A low molecular weight polysaccharide isolated from *Agaricus blazei* suppresses tumor growth and angiogenesis *in vivo*

Y.C. NIU[1*], J.C. LIU[1*], X.M. ZHAO[1] and X.X. WU[2]

[1]Institute of Medicine, Qiqihar Medical College, Qiqihar, Heilongjiang, P.R. China;
[2]Department of Urology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Received June 10, 2008; Accepted September 5, 2008

DOI: 10.3892/or_00000201

Abstract. Previous studies indicated that the low molecular weight polysaccharide extracts from *Agaricus blazei* are potential antitumor agents or adjuvant in tumor treatment. In this study, we investigated the antitumor activity of LMPAB, a low molecular weight polysaccharide isolated from *Agaricus blazei*, and the molecular mechanisms of its antitumor activity. The antitumor effect of LMPAB was examined using mouse sarcoma 180 (S180) xenograft models. Antiangiogenic effect of LMPAB was determined by chicken embryo chorioallantoic membrane (CAM) angiogenesis and Matrigel-induced neovascularization *in vivo* models. The mRNA and protein levels of vascular endothelial growth factor (VEGF) were assessed using real-time reverse transcription-polymerase chain reaction, immunohistochemistry, and enzyme-linked immunosorbent assays. Tumor inhibitory rates in the S180 xenograft models were 9.7, 23.9, and 33.0%, respectively, after administration of LMPAB at dose of 50, 100, and 200 mg/kg/day for 2 weeks. LMPAB also inhibited angiogenesis in the CAM model and Matrigel-induced neovascularization in C57BL/6 mice. The mRNA and protein levels of VEGF in tumor tissues were significantly down-regulated in the BALB/c mice received LMPAB treatment. Furthermore, significant down-regulation of serum VEGF levels was also observed in the mice. Our data suggest that LMPAB might be a promising agent for tumor therapy, and the antitumor and antiangiogenic effects of LMPAB may be related with down-regulation of VEGF.

Introduction

The basidiomycete fungus *Agaricus blazei* has traditionally been used as a health food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis (1). Previous studies have revealed that both water and lipid exacts from *Agaricus blazei* exert antitumor activity (2). They have been shown to have antitumor activity against a variety of tumors (3-5). Insight into polysaccharides highlighted it as a promising antitumor substance derived from *Agaricus blazei* (6). It was also noted that the polysaccharide extracted from *Agaricus blazei* displayed antitumor potential for their tumor-specific cytocidal and immunopotentiating effects (7,8). Ahn *et al* (9) investigated the beneficial effects of the oral daily assumption of an extract of *Agaricus blazei* on immunological status and quality of life in cancer patients undergoing chemotherapy. They observed that natural killer cell activity was significantly higher in *Agaricus blazei* Murill Kyowa-treated group as compared with non-treated placebo group. These results indicate that the low molecular weight polysaccharide extracts from *Agaricus blazei* are potential antitumor agents or adjuvant in tumor treatment.

It has been well established that all successful tumors must undergo neovascularization (angiogenesis), in order to acquire nutrients for continued growth. Tumor angiogenesis, a process of new vasculature formation, is appreciated to be an integral part of solid tumor development (10). Many angiogenic factors derived from tumor cells and tumor-infiltrating inflammatory cells stimulate endothelial cell activation and initiate angiogenesis (11). Vascular endothelial growth factor (VEGF) is one of the most important of all known inducers of angiogenesis (12). It has been demonstrated that elevated VEGF production by cancer cells directly correlates with tumor angiogenesis and tumor development (13). The blockade of the VEGF results in effective inhibition of tumor growth in xenograft mouse models, suggesting significant benefits for the therapeutic intervention in cancer patients (14).

We extracted four low molecular weight polysaccharide components from *Agaricus blazei* and identified the most potent one, designated as LMPAB. In this study, we investigated antitumor activity of LMPAB using mouse sarcoma 180 (S180) xenograft models. We further explored the molecular mechanisms that may be involved in the antitumor activity of LMPAB by analysis of VEGF expression in tumor tissues, and chick embryo chorioallantoic membrane (CAM) angiogenesis and Matrigel-induced neovascularization models.

Correspondence to: Dr Ji-Cheng Liu, The Institute of Medicine, Qiqihar Medical College, 249 Heping Road, Fulaerji District, Qiqihar, Heilongjiang 161042, P.R. China
E-mail: qyybliu@126.com

*Contributed equally

Key words: antitumor, antiangiogenesis, vascular endothelial growth factor, low molelular weight polysaccharide, *Agaricus blazei*

Materials and methods

LMPAB characterization. LMPAB was extracted from *Agaricus blazei* (Zhejiang Bo Rui Pharmaceuticals, Qingyuan, P.R. China) with a method as previously described (15). The molecular weight of LMPAB was 48 kDa based on a high-performance liquid chromatography-gel permeation chromatography analysis. The purity of LMPAB was 98.6%, determined by gel chromatography (Sephadex G-100). Sugar composition analysis showed that the polysaccharide was composed of ß-(1-3)-glucan. The contamination of proteins was excluded according to the absorption at 260 and 280 nm, respectively. LMPAB was dissolved in PBS (pH 7.2) at a concentration of 3 g/l prior to each experiment.

Tumor cells and animals. The mouse S180 cells were maintained in the peritoneal cavities of the mice obtained from Harbin Institute of Animal Parasitology, Chinese Academy of Agricultural Sciences (Harbin, P.R. China). Pathogen-free 6 weeks old female/male BALB/c mice and male C57BL/6 mice at age of 6 weeks were obtained from the Beijing Vital River Experimental Animals Technology (Beijing, P.R. China). Animals were housed in sterile cages under laminar air-flow hoods in a specific pathogen-free room with a 12-h light and 12-h dark schedule and fed autoclaved chow and water *ad libitum*.

S180 xenograft mouse model and antitumor activity assay. Cells (10^6) of S180 cells passaged 3 times in the abdominal cavity of the mice were inoculated into BALB/c mice by intraperitoneal (i.p.) injection in a volume of 0.1 ml. These model animals were randomly divided into 6 groups with 12 (6 males and 6 females) in each group. These included three groups treated with LMPAB at different doses (50, 100, or 200 mg/kg), one group was treated with fungus umbellatus polysaccharides (FUP, 5 mg/kg), one group with *Agaricus blazei* Murill polysaccharides (AbMP, 1000 mg/kg), and one model control group with 0.9% normal saline. LMPAB, FUP, AbMP or normal saline was given i.p. daily for 14 consecutive days starting on the day of S180 inoculation.

On day 14 after treatment, all mice were sacrificed under deep anesthesia. Tumor tissues were removed by dissection away from adjacent organs and weighed on an analytical balance. Tumor inhibition rate (%) was calculated by the following formula: tumor inhibition rate (%) = [(A-B)/A] x100, where A is the tumor weight average of the negative control, and B is that of the treated group. All experiments were approved by the Animal Care and Use Committee of Qiqihar Medical College.

CAM angiogenesis assay. CAM angiogenesis model was used to examine effect of LMPAB on the antiangiogenesis (16). Briefly, fertilized white leghorn chicken eggs (Shuair Poultry, Qiqihar, P.R. China) were incubated for 7 days at 37˚C. Then, a window was opened in the shell, and an avascular area of CAM was exposed to 20 μl of distilled water (control) or LMPAB (10, 20 or 40 μg/μl) by sterile filter disk. After 72 h of incubation at 37˚C, the CAMs were fixed *in situ* with methanol-acetone fixative, excised from the eggs, placed on slides, and left to dry in air. Photographs of the CAM were taken with a Canon digital camera (Tokyo, Japan) and CAM angiogenesis was analyzed by a digital medical image analysis system (Motic Med CMIAS, Beijing, P.R. China).

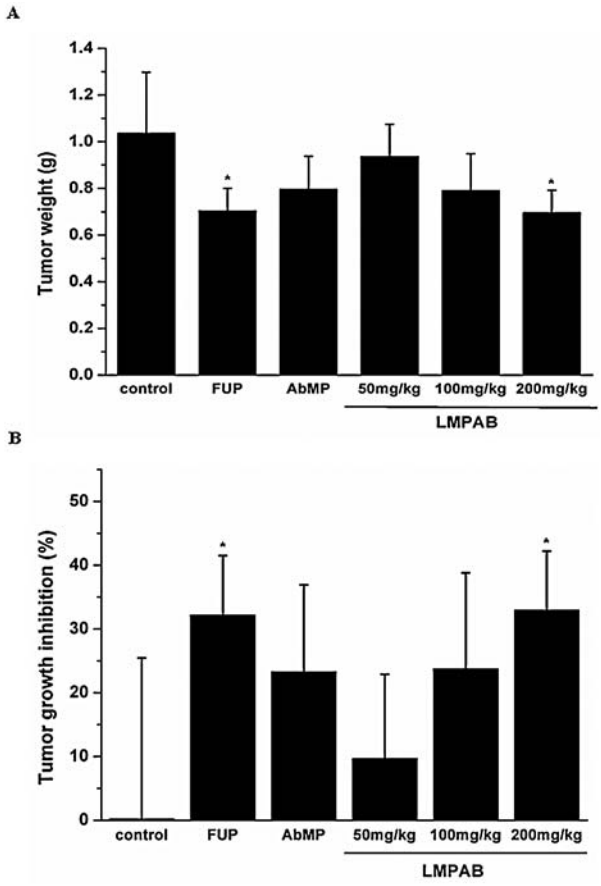

Figure 1. Effect of LMPAB on the S180 xenograft growth in mice. Treatment with LMPAB (50, 100, or 200 mg/kg/day), FUP (5 mg/kg/day) and AbMP (1000 mg/kg/day) given i.p. was initiated 24 h after the S180 i.p. into BALB/c mice for 14 days. (A) Tumor weight was measured. (B) Tumor growth inhibition rate was calculated by the formula as described in Materials and methods. The mean and SD of each group are shown (n=11-12); *P<0.05 versus control. These data represent means of three independent experiments.

Measurement of Matrigel-induced neovascularization. Matrigel-induced neovascularization was assayed *in vivo* (17). Briefly, C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel basement membrane (Becton Dickinson Labware, Bedford, MA) containing VEGF (10 ng), heparin (16 U) in the presence or absence of LMPAB (50 or 100 μg). The mice were sacrificed on day 7, and the gels were removed and weighed. Then the gel was fixed in 10% formaldehyde for 24 h, transferred to 70% ethanol, dehydrated, and embedded in paraffin. Following conventional deparaffinization, endogenous peroxidase quenching with 3% H_2O_2, 5 μm of thick sections were incubated with rat anti-mouse CD34 monoclonal antibody (mAb) (Boster Biotechnology, Wuhan, P.R. China) at 1:100 dilution in PBS for 12 h at 4˚C. This was followed by the addition of biotinylated rabbit anti-rat IgG (Boster Biotechnology) at 1:100 dilution for 30 min for 30 min, and diaminobenzidine for 5 min. An isotype-matched control IgG was used on a duplicate slide in place of

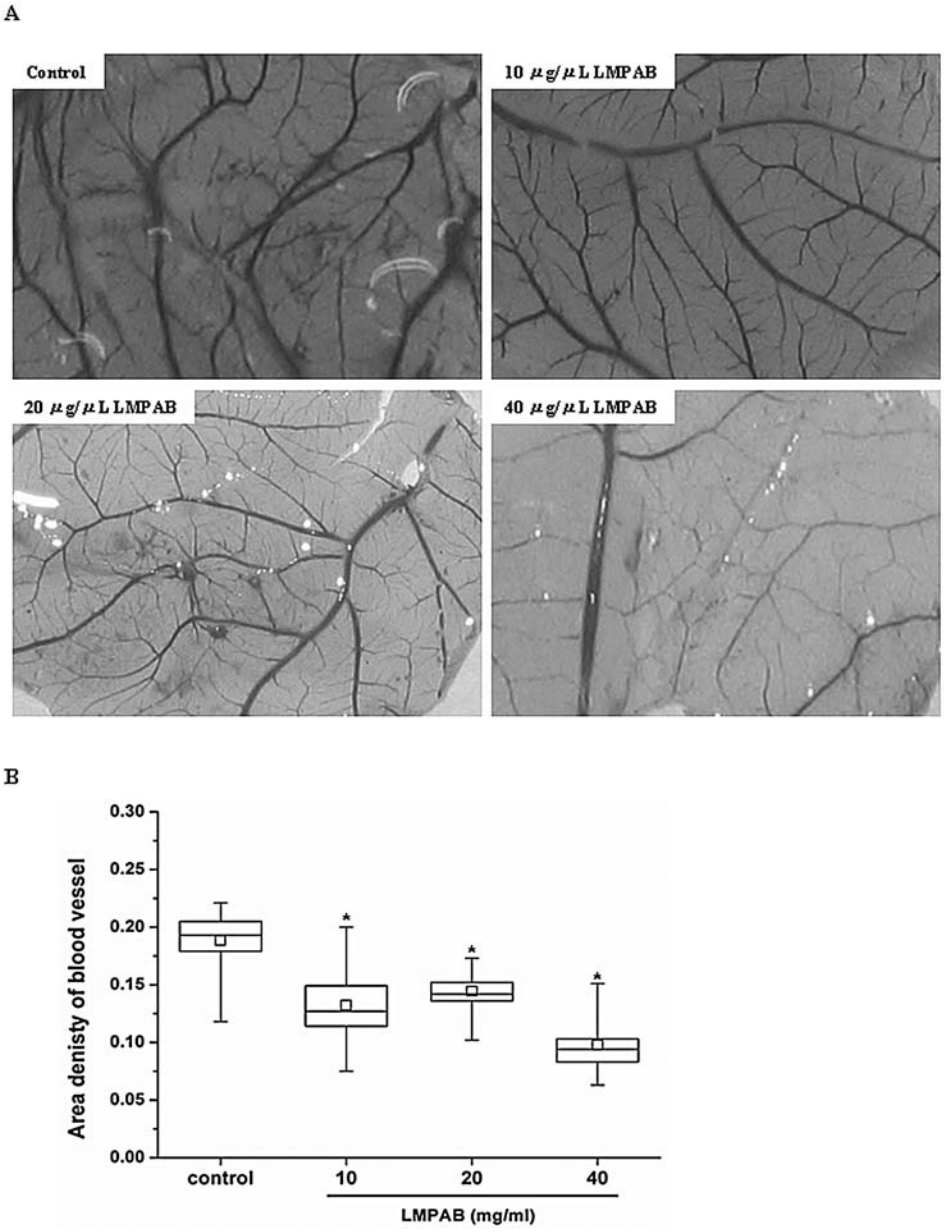

Figure 2. Effect of LMPAB on the blood vessel formation in CAM angiogenesis models. The CAM of 7-day-old chicken embryos was exposed to LMPAB (10, 20 or 40 μg/μl) for 72 h. (A) Photographs of the CAM were taken with a digital camera. (B) Average blood vessel density was analyzed by a digital medical image analysis system. Horizontal lines within boxes and error bars represent median and interquartile range, respectively. *P<0.01 versus blank control. These data are representative of three separate experiments.

the primary antibody as a negative control. Microvessels were counted on cross-sections of Matrigel under a light microscope. Three sections from each Matrigel were used to determine the average number of microvessels.

Immunohistochemical analysis. Tumor tissues were fixed in 4% buffered, freshly prepared paraformaldehyde, embedded in paraffin, and stained for routine histology. The sections were incubated at 4˚C overnight with anti-mouse VEGF mAb (Dako, Glostrup, Denmark). As secondary antibody, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Dako) was used for 30 min at 37˚C. After further washing with Tris-buffered saline, sections were incubated with complex/horseradish peroxidase (1:200 dilution) for 30 min at 37˚C. Immunolocalization was performed by immersion in 0.05% 3,3'-diaminobenzidine tetrahydrochloride as chromagen. Slides were counterstained with hematoxylin before dehydration and mounting. Incubation without the primary antibody was performed as a control for the background staining. Histological evaluation was performed by a pathologist who was blind to the pharmacological characteristics of the drugs and their postoperative outcomes.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from 100 mg of frozen tumor tissues using RNAiso reagent kit (Takara Biotechnology, Dalian, P.R. China) and cDNA was synthesized with SYBR ExScript™ RT-PCR kit (Takara Biotechnology). PCR was performed on cDNA with the use of 2X SYBR Premix Ex Taq polymerase, deoxynucleoside triphosphates,

ROX reverence dye and the corresponding primers in a total reaction volume of 50 μl. The following primers (18) were used: 5'-AAATGGTGAAGGTCGGTGTG-3' (sense) and 5'-TGAAGGGGTCGTTGATGG-3' (antisense) for the GAPDH gene; 5'-TTACTGCTGTACCTCCACC-3' (sense) and 5'-ACAGGACGGCTTGAAG-ATG-3' (antisense) for the VEGF gene. The PCR procedure was: 1 cycle of 95˚C for 10 sec, 40 cycles of 5 sec at 95˚C, 5 sec at 60˚C, 31 sec at 60˚C and a final 7 min at 72˚C. The relative levels of VEGF for each sample were calculated, as described previously (19). Fluorescence data were analyzed with reference to the standard curve in each experiment. Melting curve analysis was always included to certify amplification. Target gene expression was related to GAPDH mRNA for normalization. All tests were done in triplicate to ensure reproducibility.

Enzyme-linked immunosorbent assay (ELISA). Blood was collected directly from the mice at the time when they were sacrificed. Serum was separated by centrifugation within 1 h of blood collection and stored at -70˚C until analyzed. The levels of VEGF in serum were quantified by ELISA according to the manufacturer's protocol. Serum (100 μl) were used in each assay. Absorbance of the developed color was determined using a microplate reader at 492 nm. VEGF concentrations were extrapolated from the standard curves generated using recombinant human VEGF.

Statistical analysis. All determinations were repeated 3 times, and results are expressed as the mean ± SD. ANOVA was used to evaluate the difference among multiple groups followed by a post hoc test (Student-Newman-Keuls) when variable distributions were normal. Otherwise, the nonparametric Mann-Whitney U test was used. The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL), and $P<0.05$ was considered statistically significant.

Results

Inhibition of S180 xenograft growth in BALB/c mice by LMPAB. We examined whether treatment of BALB/c mice with LMPAB would inhibit S180 xenograft growth *in vivo*. The tumor weight was slightly reduced when the tumor-bearing mice were treated for 2 weeks with 50 or 100 mg/kg/day of LMPAB. When the concentration of LMPAB was increased to 200 mg/kg/day, however, the tumor weight was significantly reduced as well as FUP (Fig. 1A). Compared with control group, the tumor inhibitory rates in the group treated with LMPAB at the dose of 50, 100, and 200 mg/kg/day were 9.5, 23, and 32%, respectively (Fig. 1B).

Inhibition of angiogenesis in CAM by LMPAB. Next, we assessed whether LMPAB inhibits angiogenesis using a CAM model system. CAMs were exposed to filter-paper disks containing increasing concentrations of LMPAB (10-40 μg/μl) for 72 h. LMPAB treatment dramatically decreased newly sprouting angiogenic vessels and caused them to assume a 'branchless' pattern without any signs of hemorrhage and with no egg lethality (Fig. 2A). As compared with control the blood vessel density was significantly decreased in a dose-dependent manner (Fig. 2B).

Inhibition of Matrigel-induced neovascularization in C57BL/6 mice by LMPAB. We further assessed effect of LMPAB on the Matrigel-induced neovascularization in C57BL/6 mice. The gels formed by subcutaneous implantation of Matrigel alone were readily distinguished from surrounding tissue and produced little or no local reaction or angiogenic response. However, Matrigel supplemented with VEGF and heparin produced completed gels *in vivo* with an angiogenic reaction. The Matrigel/VEGF/heparin mixture significantly increased the weight of the gels and the blood vessel density in the gels compared with C57BL/6 mice treated with Matrigel alone. However, the weight of the gels and the blood vessel density in the gels were significantly decreased in the mice treated with LMPAB (0.1 and 0.2 μg/μl) (Fig. 3).

Down-regulation of protein and mRNA levels of VEGF in tumor tissues by LMPAB. To explore the underlying molecular mechanisms of LMPAB antitumor activity *in vivo*, we determined the effect of LMPAB on the VEGF protein expression in tumor tissues by immunohistochemical assay. VEGF was mainly expressed in the cytoplasm of tumor cells. The expression of VEGF in tumor tissues in BALB/c mice treated with LMPAB (50, 100, and 200 mg/kg) was significantly decreased in a dose-dependent manner (Fig. 4A).

Since LPMAB down-regulated VEGF protein expression in tumor tissues, we further determined the effect on LMPAB on the VEGF mRNA levels using quantitative real-time RT-PCR assay. The VEGF mRNA expression in tumor tissues was significantly deceased when the BALB/c mice were treated with LMPAB for 2 weeks compared with control (Fig. 4B).

Down-regulation of serum VEGF levels in BALB/c mice by LMPAB. We further determined the effect of LPMAB on serum VEGF levels in BALB/c mice by ELISA. On the 14th day, the VEGF levels in serum of tumor-bearing BALB/c mice without any treatment were highly elevated, compared with the levels in the normal control group. However, the VEGF levels in serum in tumor-bearing BALB/c mice received LMPAB treatment were significantly decreased (Fig. 5).

Discussion

Accumulating evidence suggests that low molecular weight polysaccharide extracts from *Agaricus blazei* are potential antitumor agents. However, the antitumor potentials of the low molecular weight polysaccharide extracts from *Agaricus blazei in vivo* and the underlying mechanism remain to be fully elucidated. In this study, we provide for the first time evidence demonstrating that LMPAB has antitumor and anti-angiogenic effects *in vivo*. We also provide evidence demonstrating that LMPAB has a down-regulative effect of VEGF, which play critical roles in tumor angiogenesis and tumor development. These findings suggest that LMPAB might be a promising agent for tumor therapy, and the antitumor and antiagiogenic effects of LMPAB may be related to down-regulation of VEGF.

In a previous study, we reported that LMPAB had a significant antiproliferative effect in leukemia cell line K562

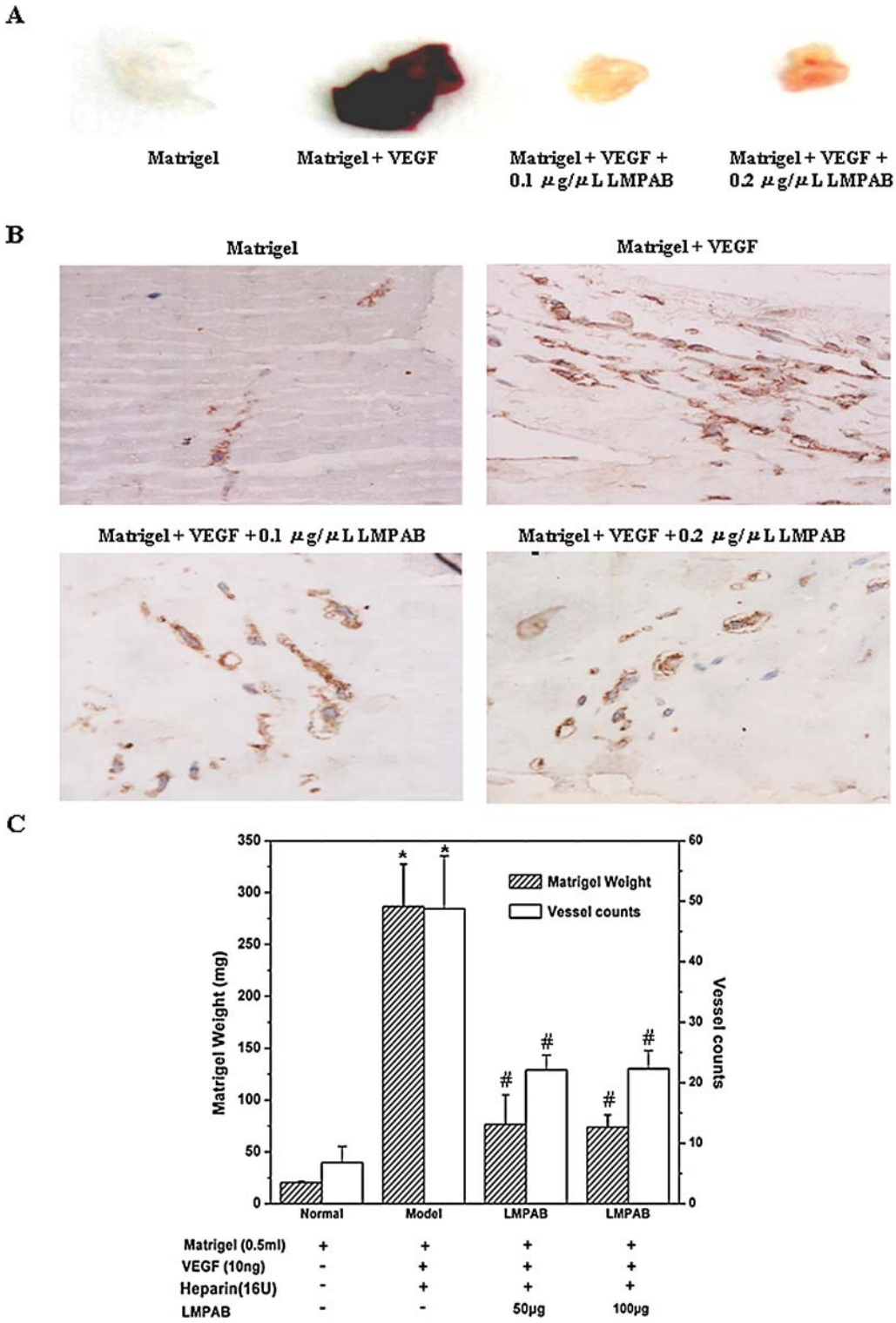

Figure 3. Effect of LMPAB on the neovascularization in Matrigel *in vivo*. C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel basement membrane containing VEGF (10 ng), heparin (16 U) in the presence or absence of LMPAB (50 or 100 μg). The mice were sacrificed on day 7, and the gels were removed, weighed, and prepared sections as described in Materials and methods. All microvessels in Matrigel were immunohistochemically stained using anti-CD34 mAb. (A) Matrigel weight was measured. (B) The number of microvessels in the sections was counted under the high-power field of a light microscope (original magnification, x400). (C) Open column represents the mean of vessel counts; striped column represents the mean of Matrigel weight (n=5); bars, SD; $^{*,\#}$P<0.05 versus model control.

at 1-10 μg/ml of concentrations *in vitro* (15). In the current study, we found the antitumor effect of LMPAB in mouse S180 xenograft models without toxicity to normal tissues *in vivo*. After administration of LMPAB at doses of 50, 100, and 200 mg/kg/day for 14 days, the tumor inhibitory rates in the S180 xenograft models were 9.7, 23.9, and 33.0%, respectively. These findings suggest that treatment using LMPAB is promising from a clinical perspective.

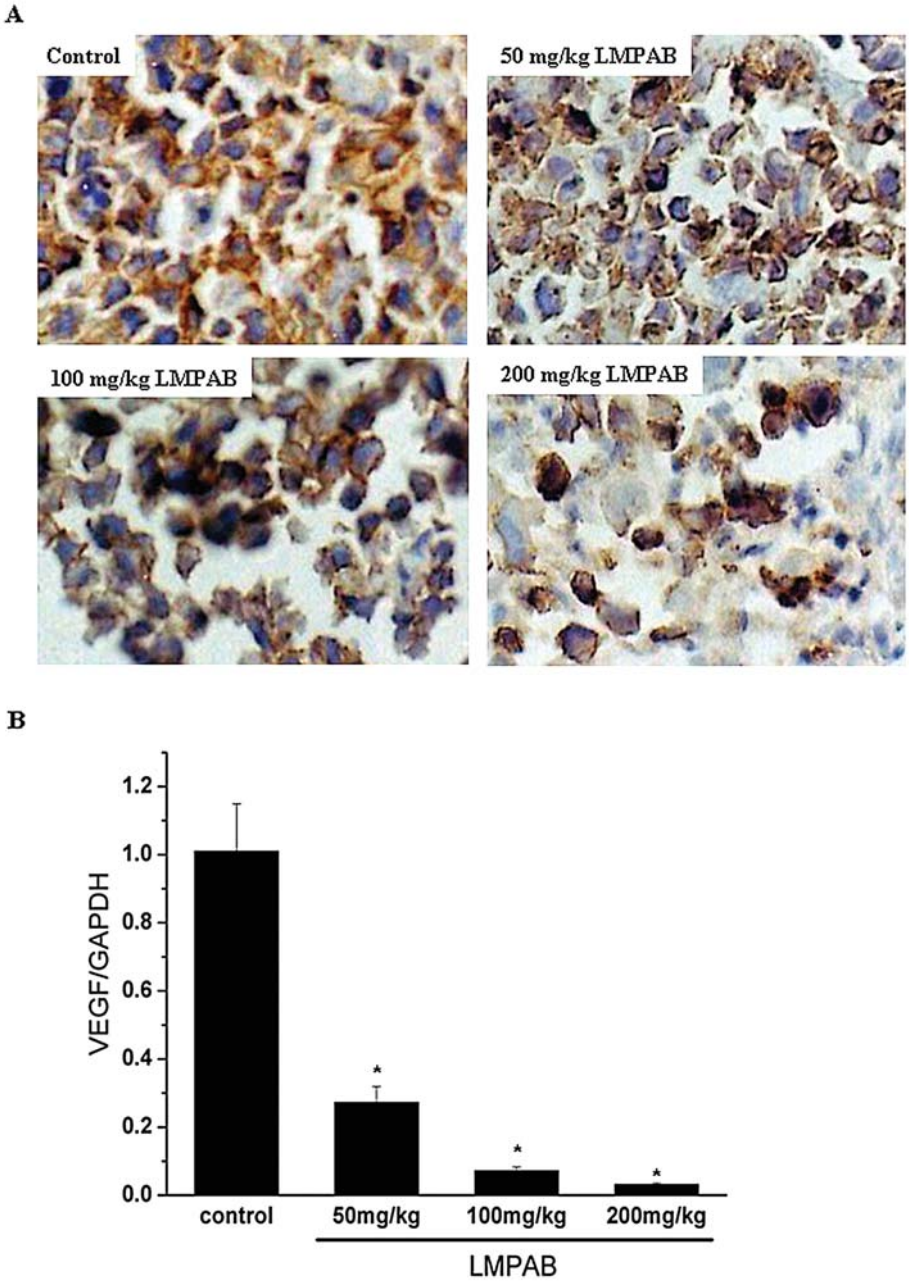

Figure 4. Effect of LMPAB on the protein and mRNA levels of VEGF in tumor tissues. (A) Paraffin sections of tumor tissues derived from BALB/c mice were immunohistochemically stained for VEGF using anti-mouse VEGF mAb. These figures are representative of three different experiments (magnification, x400). B, Total RNA was isolated from tumor tissues using RNAiso reagent and used for cDNA synthesis. The mRNA levels of VEGF were detected by real-time RT-PCR. *P<0.05 versus control. These data are representative of three separate experiments.

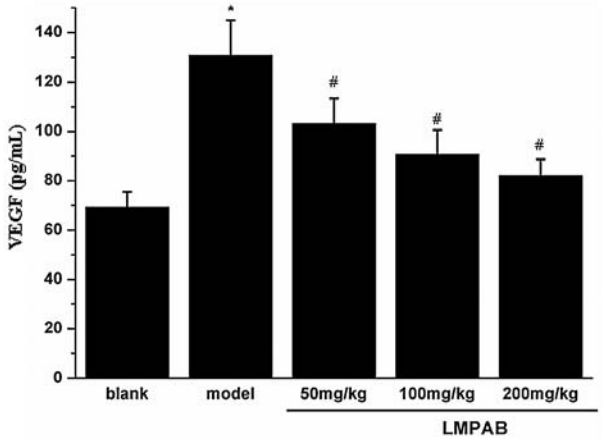

Figure 5. Effect of LMPAB on the serum VEGF levels in BALB/c mice. Serum was separated from blood collected from the mice by centrifugation and used for ELISA of VEGF. The mean and SD of each group are shown. *P<0.05 versus blank control; #P<0.05 versus model control. These data are representative of three separate experiments.

Angiogenesis is required for tumor growth and the tumor cannot grow >1-2 mm in diameter without new blood vessel formation (20). It is well demonstrated that an increase in tumor growth must be preceded by an increase in the microvasculature that supplies the neoplasm (21). The inhibition of tumor angiogenesis is an important strategy for cancer therapy (22). Therefore, the development of antiangio-

genetic reagents has become a new attractive strategy to combat cancer.

CAM angiogenesis model is particularly convenient due to its hierarchical organization, optical transparency, and availability to *in vivo* imaging techniques for anatomical characterization. CAM assay is a well-established model of angiogenesis that can be used to test pro- and anti-angiogenic conditions and molecules (23). The allantois is an extra-embryonic membrane, composed of endoderm and mesoderm, in which primitive blood vessels begin to take shape on day 3 of incubation. Primitive vessels continue to proliferate into an arteriovenous system until day 8, and the vascular system attains its final arrangement on day 18, just before hatching. The main function of CAM is to mediate gas exchanges with the outer environment. The CAM model has several advantages over mammalian models. The vascular system of CAM is directly accessible to observation and experimentation, and there are no metabolic or hormonal influences from the mother. In addition, it is a more physiological model than *in vitro* models because vascularization of CAM is subject to regulations through fluxes, pressure, shear stress, growth factors, and so on. Furthermore, in contrast to mammalian models of embryonic development, there is no external (i.e., maternal) influence (24).

To our knowledge, this is the first study showing that LMPAB has an antiangiogenic activity *in vivo*. In this study, we used the CAM model to study the direct effects of LMPAB on physiological embryonic angiogenesis. The results obtained demonstrated that the angiogenesis was significantly suppressed by LMPAB in a dose-dependent manner. We further observed the inhibitory effects of LMPAB on Matrigel-induced neovascularization using an *in vivo* model. LMPAB significantly inhibited the Matrigel-induced neovascularization in C57BL/6 mice. These findings indicate that the antitumor activity of LMPAB may be due to direct inhibition of angiogenesis.

The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by both tumor and normal cells (25). VEGF is an angiogenic factor that plays a pervasive role in sustaining the growth of many solid tumors. Inhibition of VEGF production is a promising therapeutic approach for cancer (26). Hypoxia, growth factors and cytokines appears to be important stimulus for VEGF production in both malignant and normal cells (27). VEGF plays a role in endothelial sprouting, increasing vascular permeability and the expression of tissue matrix metalloproteinases, and the digestion of matrix, which is required for the endothelial cell to move. The increased endothelial cell mitogenesis and spread and activation of other factors lead to the formation and movement of endothelial cells, including other supporting cells such as pericytes, and eventually lead to vessel extension, increased capillary integrity, differentiation of microvessel support cells, and formation of the vascular network (28). Many studies have shown a significant correlation between VEGF expression and vessel density in several malignancies including breast, liver, pancreas, colon, and gastric carcinoma (29-33). Recent evidence suggests that VEGF may not only play a role in inducing angiogenesis but also is important in promoting the survival of new vessels formed in tumors (34). The importance of VEGF as a potential target for antineoplastic therapy has been demonstrated in several studies in which neutralizing antibodies to VEGF inhibited tumor growth and vascularization *in vivo* (35).

The present study showed that LMPAB down-regulated both the mRNA and protein levels of VEGF expression in tumor tissues. Furthermore, we found that VEGF levels in serum of tumor-bearing BALB/c mice without any treatment were significantly higher than in the normal control group. Interestingly, the VEGF levels in serum of tumor-bearing BALB/c mice were significantly down-regulated after LMPAB treatment in a manner similar to tumor inhibition. These findings imply that LMPAB might inhibit tumor growth and angiogenesis through down-regulation of VEGF expression *in vivo*. Further study is needed to clarify whether LMPAB induce cytotoxity and antiangiogenetic activity by down-regulating VEGF expression in other type of tumors.

In summary, we demonstrated that LMPAB has significant antitumor and antiangiogenic effects *in vivo*. LMPAB also has a down-regulating effect on VEGF expression in tumor tissues. Furthermore, serum VEGF levels in the tumor-bearing BALB/c mice were significantly down-regulated by LMPAB treatment. Our results provide new insights into LMPAB for tumor treatment and highlight its potential as anticancer agent.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 30772751) and of the Heilongjiang province (No. D200674).

References

1. Chang R: Bioactive polysaccharides from traditional Chinese medicine herbs as anticancer adjuvants. J Altern Complement Med 8: 559-565, 2002.
2. Kim GY, Lee MY, Lee HJ, *et al*: Effect of water-soluble proteoglycan isolated from *Agaricus blazei* on the maturation of murine bone marrow-derived dendritic cells. Int Immunopharmacol 5: 1523-1532, 2005.
3. Kaneno R, Fontanari LM, Santos SA, Di Stasi LC, Rodrigues Filho E and Eira AF: Effects of extracts from Brazilian sun-mushroom (*Agaricus blazei*) on the NK activity and lymphoproliferative responsiveness of Ehrlich tumor-bearing mice. Food Chem Toxicol 42: 909-916, 2004.
4. Barbisan LF, Scolastici C, Miyamoto M, *et al*: Effects of crude extracts of *Agaricus blazei* on DNA damage and on rat liver carcinogenesis induced by diethylnitrosamine. Genet Mol Res 2: 295-308, 2003.
5. Pinheiro F, Faria RR, de Camargo JL, Spinardi-Barbisan AL, da Eira AF and Barbisan LF: Chemoprevention of preneoplastic liver foci development by dietary mushroom *Agaricus blazei* Murrill in the rat. Food Chem Toxicol 41: 1543-1550, 2003.
6. Kimura Y, Kido T, Takaku T, Sumiyoshi M and Baba K: Isolation of an anti-angiogenic substance from *Agaricus blazei* Murill: its antitumor and antimetastatic actions. Cancer Sci 95: 758-764, 2004.
7. Murakawa K, Fukunaga K, Tanouchi M, Hosokawa M, Hossain Z and Takahashi K: Therapy of myeloma in vivo using marine phospholipid in combination with *Agaricus blazei* Murill as an immune respond activator. J Oleo Sci 56: 179-188, 2007.
8. Firenzuoli F, Gori L and Lombardo G: The medicinal mushroom *Agaricus blazei* Murrill: Review of literature and pharmaco-toxicological problems. Evid Based Complement Alternat Med 5: 3-15, 2008.
9. Ahn WS, Kim DJ, Chae GT, *et al*: Natural killer cell activity and quality of life were improved consumption of a mushroom extract, *Agaricus blazei* Murill Kyowa, in gynecological cancer patients undergoing chemotherapy. Int J Gynecol Cancer 14: 589-594, 2004.

10. Fontanini G, Lucchi M, Vignati S, *et al*: Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. J Natl Cancer Inst 89: 881-886, 1997.
11. Zhao X, Mohaupt M, Jiang J, Liu S, Li B and Qin Z: Tumor necrosis factor receptor 2-mediated tumor suppression is nitric oxide dependent and involves angiostasis. Cancer Res 67: 4443-4450, 2007.
12. Yan RL, Qian XH, Xin XY, *et al*: Experimental study of anti-VEGF hairpin ribozyme gene inhibiting expression of VEGF and proliferation of ovarian cancer cells. Ai Zheng 21: 39-44, 2002.
13 Barr MP, Bouchier-Hayes DJ and Harmey JJ: Vascular endothelial growth factor is an autocrine survival factor for breast tumour cells under hypoxia. Int J Oncol 32: 41-48, 2008.
14. Chesler L, Goldenberg DD, Seales IT, *et al*: Malignant progression and blockade of angiogenesis in a murine transgenic model of neuroblastoma. Cancer Res 67: 9435-9442, 2007.
15. Liu JC and Sun JN: Isolation and antitumor activity of low-molecular-weight polysaccharides from *Agaricus blazei* Murill. China J Tradit Chinese Med Phar 21: 190-193, 2006.
16. Richardson M and Singh G: Observations on the use of the avian chorioallantoic membrane (CAM) model in investigations into angiogenesis: Curr Drug Targets Cardiovasc Haematol Disord 3: 155-185, 2003.
17. Takaku T, Kimura Y and Okuda H: Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. J Nutr 131: 1409-1413, 2001.
18. Simpson DA, Feeney S, Boyle C and Stitt AW: Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. Mol Vis 5: 178-183, 2000.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
20. Lim M, Cheshier S and Steinberg GK: New vessel formation in the central nervous system during tumor growth, vascular malformations, and Moyamoya. Curr Neurovasc Res 3: 237-245, 2006.
21. Gevertz JL and Torquato S: Modeling the effects of vasculature evolution on early brain tumor growth. J Theor Biol 243: 517-531, 2006.
22. Cascone T, Troiani T, Morelli MP, Gridelli C and Ciardiello F: Antiangiogenic drugs in non-small cell lung cancer treatment. Curr Opin Oncol 18: 151-155, 2006.
23. Huan-huan C, Li-Li Y and Shang-Bin L: Artesunate reduces chicken chorioallantoic membrane neovascularisation and exhibits antiangiogenic and apoptotic activity on human microvascular dermal endothelial cell. Cancer Lett 211: 163-173, 2004.
24. Richardson M, Liu L, Dunphy L, *et al*: Viral serpin, Serp-1, inhibits endogenous angiogenesis in the chicken chorioallantoic membrane model. Cardiovasc Pathol 16: 191-202, 2007.
25. Kaygusuz G, Tulunay O, Baltaci S and Gogus O: Microvessel density and regulators of angiogenesis in malignant and non-malignant prostate tissue. Int Urol Nephrol 39: 841-850, 2007.
26. Wedge SR, Kendrew J, Hennequin LF, *et al*: AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. Cancer Res 65: 4389-4400, 2005.
27. Nakamura M, Yamabe H, Osawa H, *et al*: Hypoxic conditions stimulate the production of angiogenin and vascular endothelial growth factor by human renal proximal tubular epithelial cells in culture. Nephrol Dial Transplant 21: 1489-1495, 2006.
28. Zheng H, Takahashi H, Murai Y, *et al*: Expressions of MMP-2, MMP-9 and VEGF are closely linked to growth, invasion, metastasis and angiogenesis of gastric carcinoma. Anticancer Res 26: 3579-3583, 2006.
29. Quaranta M, Daniele A, Coviello M, *et al*: MMP-2, MMP-9, VEGF and CA 15.3 in breast cancer. Anticancer Res 27: 3593-3600, 2007.
30. Mathonnet M, Descottes B, Valleix D, Labrousse F and Denizot Y: VEGF in hepatocellular carcinoma and surrounding cirrhotic liver tissues. World J Gastroenterol 12: 830-831, 2006.
31. Morales A, Vilchis F, Chávez B, Chan C, Robles-Díaz G and Díaz-Sánchez V: Expression and localization of endocrine gland-derived vascular endothelial growth factor (EG-VEGF) in human pancreas and pancreatic adenocarcinoma. J Steroid Biochem Mol Biol 107: 37-41, 2007.
32. Doger FK, Meteoglu I, Tuncyurek P, Okyay P and Cevikel H: Does the EGFR and VEGF expression predict the prognosis in colon cancer? Eur Surg Res 38: 540-544, 2006.
33. Gao ZL, Zhang C, Du GY and Lu ZJ: Clinical significance of changes in tumor markers, extracellular matrix, MMP-9 and VEGF in patients with gastric carcinoma. Hepatogastroenterology 54: 1591-1595, 2007.
34. Yamagishi S, Abe R, Inagaki Y, *et al*: Minodronate, a newly developed nitrogen-containing bisphosphonate, suppresses melanoma growth and improves survival in nude mice by blocking vascular endothelial growth factor signaling. Am J Pathol 165: 1865-1874, 2004.
35. Jendreyko N, Popkov M, Rader C and Barbas CF III: Phenotypic knockout of VEGF-R2 and Tie-2 with an intradiabody reduces tumor growth and angiogenesis in vivo. Proc Natl Acad Sci USA 102: 8293-8298, 2005.