Research Paper

Albizia julibrissin extract inhibits tumor growth in association with anti-angiogenesis in vitro and in vivo

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ABSTRACT

Angiogenesis plays a vital role in tumor progression. Therefore, inhibiting tumor vasculature could restrict tumor growth and metastases. Albizia julibrissin extract (AJBE), which is a traditional medicine, has shown antitumor effects. However, the relationship between tumor and angiogenesis is still not fully understood. In this study, the anti-angiogenesis of AJBE in vitro and in vivo was investigated and confirmed. In vitro, AJBE remarkably suppressed basic fibroblast growth factor (bFGF)-induced proliferation and migration and tube formation of HMEC-1 with \( IC_{50} \) of 3.36 ± 0.19 μg/mL. In vivo, AJBE delivered by daily oral administration significantly inhibited tumor-induced angiogenesis and tumor growth in an intradermal inoculation mouse model. This study indicated that AJBE could be a potent anti-tumor drug, which exerts its anti-tumor effect through anti-angiogenic action.

Key words: AJBE, tumor vasculature, human microvascular endothelial cell, antitumor, anti-angiogenesis

INTRODUCTION

Albizia julibrissin, a traditional plant widely distributed in China, has been widely used in the treatments of some disease, such as inflammation, confusion, insomnia and diuresis (Peng et al., 2007). The main components of A. julibrissin bark are saponins, lignans, phenolic glycosides, triterpenes and others. One of these chemical components in the bark of A. julibrissin is triterpenoid saponin, which is known to possess a cytotoxic activity toward KB cancer cells (Zou et al., 2000). Won et al. (2006) demonstrated that saponins from A. julibrissin extract induced apoptosis in human acute leukemia Jurkat T cells. However, the relevant mechanism of anticancer activity of A. julibrissin extract has not been thoroughly studied.

A large number of researches show that endothelial cells play an important role in angiogenic process, including the degradation of extracellular matrix, migration, proliferation, tube formation and maturation (Folkman, 2006, 2007). However, the tumor’s rapid growth relies on the nutrients and oxygen provided by blood vessec (Carmeliet et al., 2000). Some reports suggested that angiogenesis is a key process for solid tumor growth and metastasis (Wang et al., 2012). So inhibiting tumor vasculature is a critical strategy and an attention of growing interest in cancer therapeutics.

Evidence now suggests that tumor vessels play an important role in tumorigenesis, tumor proliferation, angiogenesis and metastasis (Kuniaki et al, 2014). Angiogenesis involves many angiogenic molecules, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and so on. VEGF and its receptor seem, at least in part, to contribute to the difference in the tumor architecture (Kazunari et al, 1998). bFGF is known to be involved in various biological processes, such as limb and
nervous system development, wound healing and tumor growth (Im et al, 2009). It is suggested that bFGF acts as a potent angiogenic stimulator for tumor cells and as a potent angiogenic agent that fosters the growth of new blood vessels into tumors (Gospodarowicz et al, 1989). bFGF induces angiogenesis by stimulating migration, proliferation and tube formation of endothelial cells (Gotink et al, 2010). Some agents target proliferation regulators that are involved in cell proliferation (Ikuo et al, 2013).

Therefore, the inhibitory activity of AJBE with bFGF-induced angiogenesis responses in some respects of proliferation, migration and tube formation of HMEC-1 was assessed and confirmed. The impact of AJBE on inhibiting HMEC-1 proliferation in terms of cell cycle arrest and apoptosis was also tested. In vivo, the effects of angiogenesis induced by H22 and the growth of tumor in nude mice model were investigated. This study showed AJBE possesses antitumor effect through anti-angiogenesis.

MATERIALS AND METHODS

Materials

The dried A. julibrissin bark was collected from Jiangsu Province of China and was identified by Professor Chen Jianwei, who worked in Nanjing University of Chinese Medicine. HMEC-1 was obtained from the Health and Medicine Research of French National Institute. Hepatic carcinoma cells (H22) were purchased from Pharmaceutical Research Department of Shanghai Medical and Technology Institute (China). MCDB-131, rhodamine B, bFGF, FBS and trypsin were purchased from Sigma (USA). CD31 monoclonal antibody was purchased from Wuhan Boster Company (China). Masson dye came from Nanjing Jingcheng technologic company (China). Cell cycle kit was purchased from KeyGEN (Nanjing, China).

Preparation of A. julibrissin extract

A. julibrissin (1 kg) were grounded with a crushing machine to pass 0.3 mm screen and were extracted with 70% ethanol for twice. The 70% ethanol was collected and evaporated. Then it was dissolved in water and extracted with n-butanol solution. The n-butanol solution was concentrated and residue (31.6 g) was obtained eventually. The residue was labeled as AJBE and stored in refrigerator until use. The content of total triterpenoid saponins in AJBE was 63.49% (Feng, 2009).

bFGF-induced cell growth inhibition assay

Effects of AJBE on HEMC-1 growth inhibition were measured by the SRB assay. Briefly, adherent cells were seeded into each cell of 96 plates and were cultured at 37°C in 5% CO2 saturated humidity condition for 24 h, then incubated with different concentrations of AJBE for 48 h. After the culture medium (10% FBS, 10 ng/ml bFGF) was removed and 200 µl TCA solution was added into each well at 4°C for 40 min, mixture was cleaned up and dried up at 37°C, then 100 µl SRB was added into each well for 20 min. After washing them away, 150 µl Tris solution was added into each well. The mixture was shaken and measured at 450 nm. The average level was calculated by the inhibition rate formula: Percent inhibition = (High signal-test well signal) / (High signal-Low signal) ×100%. IC50 was considered to be the concentration which caused 50% inhibition of cell proliferation.

Cell migration assay

Briefly, HMEC-1 (8×103) were seeded onto per well and were cultured at 37°C in a saturated humidity containing 5% CO2 for 24 h. When the cells have attached completely, the middle of the cell plate was scraped with a line about 1 mm width, and then changed to MCD medium containing 10% FBS, 10 ng/ml bFGF and two different concentrations of AJBE (2.5 and 5 µg/ml). The cells were incubated and randomly chosen fields were photographed at 100× amplification under a microscope video system (Olympus, IX70, Japan). The width of each gap in three different places were measured and averaged.

Capillary tube formation assay

The capillary tube formation assay was performed as described previously (Min, 2004). Briefly, the 6-well plate was coated with 0.15 ml Matrigel, and the Material was allowed to solidify and polymerize at 37°C for 30 min. Every well was seeded with 5×105 HMEC-1 and incubated in MCD medium containing 10% FBS, 10 ng/ml bFGF and different concentration concentrations of AJBE (2.5 and 5 µg/ml) for 24 h. Then the tubular structures were photographed from five randomly chosen fields by microscope (Olympus, IX70, Japan).

The cell cycle progression by flow cytometric analysis

The cell cycle progression of HMEC-1 following AJBE treatment was analyzed by Flow Cytometer. Briefly, 1×10⁶ cells were suspended in 100 µl PBS, and 500 µl cold 70% ethanol was added. The cells were incubated at 4°C overnight, then washed with PBS twice, and 100 µL of RNase A was added at 37°C for 30 min before staining of the cellular DNA with 400 µl Propidium Iodide (PI) for 30 min at 4°C. The distribution of HMCE-1 was analyzed by Cell Quest software.
Detection of apoptosis by flow cytometric analysis

HMCE-1 samples were prepared for analysis according to the instruction of Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, Nanjing, China). The data were analyzed by CellQuest software. For each analysis, 10,000 events were recorded.

Hoechst staining

In the 6-well plate with cover slips, after $5 \times 10^4$ HMEC-1 were seeded onto each well and cultured for 24 h, AJBE was added into each well and cultured for 48 h. After throwing the culture medium, the cells were fixed with 0.5 ml 4% paraformaldehyde, and then washed with PBS twice. After treatment with Hoechst dyes (Wuhan Boster, China) for 5 min, the cells were rinsed with PBS twice. The stained cells were immediately observed under fluorescence microscope (Olympus, IX70, Japan).

Establishment of mouse model and treatment

This experiment was conducted in accordance with the guideline issued by the State Food and Drug Administration (SFDA, China). Male C57BL/6 mice were anesthetized with 5 mg/kg sodium pentobarbital and then were injected with $10^7$ H22 cells into the right axilla subcutaneously. In addition, mice were divided into 4 groups (8 mice per group) randomly, and three groups were treated with oral AJBE at the dose of 2, 4 and 8 mg/kg, respectively. The fourth group was treated with saline. Two weeks later, the mice were sacrificed, and the solid tumors were removed and weighted. Tumor size was measured in two perpendicular dimensions with Vernier Calipers and converted to tumor volume (TV) using the formula: $(ab^2)/2$, where $a$ and $b$ referred to the length and width, respectively.

Immunohistochemistry

Tumor tissue section of the same volume was put onto treated slides. According to sequences, sections were fixed, deparaffinized and rehydrated to the distilled water. Tissues were boiled in 2% citrate buffer at 95°C for 20 min, then treated with 3% hydrogen peroxide in order to block the activity of endogenous peroxidase. The slides were incubated with protein-blocking agent, then treated with the primary antibodies including CD31, P-bFGFR1 and P-Akt at 4°C overnight. The tissues were then incubated with the secondary biotinylated antibodies and finally stained with DAB (diaminobenzidine) according to the manufacturer’s instructions (Eno Gene). Counter staining was dealt with hematoxylin. Microvascular density (MVD) was estimated by Weidner (Weidner, 1995). p-bFGFR1 and p-Akt expressions were determined using Image-Pro Plus software (Media Cybernetics, America)

Statistical analysis

The results were given by mean±SD. Statistical analysis was performed by ANOVA/Dunnet t-test for multiple group comparisons and Student’s t-test for two group comparisons. $P <0.05$ was considered as statistically significant.

RESULTS

The cell inhibition

The effect of AJBE on the HMEC-1 was evaluated. The results showed that AJBE caused a decrease on the HMEC-1 growth. The IC$_{50}$ of AJBE was estimated to be 3.36 ± 0.19 μg/ml.

The AJBE inhibit the HMEC-1 migration and tube formation

According to Figure 1A-B, the HMEC-1 migration with the treatment of AJBE was observed. It showed that the AJBE could inhibit bFGF-induced migration significantly treated with the concentrations of 2.5 and 5 μg/ml for 24 h. As shown in Figure 2A-B, the control group formed an enclosed and robust network of tubes, and bFGF stimulated the angiogenic differentiation of HMEC-1(tube formation) on Matrigel. However, AJBE effectively inhibited the tube formation induced by bFGF at the doses of 2.5 and 5 μg/ml at 24 h.

The results of the flow cytometry

A flow cytometry analysis was applied to detect the effect of AJBE on cell cycle. It was found that AJBE reduced cell proliferation through the inhibition of cell cycle and induced apoptosis. Figure 3A showed the flow cytometric distribution of HMEC-1 treated with AJBE for 48 h. The 3D histograms showed the comparison of groups with AJBE treatment and control group (Figure 3B). Quantitative analysis of flow cytometric data showed that AJBE caused cell cycle arrest in S-phase (Table 1), where the proportion of cells was increased.

Induction of apoptosis in HMEC-1

Induction of apoptosis was measured by annexin V and
Figure 1. The effects of the AJBE on migration and tube formation of HMEC-1. A HMEC-1 were cultured for 24 h with different concentrations of AJBE. (a) control; (b) control +bFGF; (c) AJBE, 2.5 µg/ml; (d) AJBE, 5 µg/ml. B The migration degree was analyzed by averaging width of each gap in three places. # $P<0.05$, ## $P<0.01$ compared with bFGF-untreated control; * $P<0.05$, ** $P<0.01$ compared with bFGF-treated control.

Figure 2. The effects of the AJBE on tube formation of HMEC-1. A The tube formation picture of HMEC-1 with AJBE treatment at various concentrations for 24 h. (a) control; (b) control+bFGF; (c)AJBE, 2.5 µg/ml; (d)AJBE, 5 µg/ml B shows quantitation of tube formation on HMEC-1 with the treatment of AJBE at the concentrations of 2.5 and 5 µg/ml # $P<0.05$, ## $P<0.01$ compared with bFGF-untreated control; * $P<0.05$, ** $P<0.01$ compared with bFGF-treated control.

propidium iodide (PI) double staining. The flow cytometric analysis of AJBE at 2.5 and 5 µg/ml showed an increase in the HMEC-1 apoptosis at 24 h. The ratio of prophase and late apoptosis reached (4.10 ± 1.02) % and (6.86 ± 0.89) % respectively with AJBE (5 µg/ml). Whereas the treatment of HMEC-1 with AJBE at 5 µg/ml for 48 h resulted in (9.34 ± 0.96) % of prophase apoptosis and (9.73 ± 2.05) % of late apoptosis (Figure 3C).

AJBE induce HEMC-1 apoptosis analysis by Hoechst staining

In order to further investigate the condition of AJBE on inducing apoptosis, Hoechst staining was applied to detect the effect. As shown in Figure 3D, the nuclei appeared to be balanced blue without bright apparent spots in the control group. It indicated that the control group exhibited no DNA damage. However, with the treatment of AJBE, it showed
Figure 3. AJBE arrests HMEC-1 cycle and induces apoptosis. HMEC-1 were incubated with AJBE for 48 h. Both floating and adherent cells were collected and analyzed by Flow cytometry. A Flow cytometry data of HMEC-1 treated with AJBE and control group. (a) Control group; (b) AJBE, 2.5 μg/ml; (c) AJBE, 5 μg/ml. B 3D view histograms showed the comparison of groups with AJBE treatment and control group. (a) Control group; (b) AJBE, 2.5 μg/ml; (c) AJBE, 5 μg/ml. M1: apoptosis M2:G0/G1 M3:S M4: G2/M. C HMEC-1 were cultured for the indicated times before staining with FITC-annexin V and PI and analyzed by flow cytometry. The percentage of FITC-annexin V+/PI- and FITC-annexin V+/PI+ HMEC-1 was shown. D Apoptotic bodies were stained with Hoechst solution and observed under a fluorescent microscope using a blue filter. Apoptotic bodies are shown by white arrows.
there were many bright blue spots in smaller size or cell fragmentations obviously, which referred to be cell damage.

**AJBE inhibit the tumor growth and angiogenesis**

To examine whether antiangiogenic function of AJBE could exhibit response to the anti-tumor activity in vivo, H22 allografts were implanted subcutaneously into C57BL/6 mice with oral AJBE every day for 14 days. Tumors grew rapidly and reached an average volume of 400 mm³ on 14th day. However, compared with saline-treated control group, the tumor volumes and weights were remarkably inhibited with the treatment of AJBE at the concentrations of 4 and 8 mg/kg (Figure 4A-C).

To further examine whether AJBE could inhibit H22 tumor growth by anti-angiogenesis, tumor tissues were stained with antibodies against CD31, P-bFGFR1 and P-Akt (Figure 4D). The antiangiogenic effect was measured by the microvascular density (MVD). Compared with controls, the MVD in tumors with the AJBE treatment at the concentration of 8 mg/kg showed a remarkable reduction (Figure 4E). In addition, activation of FGFR1 and its downstream signaling molecule, Akt, has been verified to have the correlation with angiogenesis and tumor growth (Sun et al., 2013). It was found that AJBE treatment could obviously down-regulate expressions of P-bFGFR1 and P-Akt of tumor vessels (Figure 4E), further indicating that AJBE could suppress angiogenesis probably at least through bFGFR1 signaling pathways.

**DISCUSSION**

Angiogenesis plays very important roles in biological processes, including the endothelial proliferation, migration, tube formation and survival (Goñi et al., 2010). Furthermore, there is evidence that the growth and metastasis of tumor is directly related to angiogenesis (Sathornsumetee et al., 2007). Angiogenesis is an attractive target for novel anti-cancer therapies or cancer chemoprevention because of the advantages that it may offer, including better agent accessibility to endothelial cells than to tumor cells, independence of tumor cell drug resistance mechanisms and broad applicability to many tumor types (Tosetti et al., 2002). Using natural medicine to treat various diseases is a long tradition in China. This study focused on exploring the potential antiangiogenic compounds from the traditional medicine.

This study focused on inhibitory effects of AJBE on HMCE-1 proliferation, migration and tube formation, which were key characteristics of endothelial cells in angiogenesis. In previous study, compared with AJBE, water extract from *A. Julibrissin* exerted lower inhibition on HMCE-1 proliferation (IC₅₀=30 μg/ml). AJBE suppressed HMCE-1 proliferation and IC₅₀ value was 3.36±0.19 μg/ml In this study, AJBE inhibited the HMEC-1 migration significantly, which is an important and early event in angiogenic process. Tube formation assay is a widely used model in order to test the angiogenic activity (Goodwin et al., 2007). Further studies using flow cytometric analysis implied that AJBE (2.5 and 5 μg/ml) inhibited cell proliferation via causing HMEC-1 cycle arrest at S-phase to suppress cell cycle progression.

Apoptosis is the process of programmed cell death (PCD). Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that can be engulfed and quickly removed by phagocytic cells before the contents of the cell can spill towards surrounding cells and cause damage (Albers et al., 2008). Annexin V/PI double staining showed that AJBE could induce apoptosis of HMCE-1 both at 24 and 48 h, and there was an increased ratio of prophase and late apoptosis at a dose-dependent manner. The cell-permeable DNA dye Hoechst 33342 was also used to further detect the apoptosis. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation or fragmentations was indicative of apoptosis (Gschwind et al., 1995; Guan et al., 1995). Based on these observations, it was shown that AJBE could induce HMEC-1 apoptosis.

In order to investigate the effect of AJBE on inhibiting the angiogenesis of tumor, the mouse models transplanted with hepatic carcinoma was used. By intragastric administration of AJBE per day, the tumor size, volume and weight had remarkably decreased compared with control group. Microvascular density (MVD) is a marker to assess the level of tumor angiogenesis, and an increase of MVD in tumor tissue suggests a fast-growing and potentially more metastatic tumor (Liu et al., 2011). After treatment with AJBE *in vivo*, its inhibitory effect against the growth of H22 allograft was correlated with decreased MVD. The MVD has been dramatically decreased at the concentrations of 4 and 8 mg/kg each day.

bFGF acts as a potent angiogenic agent that exerts the effect on cells through transmembrane receptor with tyrosine kinase activity, which in turn activate a variety of intracellular signaling cascades to regulate cellular functions. PI3K and its downstream activated serine/threonine kinase Akt are involved in several processes of angiogenesis, including endothelial cell migration, proliferation and survival (Pratsinis et al., 2007; Kowanet et al., 2006). According to block FGFR1 phosphorylation, AJBE inhibited the Akt signaling pathways. It is noteworthy that AJBE was potent to tumor treatment correlated with anti-angiogenesis.

In summary, the AJBE inhibited the angiogenesis *in vitro* and *in vivo*. Based on the results, AJBE might be used as a potent antiangiogenic and anticancer drug. In addition, research is continued.

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Figure 4. Effects of AJBE on cancer-induced angiogenesis. A. Tumor inoculated sites were dissected from control and AJBE-treated mice (Day 14 after the tumor inoculation). The picture showed the appearance of tumors dissected. B. Tumor volume of control and AJBE-treated groups. C. The weight of tumors from control and AJBE treated groups. D. Inhibition of AJBE on the angiogenesis of transplantable tumor was evaluated by microvascular density (MVD), p-bFGFR1 and p-Akt expression. E. Compared with the control group, MVD, p-bFGFR1 and p-Akt expression were lower with the treatment of AJBE. * P<0.05, **P<0.01 compared with control group.
REFERENCES


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