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Betulinic acid exerts immunoregulation and anti-tumor effect on cervical carcinoma (U14) tumor-bearing mice

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Phytochemicals used in cancer therapy and prevention are an important source. Betulinic acid (BetA), a lupine-type pentacyclic triterpenoid saponin from plants, has shown anti-tumor activity in some cell lines in previous studies. In this paper, its anti-tumor effect and the possible mechanisms were investigated in U14 tumor-bearing mice. The results showed that BetA (100 mg/kg and 200 mg/kg) effectively suppressed tumor growth *in vivo*. Compared with the control group, BetA significantly improved the levels of IL-2 and TNF-α in tumor-bearing mice and increased the number of CD4+ lymphocytes subsets, as well as the ratio of CD4+/CD8+ at a dose of 200 mg/kg. Furthermore, treatment with BetA induced cells apoptosis in dose-dependent manner in tumor bearing mice, and inhibited the expression of Bcl-2 and Ki-67 protein while upregulated the expression of caspase-8 protein. The mechanisms by which BetA exerted anti-tumor effects might involve the induction of tumor cell apoptosis. This process is also related to improvement of body's immune response.

1. Introduction

Natural products have been demonstrated to be reliable and excellent source for the development of new drugs. More and more natural products are applied to cancer chemoprevention and chemotherapy for fewer side effects (Graham et al. 2000). Betulinic acid (BetA) (Fig. 1), which is clarified into common secondary metabolites abundant commonly in some plants of Betulaceae family, especially in the bark of *Betula alba* (birch), has been paid more attention due to the characteristics of more pharmacological activities and less toxic than synthetic drugs (Reyes et al. 2006; Choi et al. 2006). BetA and derivatives have been shown to exert antiviral, anti-inflammatory, anti-oxidant and chemopreventive properties in earlier studies (Pavlova et al. 2003; Wada and Tanaka 2005; Chiang et al. 2005). Relevant to its anti-tumor effects, BetA has been demonstrated to play an important role in the inhibition of carcinogenesis through the regulation of various intracellular molecules involved in signal transduction or in apoptosis pathway that is essential for growth, differentiation and apoptosis (Kommera et al. 2010; Kessler et al. 2007). Thus, BetA exerts anti-tumor effects in some tumor cell lines and, although different mechanisms have been proposed, the actual regulation mechanism is still not clear.

In this study, we investigated BetA's toxic effect on kidney cells and liver cells in U14 tumor bearing mice, and then examined its immunomodulatory activity via measuring the ratio of lymphocytes subsets and the levels of cytokines in host peripheral blood. Furthermore, we investigated the effect of BetA on tumor growth through stimulating caspase-dependent or -independent apoptotic signaling pathways. According to our studies, the effect of BetA on tumor growth could be determined by evaluating immune response and induction of apoptosis as well as the relationship between immune regulation and apoptosis. These results can also provide a scientific explanation for the traditional application of phytochemicals in cervical cancer therapy.

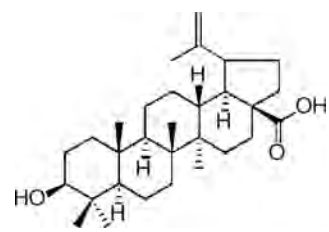

Fig. 1: Molecular structure of betulinic acid

2. Investigations and results

2.1. Effect of BetA on tumor growth

To explore the anti-tumor activity of BetA *in vivo*, we treated tumor-bearing mice with various doses of BetA. BetA administration slightly increased body weight of tumor-bearing mice ($p > 0.05$). On the other hand, we found that BetA significantly inhibited tumor growth in a dose-dependent manner. Moreover, the mice treated with BetA in dose of 200 mg/kg had a similar tumor growth rate compared to the CTX (25 mg/kg body weight, i.p.) group (Table 1).

2.2. Histopathological analysis of effect of BetA on liver and kidney

We next determined the effect of BetA on liver and kidney of tumor-bearing mice by H&E. staining. There were no marked

Table 1: Inhibitory effect of BetA on tumor growth in mice ($\bar{x} \pm s$)

Groups	Doses (mg/kg)	Body weight of mice (g)		Mean weight of tumor(g)	Inhibition (%)
		Beginning	End		
Control	Vehicle	21.43 ± 2.21	23.31 ± 1.93	3.36 ± 0.76	0
BetA	100	21.32 ± 1.71	23.84 ± 2.09	$2.09 \pm 0.42^*$	37.79
	200	20.96 ± 1.79	22.47 ± 1.51	$1.65 \pm 0.52^*$	50.89
CTX	25	20.45 ± 1.57	20.98 ± 2.34	$1.43 \pm 0.34^*$	57.45

* $p < 0.05$ as compared with control group, values are mean ± SD. CTX: Cyclophosphamide

Table 2: Inhibitory effect of BetA on the number of peripheral blood T-lymphocyte subpopulations of tumor bearing mice ($\bar{x} \pm s$)

Groups	Doses (mg/kg)	CD4+ ($\times 10^6$)	CD8+ ($\times 10^6$)	CD4+/CD8+ ratio
Control	Vehicle	21.82 ± 2.43	25.81 ± 1.56	0.85
BetA	100	$25.42 \pm 2.12^*$	$9.31 \pm 1.56^*$	2.73
	200	$30.65 \pm 3.20^*$	$8.45 \pm 2.71^*$	3.62
CTX	25	$15.65 \pm 3.12^*$	$16.87 \pm 1.01^*$	0.93

* $p < 0.05$ as compared with control group, values are mean ± SD. CTX: Cyclophosphamide

differences between vehicle and BetA group under light microscope. All levels of vascular, hepatic lobule arranged regularly, and hepatic cell scattered around the center vein in liver samples; The structures of glomeruli, nephric tubuli, renal interstitium and renal vessels in the treated groups maintained the normal situation by comparing the images with that of the normal mice. The results suggested that BetA (200 mg/kg body weight, p.o.) had no toxic effect on liver and kidney (Fig. 2).

2.3. Histopathological analysis of tumor tissues

Cells in tumor tissues displayed various shapes, sizes and structures shown in Fig. 3. In control group, tumor cells arranged closely with irregular shape and disequilibrium nuclear-cytoplasmic ratio, as well as chromosomes abnormity. However, in treated groups, tumor cells appeared to be induced to apoptosis and necrosis by BetA. The nucleus was broken but encapsuled by intact membrane, containing membrane blebbing, formation of apoptotic bodies, and in most cases, DNA fragmentation. For some necrosis places, the tumor cells died followed by the nucleus atrophy and the disruption or the dissolution.

2.4. Effect of BetA on peripheral blood T-lymphocyte subpopulations of tumor bearing mice

The effect of BetA on immune response in cervical cancer bearing mice are shown in Table 2, compared with the control group. According to the results, we find the treatment with BetA following tumor implantation caused a significant increment in the number of CD4+ T lymphocyte ($P < 0.05$, 200 mg/kg) and a decrease in the number of CD8+ T lymphocyte ($P < 0.05$, 100 mg/kg and 200 mg/kg) of tumor bearing mice peripheral blood, respectively. Furthermore, CD4/CD8 ratios of the U14-bearing mice treated with dose of BetA (100 and 200 mg/kg) were significantly higher than that of the control group without BetA. While the treatment with CTX caused a significant decrease in the number of CD4+, CD8+ T lymphocyte subpopulation of peripheral blood, and the ratio of CD4+/CD8+ T lymphocyte subpopulation of tumor bearing mice peripheral blood has no significant change compared with that of control group (Table 2).

2.5. Effect of BetA on IL-2 and TNF-α in serum

It has been well documented that IL-2 and TNF-α play an important role in anti-tumor defense. We tested whether the anti-tumor effect of BetA was mediated via modulation of IL-2 and TNF-α. Levels of IL-2 and TNF-α in serum were measured. According to the results, shown in Table 3, BetA treatment elevated levels of IL-2 and TNF-α in tumor-bearing mice in a dose-dependent manner. Levels of IL-2, TNF-α were significant reduction in tumor-bearing mice in CTX group as compared with control mice in serum (Table 3).

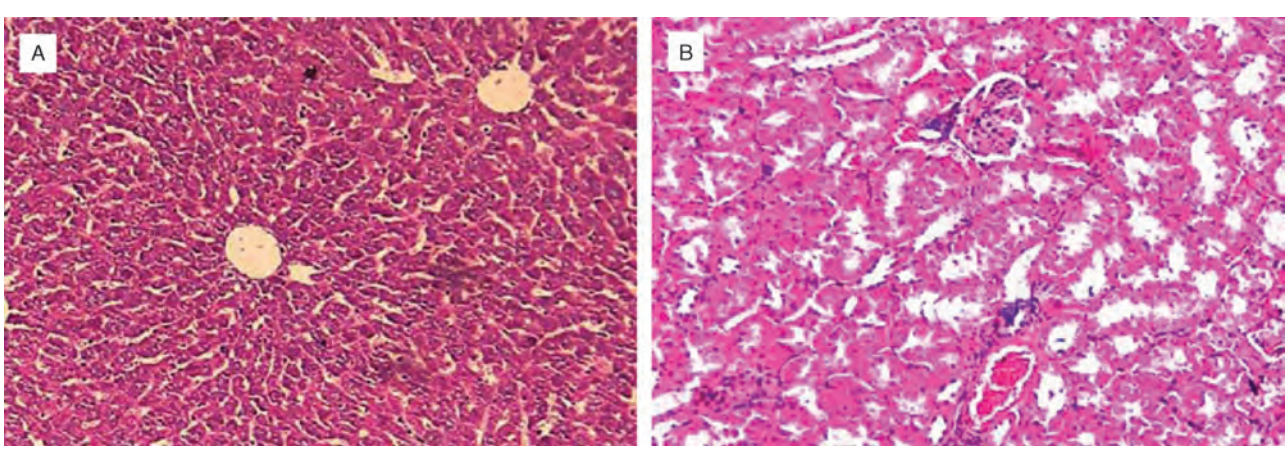

Fig. 2: Microscopic photos of the histological sections of liver (**A**) and kidney (**B**) in BetA group (H&E × 200)

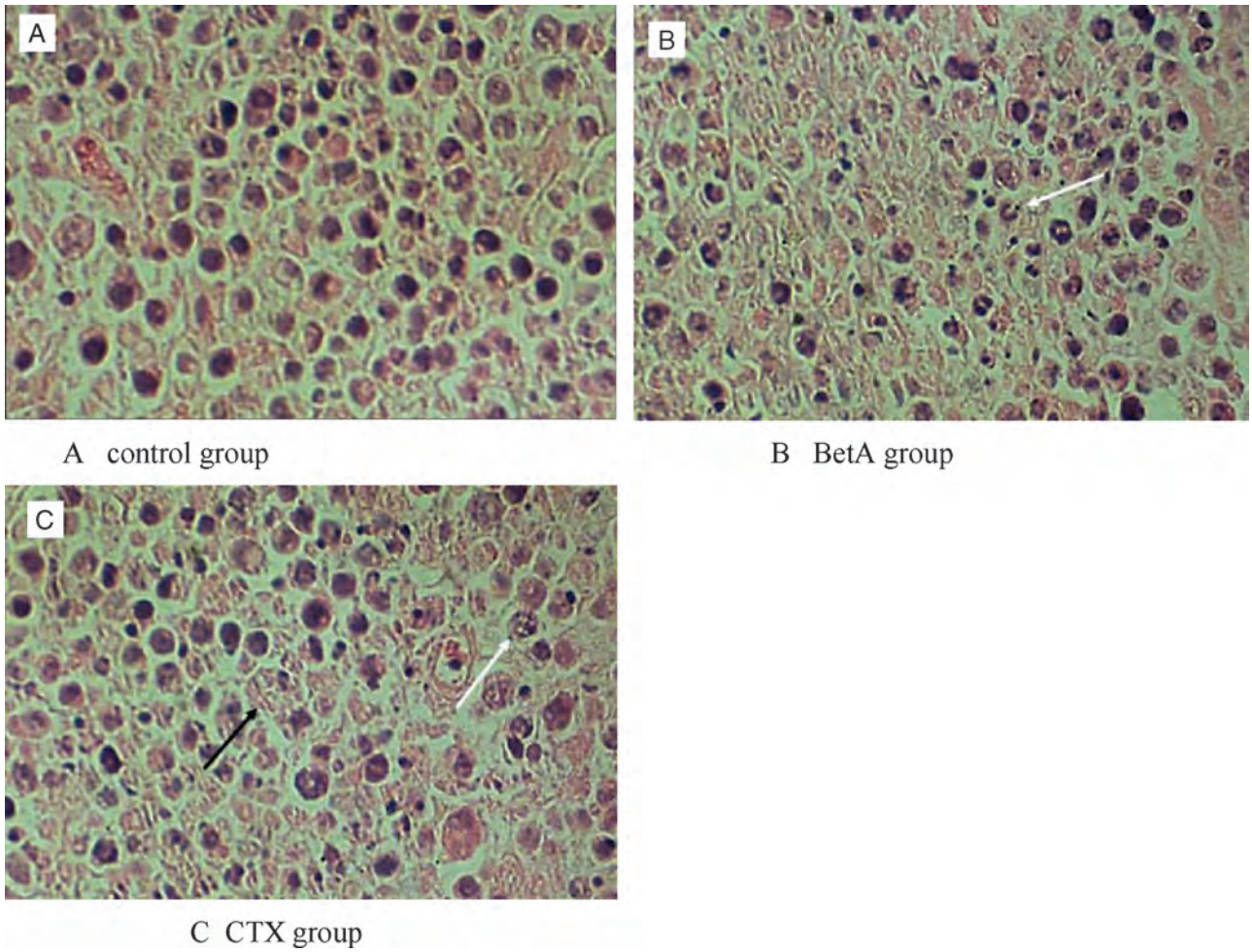

Fig. 3: Morphological change of cells of tumor in the U14 bearing mice (H&E × 200). A: control group. B: BetA group treated at dose of 200 mg/kg for 14 days. C: CTX group treated at dose of 25 mg/kg for 14 days. White arrows show apoptotic cells and black arrows show necrotic cells

2.6. Treatment with BetA induces cell apoptosis of tumors in vivo

Apoptosis was first determined by the immunohistochemical detection of DNA strand breaks by TUNEL staining. As shown in Table 4, the ratio of TUNEL-positive cells treatment with BetA (200 mg/kg body weight, p.o.) reached to 17.61%, which is significantly higher than that in control group and CTX group. The results indicate that the activity of BetA on the inhibition of tumor growth can play an important role in inducing apoptosis (Table 4, Fig. 4).

2.7. Effect of BetA on the expression of Bcl-2, Ki-67 and Caspase-8 in tumor cells

The streptavidin-peroxidase method was used to examine the expression of Bcl-2, Ki-67 and Caspase-8. Positive Bcl-2, Caspase-8 staining was identified by brown and yellow staining mainly in cytoplasm or membrane and Ki-67 staining in nuclear. According to results, treatment with BetA (200 mg/kg body weight, p.o.) and CTX reduced the expression of Bcl-2 and Ki-67 proteins respectively, while the expression of Caspase-8 increased. The percent of the positive cells of Bcl-2 was 68.82% in the control group; treatment with BetA (200 mg/kg body weight, p.o.) and CTX, the percent of the positive cells of Bcl-2 decreased to 23.23% in the BetA group and 45.61% in the CTX group (Table 4, Fig. 5). The number of the positive cells of Ki-67 was 64.44% in the control group, but the percent of the positive cells of Ki-67 treated with BetA and CTX reduced significantly to 25.81% and 36.82% (Table 4, Fig. 6). Meanwhile, the percent of the positive cells of Caspase-8 was 11.11 % in the control group, and the treatment with BetA and CTX significantly increased the number of Caspase-8 positive cells to 73.85% in BetA group and 31.26% in CTX group (Table 4, Fig. 7).

Table 3: Inhibitory effect of BetA on level of IL-2 and TNF-α in serum of tumor bearing mice ($\bar{x} \pm s$)

Groups	Doses (mg/kg)	IL-2 (μg/l)	TNF-α (μg/l)
Control	Vehicle	5.33 ± 1.87	6.02 ± 0.65
BetA	100	$7.87 \pm 1.40^*$	$7.11 \pm 1.23^*$
	200	$9.82 \pm 1.76^*$	$7.66 \pm 1.45^*$
CTX	25	$3.67 \pm 1.18^*$	$7.07 \pm 0.98^*$

* $p < 0.05$ as compared with control group, values are mean ± SD. CTX: Cyclophosphamide

3. Discussion

Cervical cancer, the second most common in women cancer worldwide, takes the lives of more than 250000 women each year globally. The effective therapeutics to cervical cancer is still radical hysterectomy with bilateral pelvic node dissection or radical pelvic radiation therapy following by concurrent chemotherapy. This can significantly improve the patients' survival rate to approximately 80% (Cheng et al. 2005). However, the prognosis of these patients is poor because it often recurs in several

Table 4: Effect of BetA on the expression of Bcl-2, Ki-67, Caspase 8 and apoptosis cell in cervical cancer tissue ($\bar{x} \pm s$, %)

Groups	Doses (mg/kg)	Bcl-2	Ki-67	Caspase8	Apoptosis cells
Control	Vehicle	68.82 ± 6.13	64.44 ± 7.14	11.11 ± 2.03	1.93 ± 1.57
BetA	100	51.34 ± 4.54	55.31 ± 6.33	$32.60 \pm 3.64^*$	$10.85 \pm 2.55^*$
	200	$23.23 \pm 3.04^*$	$25.81 \pm 3.31^*$	$73.85 \pm 7.69^*$	$17.61 \pm 3.12^*$
CTX	25	$45.61 \pm 4.67^*$	$36.82 \pm 4.64^*$	$31.26 \pm 3.42^*$	$6.42 \pm 2.89^*$

* $p < 0.05$ as compared with control group, values are mean ± SD. CTX: Cyclophosphamide

years (Hong et al. 2004). Therefore, it is necessary to investigate novel anti-tumor substances with higher efficiency which can not only kill cancer cell or inhibit tumor growth but also can improve immunity potential.

BetA is a potent bioactive molecule that possesses anticarcinogenic effects since it can interfere with the initiation, development and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis. Meanwhile, it can also act as key player in regulating the immune response through stimulation of immune organs. We aimed to focus the activity of BetA on treatment of U14 tumor-bearing mice, and explored the possible mechanism in immunoregulatory effect and inducing apoptosis.

BetA can improve the function of immune organs with no obvious side effects on body, liver, and kidney tissue in animals. It exhibits biological activities by stimulating the body to produce more CD4+ T lymphocyte subsets and raise the ratio of CD4+/CD8+ in treated U14 bearing mice in dose-dependent manner. Furthermore, BetA increased the levels of IL-2 and TNF-α in serum. It has been reported that IL-2 and TNF-α play important roles in cancer therapy (Chada et al. 2003; Antony and Restifo 2005; Sun et al. 2008). A synergistic anti-tumor effect has been observed when TNF-α is combined with IL-2 (Su and Wu 2000). In this study, it is likely that BetA might be effective immune organs directly and regulate the immune response in U14 bearing mice. Tumor cell proliferation and tumor growth were inhibited with raising levels of IL-2 and TNF-α which exert the function of the immune system and a direct cytotoxic effect on tumor cells. However, results of the CTX treated group were different from those in the BetA group. Thŭs, BetA and CTX might have different mechanisms of anti-tumor activities.

BetA induced apoptosis by activation of caspases-8 and down-regulation expression of Bcl-2 and Ki-67. Apoptosis is characterized by typical variations such as chromatin condensation in early stage, then cell shrinkage, DNA fragmentation and appearance of apoptotic bodies, as well as the radical reason of the activation of cysteine aspartic acid specific protease known as caspase. Once apoptosis starts, there are two main pathways which converge on caspase-3. One is the caspase-dependent pathway involving caspase-8, the other is a caspase-independent pathway involving activation of caspase-9 and mitochondrial release of cytochrome *c* (Sun et al. 1999). Our results showed that caspase-8 was involved in this course of apoptosis stimulated by BetA.

It is well-known that TNF-α can specifically induce apoptosis of cells through the receptor pathway (TNFR1) apart from immunomodulatory activity (Pober 1998). The interaction between TNF and TNFR1 combines with FADD through mutual recognition of Death domain, and then combines with caspase-8. Caspase-8, the initiator of the caspase (cysteinyl-aspartate protease) family, plays a critical role in apoptotic cell death pathway and is present in most cells as proenzyme(zymogen) in an inactive state. Upon the induction of apoptosis, caspase-8 becomes activated via oligomerization and bind to FADD in the form of a complex (Boatright and Salvesen 2003). Then cascade reactions take place in cells resulting in apoptosis. Recent reports suggest that caspase-8 also plays a role in maintaining homeostasis of peripheral T lymphocytes via the regulation of cytokine production (Schimnich et al. 2002; Launay et al. 2005). IL-2 is a product of T-cells which plays an important role in promoting the function properties of B cells, macrophages, and NK cells. Treatment U14 tumor bearing mice with BetA improved the levels of IL-2, TNF-α and caspase-8. The results indicated that high expression of caspase-8 in tumor cells participated in the mediating caspase-dependent apoptosis pathway directly. It is also related to upregulation of levels of IL-2 and TNF-α during promoting the apoptosis of cervical cancer cells. These results proved that BetA cannot only stimulate immune response directly but also induce apoptosis associated with the production of cytokines.

Bcl-2 is the founding member of a large family of apoptosis regulating proteins. It plays a key role as a proto-oncogene in inhibiting apoptosis rather than promoting proliferation (Cory et al. 2003; Letai 2008). Some studies reported that overexpression of bcl-2 can strengthen the viability of cells under various adverse circumstances such as cytokine withdrawal and loss of cell adhesion (Lu et al. 1995). Therefore, in the course

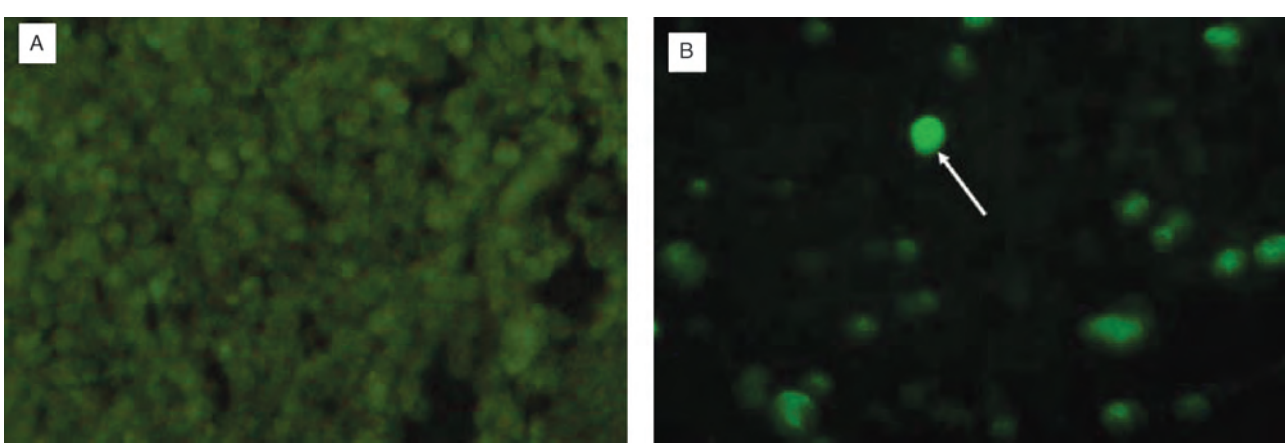

Fig. 4: Apoptosis cells in tumor tissue shown in the U14 bearing mice treated with BetA (TUNEL × 200) A: control group (under the light microscope). B: BetA group treated at dose of 200 mg/kg for 14 days (under the fluorescence microscope). White arrows show apoptotic cells

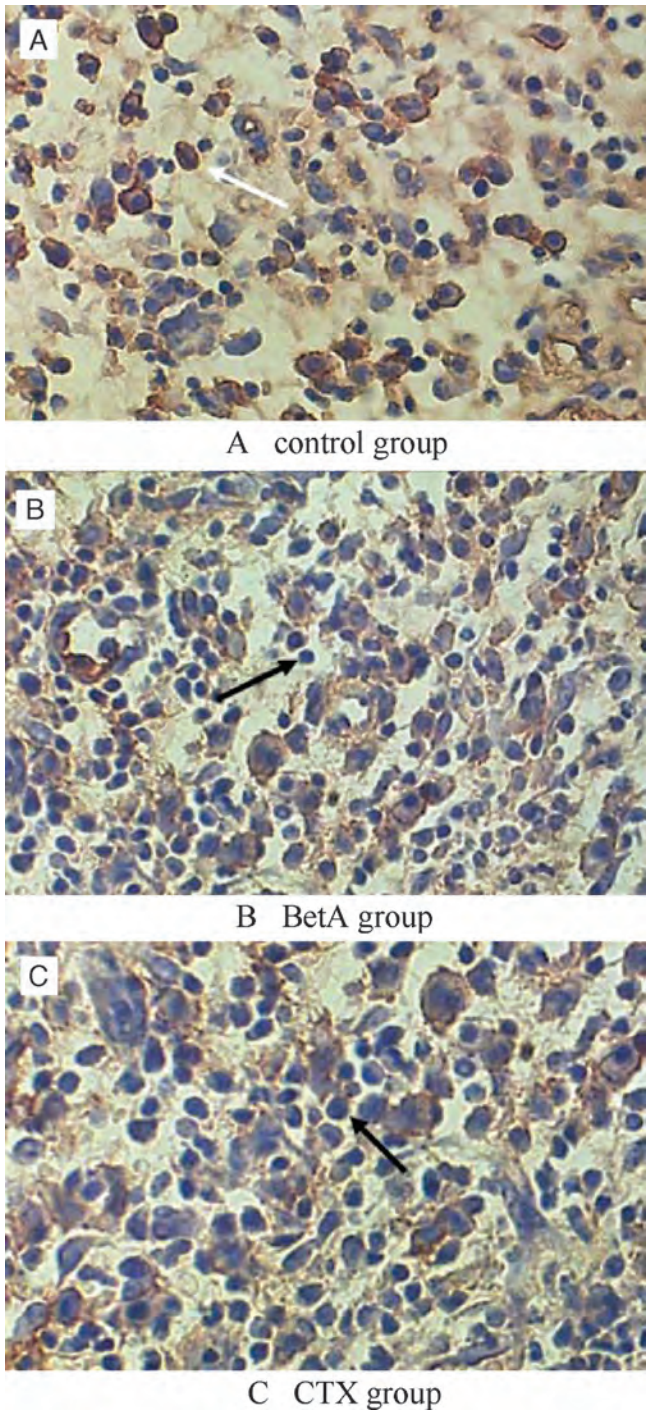

Fig. 5: Effect of BetA on the expression of bcl-2 protein in U14 tumor tissues (S-P × 200). White arrows indicate that cytoplasm is stained brown, which is a bcl-2 protein positive nucleus. Black arrows indicate that nucleus is stained blue, which is a bcl-2 protein negative nucleus

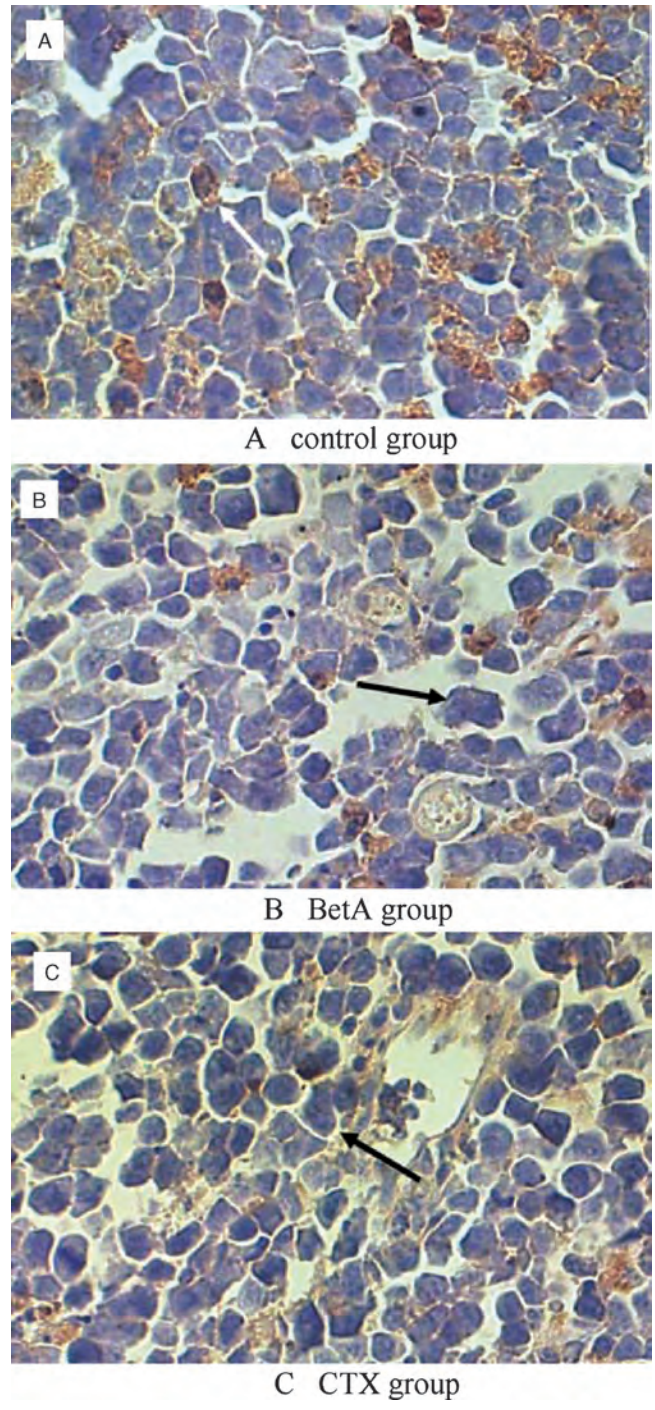

Fig. 6: Effect of BetA on the expression of Ki-67 protein in U14 tumor tissues (S-P × 200). White arrows indicate that nucleus is stained brown, which is a Ki-67 protein positive nucleus. Black arrows indicate that nucleus is stained blue, which is a Ki-67 protein negative nucleus

of inducing apoptosis, decreased expression of bcl-2 observed in tumors treated with BetA was associated with caspase-8 up-regulation. So we demonstrated that activity of BetA induced tumor cell apoptosis through the caspase-dependent pathway.

In order to interpret the mechanism of anti-tumor effect of BetA *in vivo*, we also detected the expression of Ki-67 in cervical cancer cells. As well known, Ki-67 is a marker of cell proliferation, which can quickly reflect the proliferation rate of malignant tumors (Gerdes et al. 1984). Ki-67 belongs to a nuclear antigen that is expressed predominantly in the S and M phases of the cell cycle, and it has been used for judgment of tumor growth, invasion and metastasis. Our results showed that treatment with BetA reduced the number of Ki-67 positive cells similar to CTX, which indicated BetA might participate in course of tumor cells apoptosis by regulation of cell cycle.

In summary, the current study demonstrates that BetA can significantly inhibit the growth of tumors in U14 tumor bearing mice through inducing tumor cell apoptosis in a dose-dependent manner. The molecular mechanism may be related to up-regulating caspase-8, down-regulating Bcl-2 and Ki-67 expression. Meanwhile, BetA plays a crucial role in regulating the immune response *in vivo*. Further investigations are needed to determine the specific pathway which is mainly responsible for BetA-induced tumor suppression in U14 bearing mice.

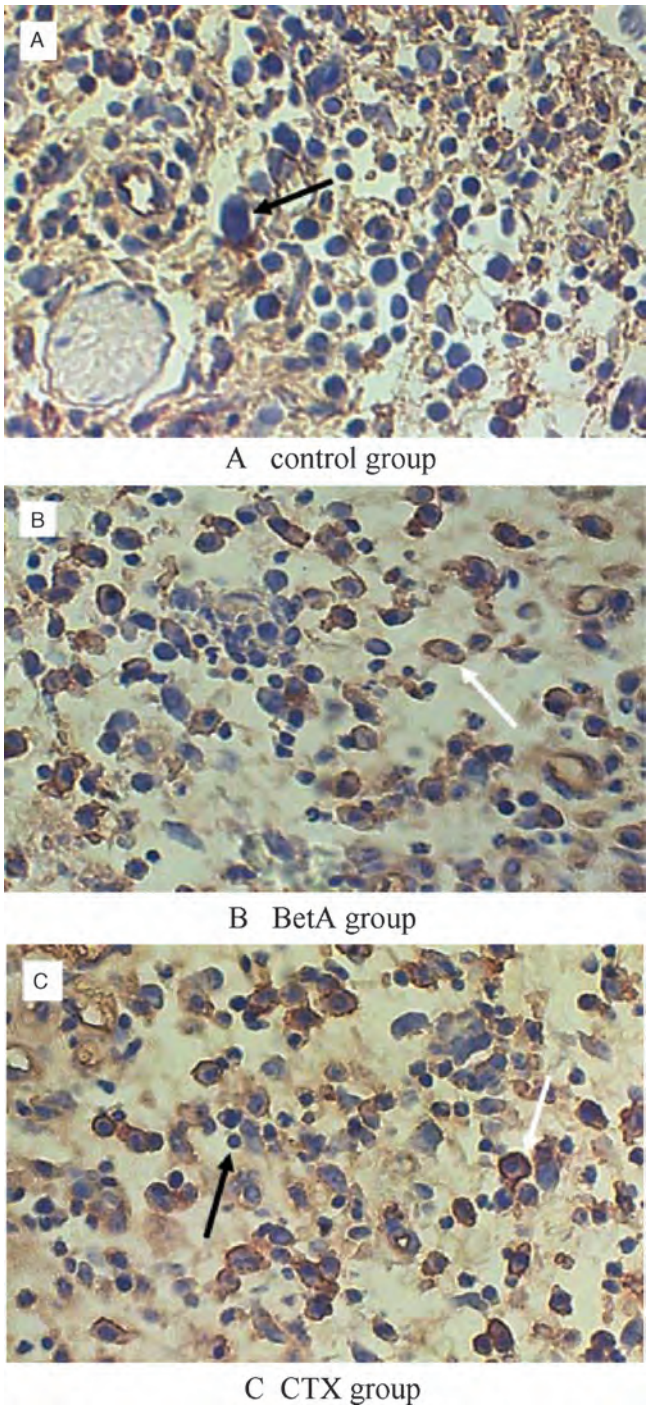

Fig. 7: Effect of BetA on the expression of Caspase-8 protein in U14 tumor tissues (S-P × 200). White arrows indicate that cytoplasm is stained brown, which is a caspase-8 protein positive nucleus. Black arrows indicate that nucleus is stained blue, which is a caspase-8 protein negative nucleus

4. Experimental

4.1. Chemicals and instruments

Betulinic acid (BetA) was obtained from Beijing Pharmaceutical University (Beijing, China). Cyclophosphamide (CTX) was obtained from Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China). Mouse anti-caspase-8, anti-Bcl-2 antibodies and anti-Ki-67 monoclonal antibodies were purchased from Beijing Biosynthesis Biotechnology Co. Ltd., (Beijing, China) and streptavidin-biotin-peroxidase (S-P) reagent kit was obtained from Santa Cruz Bio Inc. (Santa Cruz, CA, USA). Enzyme linked immunosorbent assay (ELISA) used for determining interleukin-2 (IL-2) and TNF-α were obtained from Sigma Chemical (St. Louis, Mo). TUNEL apoptosis assay system was purchased from KeyGen Biotech. Co. Ltd (Nanjing, China). Phycoerthrin (PE) anti-mouse CD3e, Fluorenscein isothiocyanate (FITC) anti-mouse CD4, Fluorescein isothiocyanate (FITC) anti-mouse CD8a were purchased from eBioscience, Inc. All other reagents were of analytical grade made in China.

4.2. Animals

Female Kunming mice 6–8 weeks old (20 ± 2.0 g) were provided by the Laboratory Animal Center of the Academy of Military Medical Sciences. They were maintained under standard environmental conditions and fed with a standard pellet diet and water *ad libitum*. All animal experiments were conducted in accordance with the NIH Guide for the care and Use of Laboratory Animals (NIH Publication No. 80–23; revised 1978 and the number approved by Administrated-Committee of Laboratory Animals was 062310).

4.3. Cell lines

Uterine cervical carcinoma (U14) was obtained from Institute of Medical Material, Chinese Academy of Medical Sciences and grown in RPMI 1640 medium supplemented with 10% FBS.

4.4. U14 tumor-bearing animal model and evaluation of anti-tumor activity

Prior to transplantation, animals were randomly divided into 4 groups, 10 mice per group. Under sterile conditions, 0.2 ml of U14 cells (1×10^7 cell/ml) was injected into the left axilla s.c. per mouse (day 0). After 24 h of inoculation, BetA was given orally at a dose of 100 mg/kg (low dose of BetA group) and 200 mg/kg (high dose of BetA group). The group administered with vehicle alone (sterile physiological saline, administered orally) was taken as control group and the group treated with cyclophosphamide (CTX, 25 mg/kg, administered intraperitoneally) was considered as the standard reference drug (CTX group). All groups were continuously treated for 2 weeks. Animal survival was monitored three times daily. On day 15, the mice were sacrificed and autopsies were made. The total body weight of each mouse was firstly measured following the collection of peripheral blood. Major organs such as thymus, spleen and tumor mass were then dissected and their weight was measured. Portions of each tissue were fixed in 10% formalin (pH 7.4) for histology, snap frozen in liquid nitrogen for other analysis. The rate of tumor inhibition was calculated as follows: The rate of inhibition (%) = (mean tumor weight of control group-mean tumor weight of treated group) /mean tumor weight of control group × 100%.

4.5. Toxic effect of BetA on liver and kidney

The liver and kidney tissues from treated and control mice were collected followed by autopsies and processed as slides for histopathological analysis under light microscope.

4.6. Morphology analysis of tumor tissues

Tumor specimens collected from the control group, CTX group and BetA group (200 mg/kg) were fixed in 10% (v/v) neutral formalin solution and embedded in paraffin. Subsequently, Tissues from sacrificed mice were sliced into 4 μm thick serial sections and stained with hematoxylin and eosin and then examined under the light microscope.

4.7. Effect of BetA on peripheral blood T-lymphocyte subpopulations of tumor bearing mice

By the end of the experiment on day 14, the thymus, spleen and peripheral blood of all groups were collected for examination immediately after the mice had been killed. The collected anticoagulated blood was diluted to 1×10^6 cell/ml, labeled with anti-mouse monoclonal antibodies 0.1 ml for 30 min, washed with 10 ml PBS and then incubated in the dark for 30 min at 4 °C with 100 μl of rabbit anti-mouse FITC-IgG monoclonal antibodies to CD4+(1 μg) and CD8+(1 μg). Stained cells were examined with an FACS Calibur flow cytometer (BD, America) using CellQuest Analysis Software.

4.8. Effect of BetA on IL-2 and TNF-α in serum

The serum of peripheral blood from treated groups and control group was isolated by centrifugation of coagula in eppendorf tubes at 6000 rpm for 4 min on a microcentrifuge. IL2 and TNF-α levels were assessed using commercial enzyme linked immunosorbent assay kits. Assays were carried out according to the manufacturers' instructions. All samples were analyzed in duplicate.

4.9. TUNEL assay for apoptosis

Apoptotic cells in the tumor sections were visualized by the terminal deoxyncleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique

according to the manufacturer·s recommendations. Tissue sections were treated by dewaxing and hydration firstly according to a conventional method, and then processed with 10 μg/ml protease K for 30 min at room temperature. The slides were immersed in a 2% H_2O_2 solution to block endogeneous peroxidase activity after rinsing two times in PBS. TdT was used to catalyze the addition of fluorescein-conjugated dUTP to the 3'-OH ends of DNA fragments. So each sample was added to 50 μl TdT enzyme reaction solution except for control group and covered for 60 min at 30 °C. Apoptotic cells which could emit fluorescence from the excitation light were detected by fluorescence microscope.

4.10. Immunostaining on expression of Bcl-2, Ki-67 and caspase-8 expression

The expression of Bcl-2, Ki-67 and Caspase-8 in tumor tissue was analyzed and used to reveal the level of apoptosis during the tumor development. Tumor tissue sections were stained with standard streptavidin-peroxidase method described in the procedure program of streptavidin-peroxidase reagents kit (Sigma, St. Louis, Mo), and observed with light microscope. A previously known positive tumor tissue was used as a positive control for Bcl-2, Ki-67, and Caspase-8. Bcl-2, Ki-67, and Caspase-8 expression indices were calculated as percentages, dividing the number of positively stained tumor cells (nuclear staining for Ki-67expression and cell cytoplasm staining for Caspase-8 expression and Bcl-2 expression) by the total number of cells in 10 randomly selected high power fields (× 400). At least 500 tumor cells were counted per section.

4.11. Statistical analysis

Data were expressed as mean ± S.D. One way analysis of variance and Duncan's multiple range tests were used for determining differences between groups, and $P < 0.05$ was regarded as statistically significant.

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