method, DNA fragmentation (Fig. 2). In the present study, two types of cells were used, and the two parameters of cell death and cell proliferation were evaluated. All these measurements showed no significant prevention of bFGF against radiation-induced cell death and proliferation inhibition.

A second issue is whether the dose of bFGF used in the present study is too low to show significant protection against radiation-induced cell death and proliferation inhibition. Although we can not exclude this possibility, other data appear not to support this. We performed a study in which apoptotic cell death was induced by Dex, FGF in the dose range of 0.1–0.2 $\mu$g/ml significantly reduced Dex-induced thymocyte apoptosis, and the maximal reduction was found in the group with FGF 0.15 $\mu$g/ml.[5] In addition, we also demonstrated that FGF at dose of 40 ng/ml significantly reduced hydrogen peroxide-induced apoptosis in cardiac cells (32.9 ± 3.3% in the hydrogen peroxide group vs 19.7 ± 1.9%1.1 in the hydrogen peroxide plus FGF group).[15] Other studies also showed that FGF at a dose range of 0.1–100 ng/ml also significantly prevented radiation-induced murine and human colony-forming unit (CFU)-granulocyte-macrophage (GM) formation.[6]

Third, bFGF was given *in vitro* after irradiation in the present study, while previous studies that indicated protective effects were predominantly obtained in *in vivo* animal studies.[6,7,9,16] For example, Gallicchio *et al*.,[6] demonstrated that when combined with adherent cell-depleted normal murine marrow cells, FGF increased the number of both day 9 and day 12 spleen CFUs from lethally irradiated animals. In adherent cell depleted murine and human marrow cultures, the addition of FGF showed synergistic activity in combination with the optimal concentration of GM-colony-stimulating factor (CSF) for CFU-GM. No *in vitro* colony formation was observed when cells were cultured in the presence of FGF but in the absence of the specific hematopoietic growth factor. These data demonstrate that FGF influences early- and late-stage hematopoietic progenitors and shows synergistic activity with hematopoietic growth factors under *in vivo* conditions. Takahama *et al*.[16] also found that intraperitoneal injection of a replication deficient adenovirus containing the FGF gene increased peripheral platelet counts in mice, effectively prevented experimentally induced thrombocytopenia and provided a significant protection of mice subjected to lethal irradiation (9 Gy). These data demonstrate the *in vivo* protective effects of FGF against irradiation. In mice deficient for FGF, a block in thymic growth after embryonic day 12.5 was observed, suggesting that FGF signaling is essential for thymic epithelial proliferation.[10] Although these studies provided evidence indicating that administration of FGF *in vivo* offers a significant protection against radiation-induced tissue injuries or animal mortality, whether

administration of bFGF *in vivo* will protect against radiation-induced apoptotic cell death and inhibition of cell proliferation of splenocytes or thymocytes remains to be investigated in future studies.

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