Synergistic effect of baculovirus-mediated endostatin and angiostatin combined with gemcitabine in hepatocellular carcinoma

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ABSTRACT

Anti-angiogenic gene therapy is a promising strategy in treating cancer. Endostatin and angiostatin are widely used in tumor anti-angiogenesis therapy. Our previous studies have shown that the BDS-hEA, a baculovirus long-term expressing the fusion protein of human endostatin and angiostatin, has a favorable effect in inhibiting the growth and angiogenesis of hepatocellular carcinoma. The purpose of this study was to further investigate its synergistic antitumor efficiency in combination with low-dose chemotherapeutic gemcitabine (GEM) on the subcutaneous hepatocellular carcinoma xenograft model in nude mice. The results showed that the combined group significantly inhibited ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) the growth of tumor weight and volume, reduced the expression of ki67 (cell proliferation marker), CD31 (angiogenic marker) and Matrix metalloproteinase 9 (MMP-9, tumor invasion and metastasis marker) and increased the apoptosis of tumor cells compared with the monotherapy and control groups, respectively. Synergistic index results showed that BDS-hEA combined with GEM had a synergistic effect in inhibiting tumor volume, proliferation, microvessel density, metastasis and promoting tumor apoptosis. Furthermore, there were no metastatic nodules and obvious pathological changes in liver tissue of the combined group, and the serum liver function indicators aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-BIL), alkaline phosphatase (ALP) and glutamyl transpeptidase (GGT) were significantly reduced ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) in the BDS-hEA or GEM groups compared with the control group. Notably, the combined therapy showed lower levels of liver function indicators than the GEM group. These data support the view that the combination of BDS-hEA and GEM has a synergistic anti-tumor properties and can reduce the damage of liver to certain extent.

Key words Baculovirus; Endostatin-Angiostatin; Anti-angiogenesis; Gemcitabine; combination therapy
INTRODUCTION

Liver cancer has become one of the most common malignant tumors with the third highest mortality rate worldwide, and hepatocellular carcinoma (HCC) accounts for 85%-90% of all cases.\(^1,2\) In China, the mortality rate of liver cancer ranks second, with 18.43 per 10,000 patients suffering from this disease.\(^3\) An important reason for the poor prognosis of liver cancer is the rapid growth of tumors and the prone to metastasis, which is related to the angiogenesis induced by tumor cells.\(^4\) Thus, inhibition of tumor angiogenesis is an effective approach for cancer treatment.

To date, a multitude of endogenous angiogenic inhibitors, including tumstatin, endostatin and angiostatin, etc., have been widely studied and confirmed to elicit antitumor angiogenesis effects.\(^5\) Among them, angiostatin (an amino-terminal fragment of plasminogen) and endostatin (a carboxyl terminal fragment of collagen XVIII) have received the greatest attention.\(^5,6\) However, protein-based therapeutic strategies using these angiogenic inhibitors have encountered difficulties with short half-life, complicated protein-purification process and long-term administration to maintain tumor suppression.\(^7\)

To circumvent this obstacle, delivery of the vectors encoding these angiogenic inhibitors, such as adeno-associated virus,\(^8\) lentivirus,\(^9\) and baculovirus,\(^10\) have been explored. In our previous work, we constructed a recombinant baculovirus vector (BDS-hEA) that long-term expresses the fusion protein of human endostatin and angiostatin (hEA), and sustained hEA expression confers stronger inhibition of human umbilical vein endothelial cells (HUVECs) proliferation, migration and angiogenesis, and significantly improves the survival rate of hepatocellular carcinoma xenograft mice.\(^11\) However, the anti-angiogenesis therapy alone is not effective in advanced tumors, which is due to its
inability to directly kill tumor cells. In recent years, angiogenesis inhibitors combined with radiotherapy, immunotherapy or chemotherapy to enhance the suppression of tumors has received more attention. In the present study, the BDS-hEA combined with low-dose chemotherapeutic GEM (a deoxycytidine analog) was used to evaluate whether the combined treatment has a synergistic effect on the treatment of hepatocellular carcinoma xenograft mouse model, and whether it can reduce the toxicity of chemotherapeutics. Our results showed that the combination regimen can synergistically inhibit the growth of liver cancer xenograft tumors without overt toxicity to the liver.

MATERIALS AND METHODS

Viruses, Cells and Reagents

The recombinant baculovirus that expressed the fusion protein of endostatin and angiostatin (BDS-hEA) has been described previously as BacSC-DAF-SB-T2ChEA. HepG2, human hepatocellular carcinoma cell line, was cultured in DMEM (high glucose, Sigma, USA) with 10% fetal bovine serum (Gibco-BRL, USA) and 1% penicillin-streptomycin solution (Gibco-BRL, USA) at 37 °C with 5% CO₂. The gemcitabine was purchased from MedChemExpress (MCE) Company, Shanghai, China and was dissolved in 0.2 ml of PBS and administrated to 20 mg/kg per animal by caudal vein. The aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-BIL) and glutamyl transpeptidase (GGT) were purchased from Hua Sin Science Company, Guangzhou, China.

Hepatocellular carcinoma mouse model

The 5-6 weeks-old male BALB/c nude mice (Weitong Lihua Test Animal Co., Beijing, China) were housed in specific pathogen free barrier facility with temperature
(22 ± 1 °C), light (12:12 h light-dark cycles), relative humidity (50 ± 5%) and food and water freely. The animal protocols (IACUC-NYLAC-2020-125) were approved by the Ethical and Welfare Committee of Ningxia Medical University, Yinchuan, China. HepG2 cells (1 × 10^7) were resuspended with 200 μl PBS and administered subcutaneously on the right side of the back of mice. The longest diameters (L) and shortest diameters (S) of the tumor were measured in a 3-day interval by dial caliper, and the volume (V) was calculated by the formula: \( V (\text{mm}^3) = (L \times S^2)/2 \).

**Grouping and administration**

When the tumor volume reached approximately 100 mm^3, the mice were randomly classified into 4 groups with 5 animals each and subjected to injections as follows: ① Control group (200 μl PBS); ② BDS-hEA group (200 μl, 1 × 10^8 pfu per mouse); ③ Gemcitabine (GEM) group (20 mg/kg per mouse); ④ Combination (BDS-hEA+GEM) group (200 μl, 1 × 10^8 pfu BDS-hEA and 20 mg/kg GEM). The BDS-hEA was administered intratumorally in a 5-day interval for three times, while the GEM through the caudal vein in a 3-day interval until the 27th day.

**Immunohistochemistry staining**

All mice were euthanized on the 27th day after administration. The tumor tissues were removed and fixed in 4% paraformaldehyde, and then the immunohistochemistry staining was performed on paraffin-embedded tissues. Briefly, the sections preparation and procedures were performed according to the protocol of the immunohistochemical kit (ZSGB-BIO, Beijing, China). Sections were subsequently incubated with the monoclonal antibodies of mouse CD31 (0.5 μg/ml, Abcam, USA), rabbit ki67 (1:150, Abcam, USA) or rabbit MMP-9 (1:200, Abcam, USA) at 4 °C overnight. Then the
sections were covered using the HRP conjugated from the corresponding species of primary antibody for 1 h at room temperature. Next, the sections were incubated with diaminobenzidine (DAB) and counterstained with hematoxylin. For apoptosis analysis, the sections were stained using the TdT-mediated FITC-dUTP nick end labeling (TUNEL) and counterstained with DAPI according to the manufacturer's protocol (in situ cell death detection kit, Roche, UK). The images were taken by the positive fluorescence microscope (Ni-U, Nikon, Japan), and the positive cells from three random areas of each section were measured by determining the integral optical density (IOD)/Area using the Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

**Macroscopic observation and hematoxylin-eosin (H&E) staining**

The liver, heart, spleen, lung and kidney tissues of all mice were removed on the 27th day after injection, the metastatic nodules were examined by macroscopic observation. Subsequently, the tissues with obvious metastatic nodules were fixed with 4% paraformaldehyde, and then embedded in paraffin. After dewaxing and rehydration, the sections were stained with hematoxylin and eosin (H&E) for histopathologic observation by the light microscopy (E200, Nikon, Japan).

**Synergistic index analysis**

Synergistic index calculation were carried out as described previously with slight modification.16) Briefly, the synergistic index of tumor volume, microvessel density, proliferation, metastasis and apoptosis were calculated by the formula: relative ratio= (Expected fraction)/(Observed fraction). Ratio > 1, indicating a synergistic effect; Ratio < 1, indicating an additive effect.

**Liver toxicity analysis**
The blood samples were collected on the 27th day after administration, and then centrifuged at 3,000 × g for 15 min. The serum were collected to measure liver function indexes of AST, ALT, T-BIL, ALP and GGT according to the manufacturer's protocol using the automatic biochemical analyzer (JCA-BM6010/C, Sysmex, Japan).

Statistical analysis

Data were analyzed by one-way of variance (ANOVA) and expressed as mean ± SD. SPSS statistical software v26.0 (IBM, New York, USA) was used for statistical analysis, when *P < 0.05, **P < 0.01, or ***P < 0.001, all were considered significant differences.

RESULTS

Anti-tumor effects of BDS-hEA combined with GEM

To evaluate the anti-tumor effects of BDS-hEA in conjunction with GEM, human HCC mouse models were created by subcutaneously inoculating HepG2 cells on the right side of the back of each mouse. When the tumor volume grew to 100 mm$^3$, the mice were injected with BDS-hEA, GEM, BDS-hEA+GEM or PBS. After feeding for 27 days, the mice were euthanized and the tumors were stripped. As shown in Fig.1a, b, the tumor size and weight in the combined group were significantly smaller ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) than those in the single group and the control group. The average tumor volume (Fig.1c) of the control group increased rapidly to 1500 mm$^3$ at 27 days after administration, while the tumor growth was significantly inhibited in BDS-hEA and GEM group, especially in the combined group, which showed a stronger inhibitory effect than that in BDS-hEA and GEM group, and the tumor volume was still less than 500 mm$^3$ until the 27th day.

Tumor suppression analysis of BDS-hEA combined with GEM
To explore the underlying mechanism of BDS-hEA combined with GEM on tumor growth suppression, the tumors were sectioned and analyzed the expression of CD31, ki67 and MMP-9 by immunohistochemical staining on day 27 after administration. As shown in Fig. 2, compared with the control group, the percentage of CD31⁺, Ki67⁺ and MMP-9⁺ cells in the BDS-hEA and GEM groups were significantly reduced ($p < 0.05$ or $p < 0.01$ or $p < 0.001$), while there was no significant difference between the BDS-hEA and GEM groups. Intriguingly, the BDS-hEA combined with GEM showed lower expression levels than the single treatment group ($p < 0.05$ or $p < 0.01$ or $p < 0.001$). In addition, to further examine whether the tumor repression stemmed from the cell death, the TUNEL and DAPI staining (Fig. 3) revealed that only weak green fluorescence was observed in the control group, while significantly increased green fluorescence were observed in the BDS-hEA and GEM groups, especially in the combined group ($p < 0.001$). These data collectively confirmed that BDS-hEA combined with GEM significantly inhibited tumor growth by suppressing tumor proliferation, angiogenesis and metastasis and promoting apoptosis.

**Synergistic analysis of BDS-hEA combined with GEM**

Although BDS-hEA combined with GEM therapy has a more significant inhibition effect on tumor growth than monotherapy, it is not clear whether there is a synergistic effect between them. As shown in Table 1, the synergistic indexes of combined therapy in tumor volume, microvessel density, proliferation, metastasis and apoptosis were 1.10, 1.04, 1.64, 1.06 and 8.91, respectively. These data collectively confirmed the synergistic effect of BDS-hEA combined with GEM on tumor growth inhibition.

**Tumor metastasis analysis of BDS-hEA combined with GEM**
When the tumor progresses to mid-to-late stage, tumor metastasis may occur. To confirm whether the BDS-hEA and/or GEM can suppress metastasis of hepatocellular carcinoma, the liver, heart, spleen, lung and kidney tissues of mice were removed on 27 days after administration, and the metastatic nodules were examined by macroscopic observation (Fig.4a) and H&E staining (Fig.4b). The results showed that the metastatic nodules were obviously seen in two out of five mice in the liver tissue of control group, while no metastatic nodules were observed in any tissues of other groups. H&E staining of metastatic nodules further showed that there are a massive of tumor cells infiltration in the liver tissue.

**Liver toxicity analysis of BDS-hEA combined with GEM**

To evaluate the toxicity of BDS-hEA and/or GEM to liver after treatment, serum liver function indicators were measured. The results were shown in Table 2, compared with the control group, the AST, ALT, T-BIL, ALP and GGT levels were significantly reduced ($p < 0.05$ or $p < 0.01$ or $p < 0.001$) in the BDS-hEA and GEM groups. Notably, the combination therapy showed lower liver function index than the GEM group, indicating that the combination therapy was effective in reducing liver function damage.

**DISCUSSION**

In recent years, tumor gene therapy has developed into the fourth major treatment option after chemotherapy, radiotherapy, and surgery.$^{17,18}$ Gene therapy strategies with continuous expression of antiangiogenic factors seem perfectly suited to surmount the shortcomings such as short half-life and long-term administration of antiangiogenic protein agents.$^{18,19}$ The selection of vector in gene therapy is crucial. Compared with the commonly used vectors of human pathogens such as adenovirus, adeno-associated virus
and lentivirus, etc., baculovirus is an insect virus that has no pathogenicity to human and mammalian cells, but it can effectively transduce various mammalian cells for transgene expression.\textsuperscript{20} However, the major limitations of baculovirus as a gene delivery vector are transient expression of transgene and its vulnerability to inactivation by serum complement system \textit{in vivo}.\textsuperscript{21,22} Based on these deficiencies, in our recent study,\textsuperscript{11} we combined BV with the Sleeping Beauty (SB) transposon for transgene integration into the host chromosome, and displayed decay accelerating factor (DAF, a protein protecting baculovirus from complement attack) on the baculoviral envelope to improve \textit{in vivo} transduction efficiency. Unsurprisingly, the hEA protein expressed by this baculovirus vector can sustain the transgenic expression for at least 90 days \textit{in vitro}, and the sustained hEA expression confer the augmented antiangiogenic properties and anti-liver cancer effect than transient hEA expression. Although anti-angiogenic gene therapy has shown some advantages over the protein agents, a myriad of preclinical and clinical evidences showed that anti-angiogenesis alone is not effective in inhibiting tumor growth and metastasis, and the inefficiency is mainly ascribe to the inhibition of tumor angiogenesis rather than directly killing the tumor.\textsuperscript{12} The commonly used anti-tumor chemotherapy agents such as carboplatin, GEM, paclitaxel, etc. can directly kill tumor cells, but the overall survival of patients has not been effectively improved in clinical practice due to the severe side effect and drug resistance.\textsuperscript{23,24} Some reports indicated that antiangiogenic agents can potentiate the sensitivity of tumor to chemotherapeutic drugs.\textsuperscript{25} Therefore, a synergistic strategy by targeting tumor blood vessels and tumor itself seems to be an ideal approach to enhance the inhibition of tumor growth.\textsuperscript{26} Moreover, in combination regimens, cytotoxic agents are routinely administered at low doses and long intervals to
reduce systemic toxicity.\textsuperscript{27,28} Therefore, the aim of present study was to clarify the anti-tumor efficacy and potential toxicity of BDS-hEA combined with low-dose GEM in hepatocellular carcinoma xenograft mouse models. Our data confirmed that the BDS-hEA combined with GEM more effectively inhibited the growth of tumor volume and weight compared with the monotherapies (Fig.1).

In order to further explore the underlying mechanism of combined therapy to suppress tumors, we analyzed the CD31, ki67, MMP-9 and apoptosis of tumor tissues by immunohistochemistry staining and TUNEL analysis. Our data clearly showed that BDS-hEA and GEM not only promoted the apoptosis of tumor tissues, but also inhibited the tumor angiogenesis, proliferation and metastasis by down-regulating the expression of CD31, ki67, MMP-9 (Fig.2-3). And further found that the combined therapy could synergistically potentiate the anti-tumor effect of the monotherapy group, as confirmed by the synergistic indexes of tumor volume, microvessel density, proliferation and apoptosis in Table 1. The synergy mechanism between BDS-hEA and GEM may be based on the following facts. First, the BDS-hEA inhibited the proliferation and migration of endothelial cells, resulting in a lack of adhesion between extracellular matrix and endothelial cells, while non-adhesion endothelial cells were more sensitive to GEM, leading to a higher apoptosis rate. Moreover, we found that GEM suppressed hepatic tumor angiogenesis by reducing CD31 levels. The research has also confirmed that GEM can inhibit the angiogenesis of pancreatic/biliary tumors by suppressing the expression of VEGF, VEGFR2, CD31 and HIF-1α protein.\textsuperscript{29} Exhilaratingly, the combination of GEM and BDS-hEA exerted a stronger ability to inhibit angiogenesis and a higher rate of apoptosis. A study also found that the endostar administered simultaneously with or
following GEM can inhibit the increase of VEGF levels, which leads to a decrease in vessel density and an increase in apoptosis in tumor tissues. However, the exact synergistic anti-angiogenesis mechanism of GEM combined with BDS-hEA is still needed to be further explored, and we suspect that the synergy mechanism may be regulated by VEGF/VEGFR signaling pathway.

GEM is currently used as a first-line treatment for non-small cell lung cancer, pancreatic cancer, bladder cancer, breast cancer, etc., but there are few reports in hepatocellular carcinoma. The reason may be due to the serious side effects of GEM chemotherapy on the liver. Intraperitoneal injection of GEM induced marked hematological, biochemical, hepatorenal and histopathological alterations. The GEM combined with FGFR-1 vaccine synergistically strengthened the anti-tumor activity via suppression of tumor angiogenesis without overt toxicity in tumor-bearing mice. In this study, we focused on whether the combination of BDS-hEA and low-dose GEM can reduce the toxic effect on the liver. Our results (Fig.4) showed that there were no metastatic nodules and obvious pathological changes were found in the combination group. Surprisingly, the same results were also obtained in the GEM group. This may be due to the reduced expression of MMP-9 and the use of low-dose GEM and less frequency. At the same time, no visible pathological changes were found in other organs such as heart, spleen, lung and kidney in any groups (data not shown). We further measured the serum liver function indicators as shown in Table 2. Compared with the control group, the AST, ALT, T-BIL, ALP and GGT levels had an obvious reduction in both the BDS-hEA and GEM groups, especially the BDS-hEA group, indicating that baculovirus-mediated antiangiogenic therapy exhibited weaker toxicity than GEM.
Surprisingly, compared with the GEM group alone, the combined treatment further reduced the level of the liver function index mentioned above, indicating that the combined treatment could reduce the toxicity to liver to certain extent. These results may provide a paradigm of achieving the same therapeutic effect as conventional chemotherapy through a combination strategy without overt toxicity.

CONCLUSION

In conclusion, the present study suggests that BDS-hEA combined with GEM in the treatment of experimental human liver cancer can synergistically enhance the anti-tumor effect by inhibiting tumor angiogenesis, proliferation and metastasis, and increasing cell apoptosis, without obvious undesired toxicity. Further studies are being implemented to clarify the molecular mechanisms of its anti-tumor effects.

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Conflict of interest

The authors declare no conflict of interest.
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Fig. 1 Antitumor effects of BDS-hEA combined with GEM. (a) Anatomy of tumor in mouse. (b) Tumor weight. (c) Tumor volume. The tumors were established by subcutaneous injection of HepG2 cells (2 × 10^6 cells per mouse) on the right side of the back of BALB/c nude mice and the tumor volume was measured in a 3-day interval. When the tumor volume reached to 100 mm^3, the mice were randomly classified into BDS-hEA, gemcitabine (GEM), GEM+BDS-hEA and PBS groups (n=5 for each group). The BDS-hEA was administered intratumorally in a 5-day interval for three times, while the GEM through the caudal vein in a 3-day interval until the 27th day, at which time all mice were euthanized and the tumors were stripped, and weighed. * P < 0.05, ** P < 0.01, *** P < 0.001.
Fig. 2 BDS-hEA combined with GEM inhibited tumor proliferation, angiogenesis and metastasis. (a) Representative images of immunohistochemical staining (400×). (b) Quantitative analysis. Tumor specimens were removed on the 27th day after administration, sectioned and stained for microvessel density by using CD31 antibody, proliferation by ki67 antibody, and metastasis by MMP-9 antibody. The positive cells (brownish yellow) were calculated in three random areas of each section using Image-Pro Plus 6.0 by determining the integral optical density (IOD)/Area. Bar, 100 μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
Fig. 3 BDS-hEA combined with GEM induced cell apoptosis. (a) Representative images of TUNEL staining (200×). (b) Quantitative analysis. Tumor specimens were removed on the 27th day after administration, sectioned and subjected to TUNEL staining and DAPI counterstaining. The apoptotic cells were detected in three random areas of each section using Image-Pro Plus 6.0 by determining the green fluorescent cells/total cells. Bar, 200 µm. *** $P < 0.001$
Fig. 4 BDS-hEA combined with GEM suppressed metastasis of hepatocellular carcinoma. The liver tissues were removed from mice on 27 days after administration and the metastatic nodules were examined by macroscopic observation (a) and H&E staining (400×) (b). The metastatic nodules are indicated by red boxes. H&E staining revealed significant accumulation of infiltrated tumor cells in the control group (i), with a clear boundary between normal liver cells and tumor cells, while no significant pathological changes were seen in the BDS-hEA (ii), GEM (iii) and BDS-hEA+GEM (iv) groups.
Table 1 Synergistic analysis of BDS-hEA and GEM

| Items               | Relative fraction<sup>a</sup> | BDS-hEA+GEM |       |       |       |
|---------------------|-------------------------------|--------------|-------|-------|-------|
|                     | BDS-hEA | GEM | Expected<sup>b</sup> | Observed<sup>c</sup> | Ratio<sup>d</sup> |
| Tumor volume index  | 0.59    | 0.64 | 0.38 | 0.35  | 1.10  |
| Microvessel density index | 0.50    | 0.57 | 0.28 | 0.27  | 1.04  |
| Proliferation index | 0.34    | 0.54 | 0.18 | 0.11  | 1.64  |
| Metastasis index    | 0.59    | 0.60 | 0.35 | 0.33  | 1.06  |
| Apoptosis index     | 27.04   | 24.20 | 649.50 | 72.86  | 8.91  |

<sup>a</sup> (Mean of separate experimental group)/(Mean of control group).

<sup>b</sup> (Mean fraction of BDS-hEA group)×(Mean fraction of GEM group).

<sup>c</sup> (Mean of Combined experimental group)/(Mean of control group).

<sup>d</sup> (Expected fraction)/(Observed fraction). Ratio > 1, indicating a synergistic effect; Ratio < 1, indicating an additive effect.
Table 2 Serum liver function indicators of subcutaneous hepatocellular carcinoma mouse models treated with various treatment regimens

| Items                | Control     | BDS-hEA     | GEM         | BDS-hEA+GEM |
|----------------------|-------------|-------------|-------------|-------------|
| AST (U/L)            | 769.73±48.56| 143.24±23.09| 418.03±14.12| 244.87±42.05|
| ALT (U/L)            | 480.91±49.77| 130.19±11.39| 263.74±23.92| 154.96±28.94|
| T-BIL (µmol/L)       | 3.77±0.54   | 0.25±0.07a  | 0.32±0.10a  | 0.32±0.08a  |
| ALP (U/L)            | 171.51±49.93| 125.27±6.96a| 157.07±4.55 | 142.94±7.60 |
| GGT (U/L)            | 6.49±0.54   | 1.21±0.16a  | 1.58±0.13a  | 1.45±0.11a  |

\(^a P < 0.05\) vs Control group, \(^b P < 0.05\) vs BDS-hEA group, \(^c P < 0.05\) vs GEM group.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-BIL, total bilirubin; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase.