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Inhibition of betulinic acid to growth and angiogenesis of human colorectal cancer cell in nude mice

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Abstract ***Objective:*** Angiogenesis plays a major role in the pathogenesis of many disorders. Vascular endothelial growth factor (VEGF) has been shown to be the key regulator of normal and pathological angiogenesis. Many studies showed that decreased expression of VEGF has been inhibited growth and migration of cancer cells. The aim of this study was to explore the effects of Betulinic acid on the VEGF expression and the growth of colorectal cell SW480 xenografts in nude mice. ***Methods:*** The xenografts derived from colorectal cell SW480 were established in BALB/C nude mice. Inoculated mice were randomly divided into negative control (corn oil), low dose betulinic acid group (20 mg/kg/d) and high dose group (40 mg/kg/d). After 22 days, the animals were sacrificed; tumor volume and weights were measured. The mRNA level of VEGF was analyzed by quantitative real-time polymerase chain reaction. The expression of VEGF protein was detected by immunohistochemistry. ***Results:*** The tumor weight was significantly lower in low and high dose groups than in corn oil group (1.12 ± 0.04, 0.43 ± 0.02 vs 2.08 ± 0.07; $P < 0.05$). The mRNA levels of VEGF was also significantly lower in betulinic acid treated groups (0.72 ± 0.02, 0.38 ± 0.01; $P < 0.05$) than in control group (1.08 ± 0.04). H&E staining showed tumor tissue necrosis was observed in treatment groups. The positive expression of VEGF was lower in low and high dose groups than in corn oil group. Gray scale increased in low dose group and high dose group (121.1 ± 2.8, 156.2 ± 3.3, $P < 0.05$). ***Conclusion:*** Betulinic acid had significant inhibitory effect on VEGF expression and tumors growth of human colorectal cancer xenografts *in vivo*, and down-regulation of VEGF expression may account for one of the molecular mechanisms of the anticancer effects of betulinic acid.

Key words betulinic acid; colorectal cancer; vascular endothelial growth factor (VEGF); nude mice

Colorectal cancer is the third most common cancer in the digestive system and one of the leading causes of cancer death in China [1]. Its incidence and death rate have continued to increase. The colorectal carcinogenesis is a multistage process composed of genetic and epigenetic alteration. Various genetic and molecular alterations have been found to be associated with the growth and the malignant transformation.

The growth and metastasis of tumors depend on the development of an adequate blood supply via angiogenesis. Vascular endothelial growth factor (VEGF) is a primary regulator of physiological angiogenesis and it is a major mediator of pathological angiogenesis, such as tumor-associated neovascularization [2]. As the key factor of angiogenesis, VEGF plays an important role in colorectal cancer development [3] and has become a hot spot of the research of cancer therapy these years [4]. So blocking the action of VEGF seems to be a promising anti-angiogenic method to treat some solid tumor including breast cancer.

Betulinic acid (BA) is a pentacyclic triterpene natural product, which was discovered in National Cancer Institute drug screening program natural plant extracts, and has been recognized to possess potent pharmacological properties [5]. And it has recently been shown to possess anti-tumor properties in many different cancer cells and exhibit low toxicity in animal models [6–8]. Therefore, the present study aims at studying the effects of BA on VEGF expression and tumor growth of colorectal cancer cells *in vivo*.

Materials and methods

Cell lines and culture

The human colon carcinoma cancer cell line SW480

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was obtained from Wuhan Boster Biological Technology (China). Cell were routinely maintained in RPMI 1640 with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 ℃ in a well humidified atmosphere of 5% CO_2. The medium was changed once every three days and subcultured when confluence was reached.

Animals and Implantation of tumor cells

A total of 24 male athymic BALB/c nude mice (age 4–6 weeks) were purchased from Suzhou Bayerd Bio-med Science and Technology Company (China). The mice were maintained under specific pathogen-free conditions in facilities approved. To produce tumors, SW480 colorectal cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin. Trypsinization was stopped with minimal essential medium containing 10% fetal bovine serum, and the harvested cells were washed once in serum-free medium and resuspended in PBS. Only single-cell suspensions with greater than 90% viability were used for the injections. SW480 colorectal cells (1×10^6) were implanted into the flank of each mouse. Ten days after cell inoculation, animals were divided into three equal groups of 8 mice each. The first group received 100 μg vehicle (corn oil) by oral gavage, and the second and third groups of animals received 20 and 40 mg/kg/d doses of BA in vehicle every second day for 14 days (seven doses). The mice were weighed, and tumor areas were measured throughout the study. After 22 days, the animals were sacrificed; final body and tumor weights were determined. For immunohistochemical and histologic staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and the other part of the tumor was snap-frozen in liquid nitrogen, and stored at –70 ℃.

Real-time PCR

Total RNA of tumor lysates frozen by liquid nitrogen was extracted by Trizol reagent according to the manufacturer's instructions. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm. For the reverse transcription polymerase chain reaction, RNA PCR kit (AMV, TakaRa Bio, Japan) was used with 10 μL reverse transcription reaction. Then 2 ng of cDNAs were used to conduct PCR to detect expression of VEGF with SYBRTM Green I. The primers for human VEGF were as follows, forward primer: 5'-TCA CAG GTACAG GGA TGA GGA CAC-3'; reverse primer: 5'-TCC TGG GCA ACT CAG AAG CA-3'. This primer sequence amplifies a 184-base pair sequence. The primers for GAPDH were as follows, forward primer: 5'-TGA ACG GGA AGC TCA CTG G -3'; and reverse primer: 5'-TCC ACC ACC CTG TTG CTG TA-3'. This primer sequence amplifies a 307-base pair sequence. GAPDH was used as the internal standard in the real time PCR system. The cycling conditions for GAPDH and VEGF were the same. The PCR reaction system was 25 μL: Fast SYBR Green Master Mix 12.5 μL, forward primer 1.0 μL, reverse primer 1.0 μL, cDNA 2.0 μL, ROX 0.5 μL. The PCR conditions were as follows: 95 ℃ 30 s, 95 ℃ 5 s, 60 ℃ 32 s, 40 amplification cycles. Data analysis was Performed by the Roche Molecular Biochemicals Light Cycler software.

Immunohistochemistry

Paraffin-embedded tumor tissues were sectioned at 4–6 μm and the slices were deparaffinized in xylene as usual, treated with a graded series of alcohol [100%, 95%, and 80% ethanol (vol/vol) in double distilled H_2O], and rehydrated in phosphate buffered saline (PBS). After wards, endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 10 min. Antigen retrieval for VEGF staining was done for 10 min in 10 mmol/L sodiumcitrate buffer (pH 6) heated at 95 ℃ in a steamer followed by cooling for 15 min. The slides were washed with PBS and incubated for 30 min at room temperature with a protein blocking solution. Excess blocking solution was drained, and the samples were incubated with rabbit polyclonal antibody (diluted with PBS, 1:100, Santa Cruz Biotechnology, USA) at 4 ℃ overnight. Primary body was removed and washed with PBS for 3 times; peroxidase labeled goat-anti-rabbit IgG (Zhongshan Bio., China) was then added at 37 ℃ for 2 h. The color was developed by exposing the peroxidase to diaminobenzidine reagent, which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. VEGF expression was identified by the brown cytoplasmic staining. Finally, the data were presented as mean ± standard deviation (SD).

Statistical analysis

One way ANOVA was used to compare data among three groups. Values were expressed as mean ± standard error of the mean. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 13.0 for Windows.

Results

BA inhibits tumor growth in nude mice bearing SW 480 colorectal cancer cells

There was no significant difference in tumor size in each group of nude mice before treatment, so the tumor size in each group were comparable. Before and after treatment, tumor volume (cm^3) and body weight of nude mice (g) were measured once every two days. Tumor growth curve was shown in Fig. 1a. Weight could be observed when mice were sacrificed on the 22th day, and this was accompanied by significantly decreased tumor

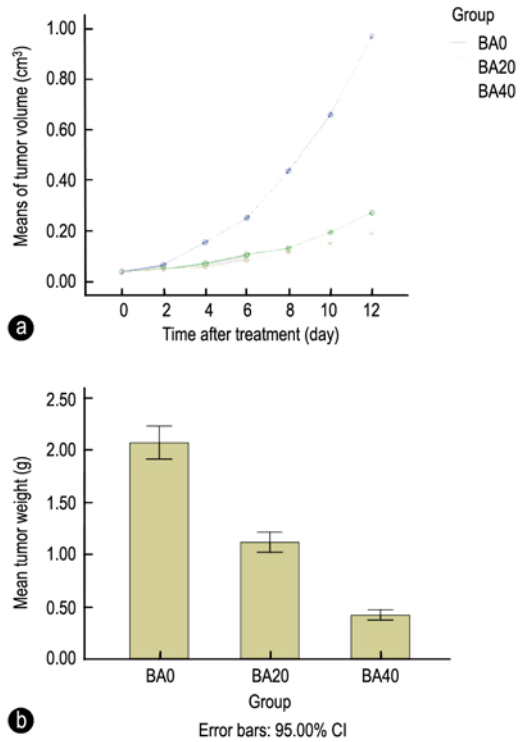

Fig. 1 (a) Tumor volume. Athymic nude mice (8 per group) bearing colorectal cells as Xenografts were treated with corn oil (control) or betulinic acid in corn oil (20 or 40 mg/kg/d). Tumor volume was calculated with the following equation: V (cm^3) = a × b^2 / 2; (b) Tumor weights. After the final treatment, animals were sacrificed, and tumor weights were determined as described in Materials and Methods. $P < 0.05$, significantly decreased tumor weights or volumes

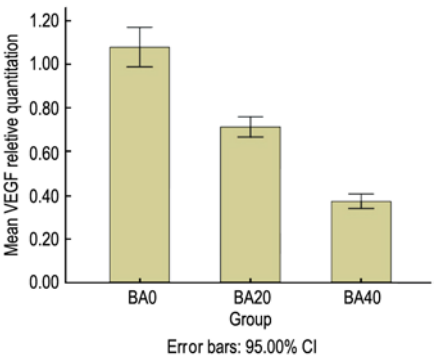

Fig. 2 VEGF mRNA relative quantitation. Effect of BA on VEGF mRNA expression in nude mice. Whole-cell lysates from coil oil and betulinic acid treated tumors were obtained from eight mice and analyzed by RT-PCR

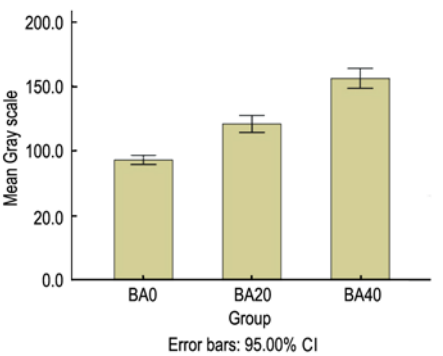

Fig. 4 Gray scale. Inhibitory effect of BA on VEGF protein expression in nude

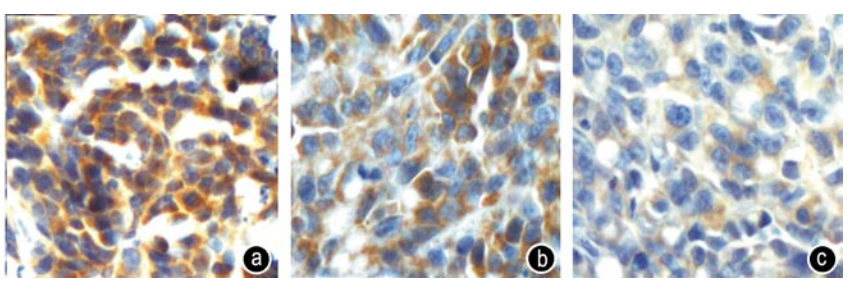

Fig. 3 Immunohistochemical expression of VEGF in nude mice of different groups (DAB staining, light microscope, original magnification × 400). (a) VEGF of expressions in coil oil were abundant and in dark brown; (b) VEGF of expressions in low dose were few; (c) VEGF of expressions in high dose were less than those in low dose

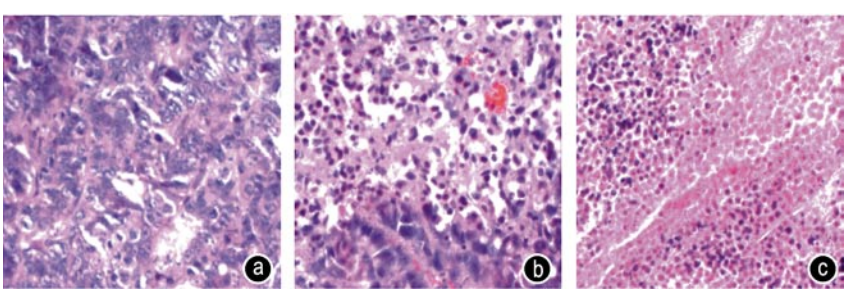

Fig. 5 H&E staining of tumor (original magnification × 200). Tumor from coil oil and betulinic acid (B and C) mice were stained with H&E and examined histopathologically. (a) Cancer cells in coil oil group arranged compactly and the shape of cancer cells is irregular; (b) Cancer cells in low dose group were crumbly; (c) there were lots of necrotic cancer cells

Table 1 Inhibitory effect of BA on the body weight of nude mice transplanted colorectal cancer cells

Grooup	Body weight (g)	
	Pre-treatment	Post-treastment
Coil oil	20.14 ± 0.64	17.09 ± 0.33
Low dose BA	20.26 ± 0.60	20.89 ± 0.74
High dose BA	19.53 ± 0.51	21.54 ± 0.54

All values were presented as mean ± SD

weights in both treatment groups (Fig. 1b). Changes in body weight of nude mice were show in Table 1. Results clearly showed that BA (20 and 40 mg/kg/d) inhibited tumor growth and final tumor weights in athymic nude mice bearing SW480 cells as xenografts ($P < 0.05$).

BA decreased VEGF mRNA expression in tumors

To investigate whether BA decreased VEGF expression at the mRNA level, we also compared expression of VEGF mRNA in tumor lysates from control and BA–treated mice (eight animals per group) by real-time PCR using GAPDH as a loading control. Relatively high level of VEGF mRNA was observed in the control tumor, in contrast, expression of VEGF mRNA was decreased in tumors from BA–treated mice (Fig. 2). Inhibition rates were 33.3% and 64.8% ($P < 0.05$). These results indicated that BA inhibited VEGF mRNA expression in tumor.

BA inhibited angiogenesis-related protein expression in nude mice transplanted tumor

The antiangiogenic activity of BA was confirmed by decreased VEGF staining in tumors from BA vs corn oil–treated mice. Immunohistochemistry showed concentrations of VEGF in the control group was significantly higher than that in the BA treatment groups (Fig. 3). Image analysis revealed gray scale of VEGF was highest in high dose group (156.2 ± 3.3) and low dose group and control group were 121.1 ± 2.8 and 93.2 ± 1.2 ($P < 0.05$; Fig. 4). H&E staining also showed that the vascularity of tumors in mice treated with BA was significantly lower than in tumors from corn oil–treated mice. There were lots of necrotic cancer cells in the cancer nest (Fig. 5).

Discussion

Invasion and metastasis of malignant tumor are the main reason for failure of cancer treatment. Cancer development requires neovascularization. It has also been suggested that inhibition of blood vessel formation could be a therapeutic target in solid tumors [9]. Blocking the neovascularization in the tumor appears to be a promising antitumorigenic approach to treat multiple types of solid tumors including colorectal cancer [10]. VEGF is a potent activator of angiogenesis as it promotes endothelial cell proliferation and new blood vessel formation [11]. It is the most important regulator of tumor angiogenesis and may play a central regulation role in a variety of angiogenic factor [12]. VEGF overexpression has been shown to be correlated to poor prognosis in a variety of different cancers and been reported to be a critical factor in tumor expansion and vascular function [13].

BA is a natural product identified in various bark extracts that exhibits a variety of biological activities including potent antitumor properties [14]. Pisha *et al* initially reported that this triterpenoid inhibited growth of several melanoma cell lines in 1995 [7]. There are an increasing number of studies showing that BA can resist the growth of tumor though inhibition of angiogenesis. In this study, we provide evidences that BA is very potent in killing colorectal cancer cells *in vivo* and can dose-dependently decrease the expression of VEGF.

In the present study, we investigate the antiangiogentic and antitumorigenic activity of BA, which down-regulate VEGF gene expression in SW480 human colon carcinoma cell xenografts in nude mice. Firstly we use nude mice injected colon cancer cells as a model. We demonstrate treatments with coil oil have no effect on tumor volume and weight. In contrast, BA (40 mg/kg) statistically significantly decreases the median tumor volume and weight. RT-PCR shows that there is a marked decreased in expression of VEGF mRNA in tumors from mice treated with BA. Decreased mRNA expression also parallels decreased VEGF proteins levels in tumors from BA-treated animals. Immunohistochemistry results clearly suggests that BA (40 mg/kg) can down-regulate VEGF protein levels, and this is consistent with the antiangiogenic activity previously reported for BA [15]. H&E staining also shows that the vascularity of tumors in mice treated with BA is significantly lower than in tumors from corn oil–treated mice, and there are lots of necrotic cancer cells.

In conclusion, these effects of BA in colorectal carcinoma cells and tumors demonstrate that VEGF may be an important target to colorectal cancer chemotherapy. BA can inhibit the expression of VEGF in athymic nude mice bearing colorectal cells as xenografts. These results may provide an insight into the control of BA to colorectal cancer.

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