



ORIGINAL ARTICLES

Recombinant vascular basement-membrane-derived multifunctional peptide inhibits angiogenesis and growth of hepatocellular carcinoma

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Abstract

AIM: To investigate the anti-angiogenic and anti-tumor activities of recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) in hepatocellular carcinoma (HCC).

METHODS: HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines were cultured *in vitro* and the inhibitory effect of rVBMDMP on proliferation of cells was detected by MTT assay. The *in vivo* antitumor efficacy of rVBMDMP on HCC was assessed by HepG2 xenografts in nude mice. Distribution of rVBMDMP, mechanism by which the growth of HepG2 xenografts is inhibited, and microvessel area were observed by proliferating cell nuclear antigen (PCNA) and CD31 immunohistochemistry.

RESULTS: MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical

vein endothelial (HUVE-12) cells in a dose-dependent manner, with little effect on the growth of L-02 cells. When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μ mol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to 5-fluorouracil (5-FU) with an IC₅₀ of 4.59 μ mol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU [SI was 1.9 (8.94/4.59)]. The VEGF-targeted recombinant humanized monoclonal antibody bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but the growth inhibitory rate of bevacizumab (100 mg/L) to HUVE-12 cells was 87.6% \pm 8.2%. Alternis diebus intraperitoneal injection of rVBMDMP suppressed the growth of HepG2 xenografts in a dose-dependent manner. rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control. Immunohistochemical staining of rVBMDMP showed that the positive area rates (2.2% \pm 0.73%, 4.5% \pm 1.3% and 11.5% \pm 3.8%) in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly higher than that (0.13% \pm 0.04%) in the control group (P < 0.01). The positive area rates (19.0% \pm 5.7%, 12.2% \pm 3.5% and 5.2% \pm 1.6%) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that (29.5% \pm 9.4%) in the control group (P < 0.05). rVBMDMP at doses of 1, 3 and 10 mg/kg significantly reduced the tumor microvessel area levels (0.26% \pm 0.07%, 0.12% \pm 0.03% and 0.05% \pm 0.01% vs 0.45% \pm 0.15%) in HepG2 xenografts (P < 0.01), as assessed by CD31 staining.

CONCLUSION: rVBMDMP has effective and unique anti-tumor properties, and is a promising candidate for the development of anti-tumor drugs.

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Key words: Hepatocellular carcinoma; Recombinant vascular basement membrane-derived multifunctional peptide; Proliferating cell nuclear antigen; CD31; Therapeutic action

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INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common cancer in the world, is responsible for over 600 000 deaths annually^[1]. The majority of patients with HCC die within 1 year after the diagnosis. Unfortunately, HCC is often diagnosed at its late stage when potentially curative therapies are least effective. For such patients, medical treatment modalities, including chemotherapy, chemoembolization, ablation, and proton beam therapy, remain disappointing. Most patients show recurrent HCC that rapidly progresses to its advanced stage with vascular invasion and multiple intrahepatic metastases and their 5-year survival rate is only 7%^[2]. Patients with surgically resectable localized HCC have a better prognosis, but their 5-year survival rate is only 15%-39%^[3], showing that new therapies for this aggressive disease are urgently needed.

Angiogenesis plays a critical role in the development of HCC. Antiangiogenesis therapy, which inhibits blood vessel formation, may be a promising treatment modality for HCC, because HCC depends on a rich blood supply^[4].

Tumstatin, a 28-kDa (244 amino acids) peptide fragment derived from the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous angiogenesis inhibitor, and has two binding sites for $\alpha v\beta 3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity^[5-7]. The peptide fragment of tumstatin consisting of amino acids 74-98 binds to both endothelial and melanoma cells, but only inhibits the proliferation of endothelial cells. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed by truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98^[5].

By targeting proliferating tumor cells and endothelial cells in a previous study^[8], we have constructed a fusion gene of the human IgG3 upper hinge region with two tumstatin-derived specific sequences, which exhibit anti-proliferation and anti-angiogenic activities. The human IgG3 upper hinge region is composed of 11 amino acids, and has a good flexibility, thus not affecting the spatial conformations of the connected peptides. The fusion sequence is named vascular basement membrane-derived multifunctional peptide (VBMDMP)^[9]. Recombinant

VBMDMP (rVBMDMP) can significantly inhibit tumor growth and metastasis in a mouse lung carcinoma model^[10]. Moreover, rVBMDMP selectively inhibits the proliferation of endothelial and human colon cancer cells, as well as induces apoptosis of endothelial cells *in vitro* and suppresses the growth of human colon cancer xenografts in Balb/c-nude mice^[11]. However, whether rVBMDMP inhibits tumor growth and angiogenesis of human HCC xenografts in a nude mouse model is unknown.

In the present study, we showed that rVBMDMP selectively inhibited the proliferation of HCC cells, using *in vitro* models of tumor growth, and also potently inhibited tumor neoangiogenesis of HepG2 xenografts in a nude mouse model, suggesting that rVBMDMP can be used as a potential agent in the treatment of human HCC.

MATERIALS AND METHODS

Cell culture and reagents

HepG2, Bel-7402, Hep-3B, HUVE-12 and L-02 cell lines, were purchased from the China Center for Type Culture Collection (CCTCC), were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin (Life Technologies) in an incubator containing 50 mL/L CO₂ at 37°C. rVBMDMP was over-expressed in *Escherichia coli* with pGEX-4T-1-VBMDMP and purified as previously described^[10] with a purity of over 95%. Synthetic peptide CNYYSNSYSFWLASLNPER (amino acid 185-203 of tumstatin, T4 peptide) and its rabbit polyclonal antibody were provided by Xi'an Huacheng Biotechnology Co., Ltd (China). Bevacizumab was purchased from Roche (Avastin®, Basel, Switzerland). Mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA) and CD31, as well as peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

MTT assay

Cells were seeded in a 96-well plate at a density of 1000 cells/well as described previously^[12]. Different concentrations of drugs were added to each well and cultured for 48 h, followed by incubation with 0.5 g/L MTT for 4 h. The supernatant was removed after centrifugation. Finally, 100 μ L DMSO was added and the absorbance at 570 nm wavelength (A_{570}) was measured with an enzyme-labeling instrument (ELX-800 type). Relative cell proliferation inhibition rate (IR) = (1-average A_{570} of the experimental group/average A_{570} of the control group) \times 100%. The IR was analyzed using the CalcuSyn program to determine the IC₅₀.

Tumor xenograft experiments

Balb/c-nude female mice (Vital River Laboratory Animal Technology Co., Ltd), used in *in vivo* study, were housed in a sterile room at Institute of Cancer Research, University of South China, with free access to food and water.

Tumors were generated by harvesting HepG2 cells

from mid-log phase cultures using 0.25% trypsin (Life Technologies). Cells were resuspended in PBS to a final cell count of $2.5 \times 10^7/\text{mL}$. A cell suspension (0.2 mL) was subcutaneously injected into the back of each mouse. The mice received a total of 10 injections of 1, 3, and 10 mg/kg body weight rVBMDMP (i.p) every other day when their average tumor volume reached 200 mm³.

Tumor dimensions and body weight were recorded every 5 d from the beginning of treatment. Tumor length and width were measured using a Vernier caliper, and tumor volume was calculated as described previously^[13]. Upon termination of treatment, the mice were weighed and sacrificed, and their tumors were excised. The mean tumor weight per group was calculated. The ratio of the mean of the treated tumor weight to the mean of vehicle control tumor weight $\times 100$ was subtracted from 100% to give the tumor growth inhibition rate for each group.

Immunohistochemical staining and quantification

Immunohistochemical staining of paraffin tumor tissue sections was done with rabbit polyclonal anti-T4 peptide antibody (Xi'an Huacheng Biotechnology) at a dilution of 1:50 using the DAB system from DAKO (Carpinteria, CA, USA) according to the manufacturer's instructions.

The tumor tissue sections were viewed at $\times 100$ magnification and images were captured with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and analyzed under four fields, excluding peripheral connective tissue and necrotic regions. The total tissue area in each section was 2.576 mm². Areas of rVBMDMP, PCNA or CD31-positive objects were quantified using ImagePro Plus version 3.0 (Media Cybernetics, Silver Spring, MD, USA). Percentage of microvessel area (MVA) in each field was calculated as (area of CD31-positive objects/measured tissue area) $\times 100$. Percentage of rVBMDMP or PCNA-positive staining in each field was calculated as (area of rVBMDMP or PCNA-positive objects/measured tissue area) $\times 100$. Mean values of MVA- or VBMDMP or PCNA-positive area in each group were calculated from six tumor tissue samples.

Statistical analysis

Experimental data in each group were expressed as mean \pm SD. Analysis of variance was performed with SPSS software for Windows 15.0 using one way ANOVA and pairwise comparison with Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of rVBMDMP on proliferation of HCC, endothelial cells (ECs) and L-02 cell lines

MTT assay showed that rVBMDMP markedly inhibited the proliferation of human HCC (HepG2, Bel-7402, Hep-3B) cells and human umbilical vein endothelial (HUVE-12) cells in a dose-dependent manner, with

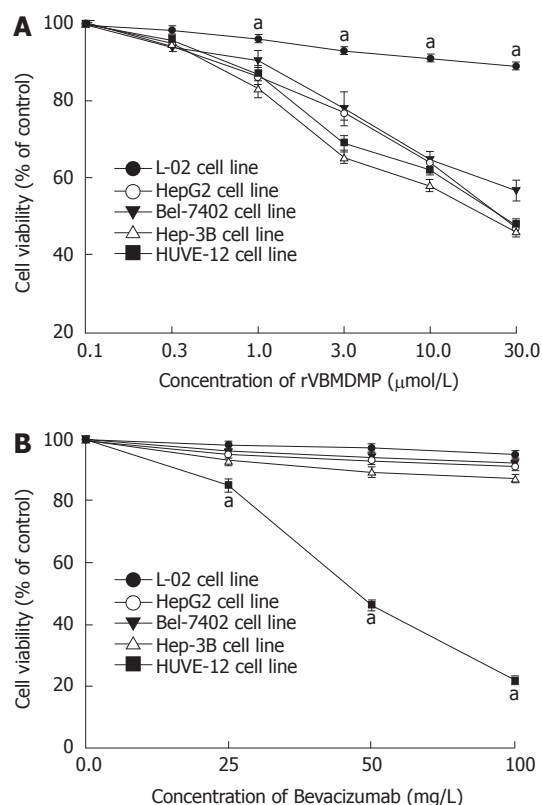


Figure 1 Proliferation of HCC (A) and ECs (B) selectively inhibited by rVBMDMP (mean \pm SD, $n = 9$). ^a $P < 0.05$ vs other cells.

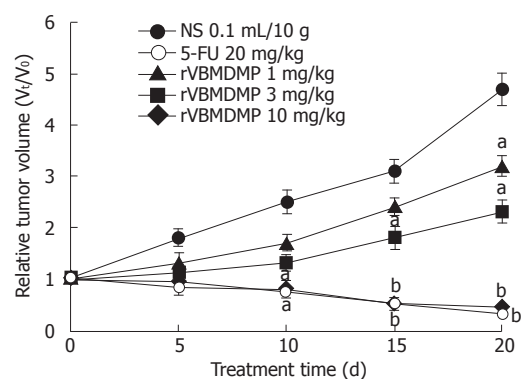


Figure 2 Robust efficacy of rVBMDMP against HepG2 xenografts in nude mice (mean \pm SD, $n = 6$). ^a $P < 0.05$, ^b $P < 0.01$ vs NS.

little effect on the growth of L-02 cells (Figure 1A). When the IC₅₀ was 4.68, 7.65, 8.96, 11.65 and 64.82 μmol/L, respectively, the potency of rVBMDMP to HepG2 cells was similar to that of 5-FU with an IC₅₀ of 4.59 μmol/L. The selective index of cytotoxicity to HepG2 cells of rVBMDMP was 13.8 (64.82/4.68), which was higher than that of 5-FU with a SI of 1.9 (8.94/4.59). Bevacizumab (100 mg/L) did not affect the proliferation of HepG2, Bel-7402, Hep-3B and L-02 cells, but its growth inhibitory rate for HUVE-12 cells was 87.6% \pm 8.2% (Figure 1B).

In vivo efficacy of rVBMDMP against HepG2 xenografts

rVBMDMP inhibited the growth of implanted HepG2 tumor xenografts in nude mice in a dose-dependent

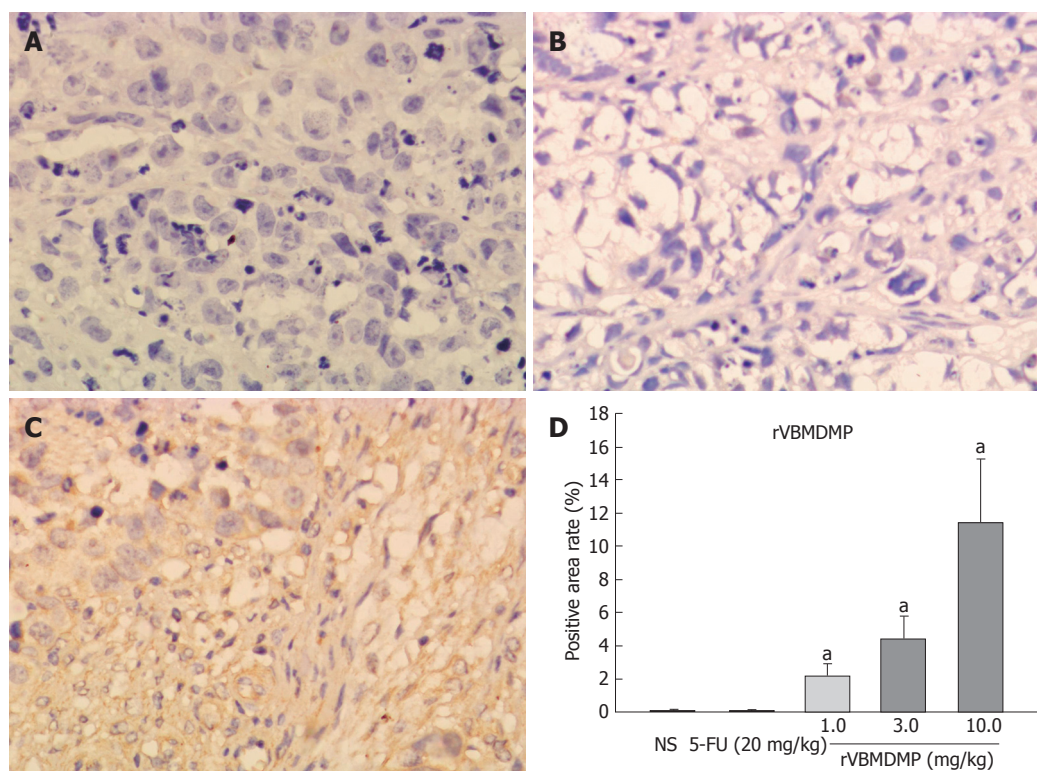


Figure 3 Accumulation of rVBMDMP in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of rVBMDMP-positive areas in HepG2 xenografts. The data are expressed as mean \pm SD ($n = 6$). $^aP < 0.05$ vs NS.

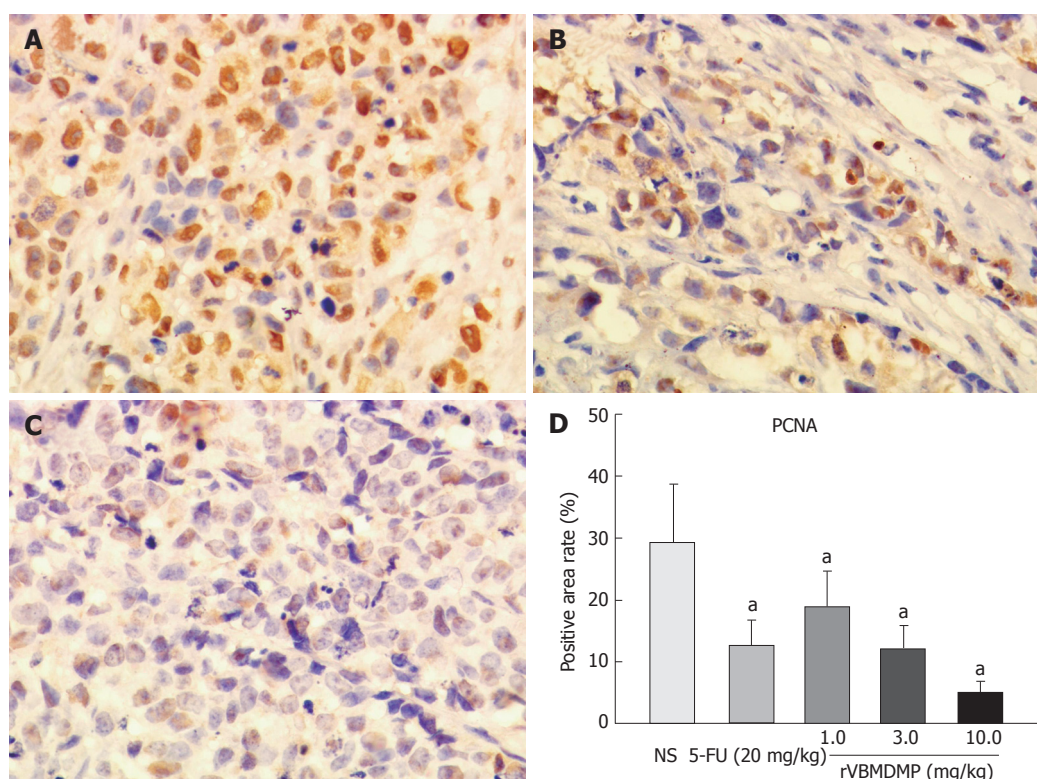


Figure 4 Expression of PCNA in HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of PCNA-positive areas in HepG2 tumors. The data are expressed as mean \pm SD ($n = 6$). $^aP < 0.05$ vs NS.

manner (Figure 2). Different doses of rVBMDMP (1, 3, 10 mg/kg) decreased the tumor weight by 12.6%, 55.9% and 79.7%, respectively, compared with the vehicle control.

Distribution of rVBMDMP in HepG2 xenografts

Immunohistochemical staining of rVBMDMP showed that the positive area rates ($2.2\% \pm 0.73\%$, $4.5\% \pm$

1.3% and $11.5\% \pm 3.8\%$) were significantly higher in rVBMDMP treated group (1, 3, 10 mg/kg) than that ($0.13\% \pm 0.04\%$) in the control group ($P < 0.01$, Figure 3), indicating that rVBMDMP can accumulate in HepG2 xenografts nude mice.

PCNA expression in HepG2 xenografts

After intraperitoneal injection of rVBMDMP every other

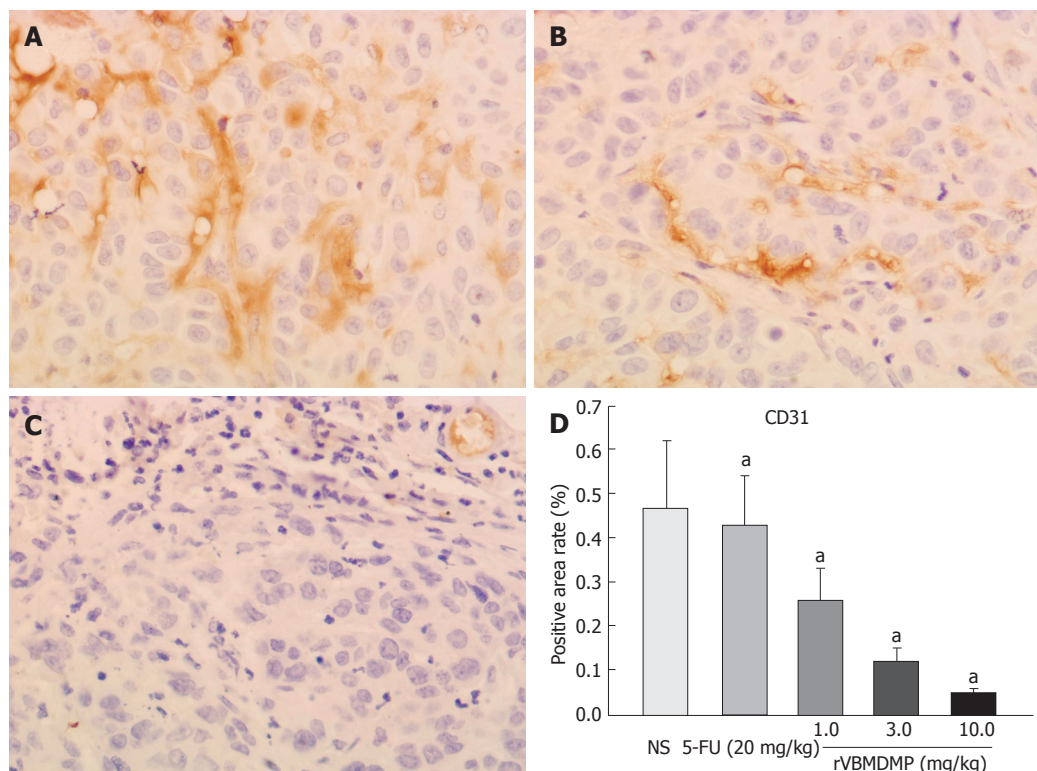


Figure 5 Microvessel density of HepG2 xenografts in nude mice after treatment. A: NS; B: 20 mg/kg of 5-FU; C: 10 mg/kg of rVBMDMP; D: Quantification of microvessel density (CD31 staining) positive areas in HepG2 xenografts in nude mice. The data are expressed as mean \pm SD ($n = 6$). ^a $P < 0.05$ vs NS.

day, the positive area rates ($19.0\% \pm 5.7\%$, $12.2\% \pm 3.5\%$ and $5.2\% \pm 1.6\%$) of PCNA in rVBMDMP treated group (1, 3, 10 mg/kg) were significantly lower than that in the control group ($29.5\% \pm 9.4\%$) ($P < 0.05$, Figure 4), suggesting that rVBMDMP inhibits the proliferation of tumor cells in HepG2 xenografts in nude mice.

Effect of rVBMDMP on angiogenesis of HepG2 xenografts

The tumor MVA rates ($0.26\% \pm 0.07\%$, $0.12\% \pm 0.03\%$ and $0.05\% \pm 0.01\%$) were significantly lower in the HepG2 xenografts of the rVBMDMP-treated group assessed by CD31 staining (1, 3 and 10 mg/kg) than that ($0.45\% \pm 0.15\%$) in the control group ($P < 0.01$, Figure 5), demonstrating that rVBMDMP inhibits angiogenesis of HepG2 xenografts in nude mice.

DISCUSSION

It was recently reported that angiogenesis inhibitors may not work well in monotherapy^[14,15]. In contrast, studies conducted in preclinical tumor models showed that angiogenesis inhibitors in combination with cytotoxic chemotherapeutic agents or radiation therapy produce additive or synergistic anti-tumor activities^[12,16,17]. The positive effects of combined chemotherapy with angiogenesis inhibitors have been reported^[18-22], suggesting that the combination therapy of a cytotoxic agent and an angiogenesis inhibitor may be a fruitful topic in future clinical research^[23,24].

In this report, rVBMDMP inhibited the proliferation of human HCC cells selectively *in vitro*. Our previously research also showed that rVBMDMP could inhibit the proliferation of colon cancer cells, but have no effect on the proliferation of normal cells^[11], suggesting that

rVBMDMP can maintain the selective anti-tumor activity of tumstatin amino acids 185-203 fragment, which is consistent with the previously reported findings^[23]. The specific inhibitory effect of rVBMDMP on the proliferation of tumor cells strongly suggests that rVBMDMP functions *via* a tumor-specific cell surface protein or its receptor.

Tumor neoangiogenesis has recently been recognized as an important factor in defining subsets of cancer patients with a poor outcome^[25-27]. A number of angiogenesis inhibitors, discovered in recent years, can inhibit tumor growth by targeting proliferating and migrating ECs. Targeting ECs supports growth of tumor rather than tumor cells directly, which is particularly promising because these ECs are genetically stable and do not develop drug resistance. In this study, rVBMDMP suppressed reduplication in human endothelial HUVE-12 cells, like bevacizumab. By immunostaining of CD31 in tumor tissues, we found that rVBMDMP significantly decreased the microvessel density of human HCC xenografts in a mouse model. It was reported that rVBMDMP can significantly inhibit the proliferation of endothelial cells, blood vessel formation, and tumor growth in *in vitro* and *in vivo* models of angiogenesis, as well as induce EC-specific apoptosis^[11]. These anti-angiogenic properties of rVBMDMP, coupled with its anti-tumor activities, strongly indicate that rVBMDMP acts as a novel inhibitor of angiogenesis and tumor growth.

Since the proliferation velocity of ECs is higher in tumor tissue than in normal tissue, angiogenesis inhibitors may be accumulated in tumor^[28]. Our results show that rVBMDMP was significantly accumulated in human HCC xenografts in a mouse model, indicating

that rVBMDMP is selectively distributed in tumor tissue.

Maeshima *et al.*^[5] demonstrated that tumstatin amino acids 185-203 fragment does not show anti-tumor activity until the peptide region is exposed to truncation, which is not required for the anti-angiogenic activity of tumstatin amino acids 74-98 fragment. A shorter fragment comprising seven N-terminal residues 185-191 (CNYYSNS) shares the same inhibitory profile. The three-dimensional structures of CNYYSNS and tumstatin amino acids 185-203 fragment show a β -turn at the YYSNS (188-191) sequence level, which is crucial for its biological activity^[29]. In our study, analysis of the structures of rVBMDMP using the AntheProt software indicated that both ends of the IgG3 upper hinge region sequence were a rarefaction structure, suggesting that rVBMDMP acts as a potent and specific agent against tumor progression^[11].

It has been shown that $\alpha_v\beta_3$ integrin is a putative receptor of tumstatin^[30,31]. Tumstatin fails to suppress neovascularization of Matrigel plugs in β integrin-deficient mice, and tumors in β integrin-deficient mice grow much faster than tumors in wild-type mice^[30,31], strongly suggesting that tumstatin acts via $\alpha_v\beta_3$ integrin as a negative regulator of angiogenesis. We speculate that the anti-tumor activity of rVBMDMP might also be mediated by $\alpha_v\beta_3$ integrin^[32].

In conclusion, rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy. The mechanism of its action requires further investigation.

COMMENTS

Background

Tumstatin, a 28-kDa (244 amino acids) peptide fragment, derived from the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous angiogenesis inhibitor. Tumstatin has two binding sites for $\alpha_v\beta_3$ integrin. One is in the N-terminal region of the molecule consisting of amino acids 74-98, which is associated with the anti-angiogenic property. The other is in the C-terminal region consisting of amino acids 185-203, which is associated with the antitumor activity. However, the anti-tumor activity of amino acids 185-203 is not realized until this peptide region is exposed to truncation, a requirement not essential for the anti-angiogenic activity of amino acids 74-98.

Research frontiers

Angiogenesis plays a critical role in the development of hepatocellular carcinoma (HCC). Antiangiogenesis therapy, which inhibits blood vessel formation, may be promising treatment modality for HCC, because HCC depends on a rich blood supply. The strategy of targeting both proliferating tumor and endothelial cells can improve the effectiveness of therapy for HCC.

Innovations and breakthroughs

It was recently reported that rVBMDMP significantly inhibits tumor growth and metastasis in a mouse lung carcinoma model and selectively inhibits the proliferation of endothelial and human colon cancer cells. In this study, recombinant vascular basement membrane-derived multifunctional peptide (rVBMDMP) selectively inhibited the proliferation of HCC cells in *in vitro* and *in vivo* models of tumor growth. Furthermore, our *in vivo* studies suggested that rVBMDMP was significantly accumulated in human HCC xenografts and potentially inhibited tumor neoangiogenesis in HepG2 xenografts in nude mice.

Applications

rVBMDMP is a novel inhibitor of angiogenesis and tumor growth. Targeting both endothelial and tumor cells can enhance the efficacy of anti-tumor therapy and can be used as a treatment modality for HCC.

Terminology

VBMDMP is a fusion gene in the human IgG3 upper hinge region with two tumstatin-derived specific sequences (amino acids 74-98 and amino acids 185-203), which exhibits anti-proliferation and anti-angiogenic activities. Recombinant VBMDMP (rVBMDMP) is produced as a recombinant molecule in *E. coli*.

Peer review

The authors demonstrated that rVBMDMP selectively inhibited the proliferation of HCC cells, and was significantly accumulated in human HCC xenografts, and potentially inhibited tumor neoangiogenesis in HepG2 xenografts in a nude mouse model by examining the effects of rVBMDMP on tumor growth and angiogenesis of HCC *in vitro* and *in vivo*. The results are interesting and may represent the strategy of targeting both proliferating tumor and endothelial cells, and provide a new treatment modality for HCC.

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