Kallikrein-binding protein inhibits growth of gastric carcinoma by reducing vascular endothelial growth factor production and angiogenesis

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Abstract
Kallikrein-binding protein (KBP) has been identified as an endogenous angiogenic inhibitor. We previously showed that KBP inhibited rat retinal neovascularization by down-regulation of vascular endothelial growth factor (VEGF) in endothelial cells. However, its antiangiogenic potential for inhibition of gastric carcinoma and the effect on VEGF in tumor cells have not been elucidated. The present study was designed to investigate the effect of KBP on growth of gastric carcinoma and the possible molecular mechanism. Recombinant KBP dose dependently inhibited proliferation and induced apoptosis of endothelial cells, but no effect on proliferation and apoptosis of SGC-7901 gastric carcinoma cells. I.p. injection of KBP resulted in growth inhibition of both heterotopic and orthotopic gastric carcinoma xenografts at 61.4% and 52.3%, respectively. Microvessel density in tumor tissues treated with KBP was significantly decreased, suggesting that KBP suppressed tumor growth by antiangiogenesis. The expression and release of VEGF, a major angiogenic stimulator, were down-regulated by KBP in SGC-7901 cells and gastric carcinoma xenografts. RNA levels of VEGF in SGC-7901 cells were also decreased by KBP, thus suggesting the regulation at the transcriptional level. Therefore, hypoxia-inducible factor 1α (HIF-1α), a crucial transcriptional factor for VEGF expression, was examined in SGC-7901 cells treated by KBP. KBP reduced HIF-1α protein level and nuclear translocation, which may be responsible for the down-regulation of VEGF transcription. Down-regulation of VEGF expression and release in tumor cells through inhibiting HIF-1α, thus attenuating the paracrine effect of VEGF on endothelial cell proliferation and vascular permeability in tumor tissues, may represent a novel mechanism for the antiangiogenic and antitumor activity of KBP. [Mol Cancer Ther 2007;6(12):3297–306]

Introduction
Kallikrein-binding protein (KBP) belongs to tissue kallikrein-kinin system. Through binding to active kallikrein, KBP exerts a variety of biological effects in physiologic and pathologic processes, such as blood pressure regulation, inflammatory response, and animal growth (1, 2). Previous studies have shown that KBP has multiple biological functions independent of tissue kallikrein-kinin system (3–5). KBP shares a considerable sequence homology with serine proteinase inhibitors (serpins), such as α1-antitrypsin and α1-antichymotrypsin, and is identified as a member of serpin superfamily (6, 7). The serpin superfamily consists of multiple proteins with diverse functions (8, 9). Many serpins, such as pigment epithelium-derived factor, antithrombin, and maspin, have been shown to have antiangiogenic activity (10–12). As a member of serpin superfamily, KBP also shows antiangiogenic property and has been identified as an endogenous angiogenic inhibitor (4, 5, 13). We previously showed that intravitreal injection of KBP inhibited retinal neovascularization and decreased vascular permeability of retina, iris, and choroid in rats with an oxygen-induced retinopathy (5).

Angiogenesis, the growth of new blood vessels from preexisting capillaries, is necessary for solid tumor growth and metastasis (14, 15). Under physiologic condition, angiogenesis is controlled by a delicate balance between endogenous angiogenic stimulators and inhibitors. Breakdown of the balance results in certain pathologic conditions, such as tumor growth and metastasis (16, 17). Antiangiogenesis therapy provides a novel approach for cancer management (18, 19). However, the antiangiogenic potential for the treatment of neoplastic diseases and the underlying mechanism of KBP has not been well
explored. Our present study was designed to test the in vitro and in vivo effects of recombinant KBP on the neovascularization and growth of gastric carcinoma, a hypervascular tumor.

The exact mechanism for antiangiogenic activity of KBP remains to be elucidated. We previously found that KBP inhibited retinal angiogenesis and decreased vascular permeability by reducing endothelial growth factor (VEGF) production in endothelial cells and blocking VEGF binding to endothelial cells (5). However, the effect of KBP on the expression and release of VEGF in tumor cells has not been investigated. Recent studies showed that pigment epithelium-derived factor, a member of the same superfamily, suppressed tumor growth by inhibiting VEGF expression in tumor cells (20, 21). Therefore, the hypothesis that KBP can regulate VEGF in tumor cells was detected for the first time in the present study.

Materials and Methods

Cell Culture

Human gastric carcinoma cells (SGC-7901 cells) were purchased from the cell bank of Sun Yet-sen University (Guangzhou, China) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (Life Technologies) and incubated at 37°C in a humidified incubator at 5% CO₂.

Human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical cords obtained from the Department of Obstetrics and Gynecology (First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China), grown in human endothelial serum-free medium (Life Technologies) supplemented with 10% FBS and endothelial cell growth supplement (Upstate), and incubated at 37°C in a humidified incubator at 5% CO₂. To maintain uniform condition, all experiments were carried out between cell passages 4 to 6.

Expression and Purification of Recombinant KBP

The KBP cDNA containing a sequence encoding the full-length mature peptide was amplified from the total RNA of rat liver by reverse transcription-PCR as described previously (5). The PCR product was cloned into the pET28 vector (Novagen) at the BamHI and SacI sites in frame with the sequence encoding the 6× His tag at its 3’ end. The KBP/pET28 construct was introduced into Escherichia coli strain BL-21/DE3 (Novagen). The expression and purification were carried out as described previously (5).

Heterotopic Tumor Growth Assay

SGC-7901 cells (5 × 10⁴/0.2 mL) in DMEM were inoculated s.c. into the dorsal area of 6- to 8-week-old female athymic mice (BALB/c nu/nu, 18-22 g, Center of Experimental Animal, Sun Yet-sen University, Guangzhou, China). When tumors reached a volume of 50 mm³, the mice were randomized into two groups (n = 5 in each group) and received i.p. injection of KBP or PBS, respectively. The KBP group received i.p. injection of KBP in PBS at the dose of 2.5 mg/kg per mouse every other day, until the overall dose reached 10 mg/kg. The control group was treated with the same volume of PBS. Tumor growth was monitored by external measurement in two dimensions every other day. Tumor volume was determined according to the following equation: volume = (length × width²) × 0.5. Four weeks after the first injection of KBP, the mice were sacrificed, and tumors were excised and weighed. The tumor inhibition ratio was calculated as follows: inhibition ratio (%) = [(C – T) / C] 100%, where C is the average tumor weight of the control group and T is the average tumor weight of the KBP-treated group. Some tumor tissues were frozen at −80°C for Western blot analysis and some fixed in 4% neutral buffered paraformaldehyde in PBS for immunohistochemistry analysis.

Orthotopic Tumor Implantation

Orthotopic tumor implantation models were developed following the methods previously described with minor modifications (22). Briefly, s.c. passage of SGC-7901 cell tumor was carried out first. When tumors reach 10 mm in diameter, they were removed and minced with scissors into pieces of ~2 mm in diameter, and reimplanted s.c. into nude mice. Passage 4 s.c. tumors at exponential growth phase were removed quickly and the healthy tumor tissues were minced into 2-mm-diameter pieces. Six- to 8-week-old female athymic mice were anesthetized with 4.3% chloral hydrate. The stomach wall was carefully exposed, and a part of the serosal membrane in the middle of the greater curvature of the glandular stomach was mechanically injured with scissors. Then, a tumor piece was fixed on each injured site of the serosal surface with a 6-0 Prolene (Ethicon) transmural suture. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed with 5-0 Prolene sutures. Seven days after implantation, the mice were assigned randomly into two groups (n = 7 in each group) and received i.p. injection of same dose and schedule of KBP or PBS as heterotopic tumor. Six weeks after the first injection of KBP, tumors were excised and weighed, and tumor inhibition ratio was calculated. Metastases were evaluated macroscopically or microscopically. Liver, lymph node, peritoneal cavity, and lung were examined macroscopically for metastatic nodules, and metastases were certified microscopically by standard H&E staining.

All animal studies were done under an institutionally approved protocol according to the USPHS guide for the care and use of laboratory animals.

Immunohistochemistry

Histologic sections, 5-μm-thick, prepared from paraffin-embedded tissue samples, were used for immunohistochemical analysis. Identification of endothelial cells was done by immunostaining using a polyclonal antibody against mouse CD34 (1:100, Santa Cruz Biotechnology). Tumor vasculature was quantified by the Weidner’s method (23). VEGF and β-actin expression in serial sections were determined by immunostaining using monoclonal antibodies against VEGF and β-actin (1:100, Santa Cruz Biotechnology). VEGF staining was scored semiquantitatively as Song’s method with minor modification (24). Briefly, the shade of staining was assigned to 0 to
were incubated under normoxic (20% O2, v/v) or hypoxic conditions (1% O2) at 37°C. The culture medium was replaced with human endothelial serum-free medium added with 2% FBS for HUVECs and supplemented with various concentrations of KBP (0, 10, 20, 40, 80, and 160 nmol/L). For SGC-7901 cells, the growth medium was replaced with serum-free medium supplemented with different concentrations of KBP (0, 80, 160, 320, 640, and 1,280 nmol/L). After incubation at 37°C for 48 h, the viable cells were quantified by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co.) assay. Absorbance was measured at 570 nm wavelength. Data represented absorbance as percentages of respective controls. The inhibitory effects of KBP on cell proliferation were expressed as IC50 values, which were determined from three independent tests.

**Quantitative Analysis of Apoptosis by Flow Cytometry**

HUVECs and SGC-7901 cells were seeded in gelatin coated or non–gelatin-coated 24-well plates in triplicate at a density of 2.5 × 104 per well. Twenty-four hours later, cells were exposed to KBP at different concentrations for another 24 h. HUVECs were treated with KBP at 40, 160, 320, and 640 nmol/L; and SGC-7901 cells were treated with 320 and 1,280 nmol/L KBP. Then, the cells were harvested for Annexin V–FITC Apoptosis Detection Kit (Sigma) following the protocol recommended by manufacturer. Cells treated with 10 μmol/L colchicine were used as positive control, and treated with PBS as negative control. The cells were subsequently counted by flow cytometry.

**Western Blot Analysis**

SGC-7901 cells were seeded in 90-mm plates and cultured in the growth medium until 90% confluency. The culture medium was replaced with DMEM added with or without KBP (640 nmol/L) and the cells were incubated under normoxic (20% O2, v/v) or hypoxic conditions (1% O2) at 37°C for 10 h. The cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 30 min. After incubation with 0.3% hydrogen peroxide to block endogenous peroxidase and subsequently with normal goat serum to inhibit nonspecific reactions, samples were incubated with the primary antibody overnight at 4°C. Anti-VEGF polyclonal antibody (1:100, Santa Cruz Biotechnology) and anti–HIF-1α polyclonal antibody (1:50, Santa Cruz Biotechnology) were used as primary antibodies, respectively. Then, samples were incubated with biotinylated anti-rabbit goat immunoglobulins for 30 min followed by incubation with horseradish peroxidase–labeled streptavidin-biotin complex for 30 min. Peroxidase reaction was visualized by the use of diaminobenzidine.
Statistical Analysis
All data were expressed as mean ± SE. SPSS 10.0 software was used for Student’s t test in all statistical analyses. A P value of <0.05 was considered statistically significant.

Results
Expression and Purification of KBP
KBP was expressed in E. coli and purified to apparent homogeneity by affinity chromatography using the His. Bind affinity column. The purified recombinant protein showed an apparent molecular weight of 45,000 Da according to Coomassie-stained SDS-PAGE, matching the calculated molecular weight from the sequence. The identity of the band was confirmed by Western blot analysis using an anti-His tag antibody (Supplementary Fig. S1).⑤ An average of 15 mg of purified KBP in soluble form was obtained from 1 liter of culture.

KBP Suppresses Growth and Metastasis of Human Gastric Carcinoma
To evaluate the effect of KBP on tumor growth, heterotopic xenografts were established. Nine days later, tumors reached a size of 50 mm³, and the mice received i.p. injection of KBP or PBS, respectively. KBP treatment significantly inhibited tumor growth when compared with PBS group, and an average of 61.4% suppression of primary tumor growth was observed (P < 0.01, n = 5; Fig. 1C). Since day 12 after KBP injection, the average tumor volume of KBP group was significantly lower than that of control group (Fig. 1D).

To mimic the growth microenvironment of gastric carcinoma, we further established an orthotopic implantation model of SGC-7901 cells to determine the in vivo efficacy of KBP. Consistent with the result in heterotopic tumor implantation model, orthotopic implanted tumor growth was also significantly suppressed by KBP, and an average of 52.3% suppression was observed (P < 0.01, n = 7; Fig. 2B). Intriguingly, metastasis in KBP group was significantly lower than that in control group. Metastasis of liver, lymph node, and peritoneum in the KBP group was found in 0, 2, and 1 mouse, respectively, versus that in the control group, in 2, 3, and 3 mice. No lung metastasis was found in two groups. All metastases were certified by tissue H&E staining (Fig. 2C).

Over the course of treatment, the KBP-treated mice showed no weight loss or unusual behavior, and histopathologic examination also did not find any detectable toxicity to liver or kidney (data not shown), suggesting that KBP, at the concentrations used, did not cause any detectable toxicity.

⑤Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
KBP Inhibits Tumor Angiogenesis

The effect of KBP on tumor angiogenesis was evaluated by CD34 immunostaining for capillaries in tumor tissues. The CD34-stained capillaries in the KBP group (Fig. 1F and I) was markedly lower than that in the control group (Fig. 1E and H). Microvessel density in tumors treated with KBP significantly reduced than that in PBS control ($P < 0.01$; Fig. 1G and J). These showed that KBP significantly reduced tumor angiogenesis and might inhibit tumor growth by antiangiogenesis.

Endothelial Cell–Specific Inhibition by KBP

KBP treatment resulted in fewer viable HUVECs at every concentration (Fig. 3A). This inhibitory effect seemed to be dose dependent, with an IC$_{50}$ of 70 nmol/L. However, KBP had no apparent effect on the growth of SGC-7901 cells (Fig. 3B). Even at the high concentration of 1,280 nmol/L, there was no apparent inhibitory effect observed. Similarly, KBP induced apoptosis of endothelial cell. As shown in Fig. 4, KBP increased apoptosis of HUVECs in a concentration-dependent manner (Fig. 4A and C). KBP did not induce apoptosis in SGC-7901 cells even at the high concentration of 1,280 nmol/L. These results showed that KBP suppressed tumor growth by block angiogenesis instead of a direct cytotoxic effect on tumor cells.

KBP Down-Regulates VEGF in Tumor Cells and Tumor Xenografts

Immunohistochemical analysis indicated that KBP inhibited VEGF expression in tumor xenografts (Supplementary Fig. S2). Western blot also showed that KBP treatment markedly down-regulated VEGF expression in cultured tumor cells and tumor tissues. Densitometric analysis of the bands showed VEGF protein levels in cultured tumor cells treated with KBP were decreased in a dose-dependent manner (Fig. 5A). KBP injection reduced VEGF protein level to ~32.5% of the control in heterotopic transplanted tumor (Fig. 5B) and 40.2% of the control in orthotopic transplanted tumor (Fig. 5C). These results suggested that the antiangiogenesis activities of KBP could be through down-regulation of VEGF expression.

We further measured VEGF in the conditioned medium by ELISA. As shown in Fig. 5D, KBP treatment resulted in reduced VEGF release in the medium, and the inhibitory effect was in a dose-dependent manner.

KBP Inhibits VEGF Expression at the Transcriptional Level

To determine the regulatory level of KBP on VEGF expression, we examined VEGF mRNA expression in tumor cells by reverse transcription-PCR. We found that VEGF mRNA expression in KBP-treated cells were significantly reduced than that in control cells (Fig. 5E). These results suggested that KBP regulated VEGF expression at the transcriptional level.

KBP Reduces Hypoxia-Inducible Factor 1α in Tumor Cells

To elucidate whether KBP inhibited transcription of VEGF through hypoxia-inducible factor 1α (HIF-1α), we...
examined the amount and location of HIF-1α in SGC-7901 cells by immunocytochemistry. As shown in Fig. 6A, HIF-1α protein mainly located in cytoplasm under normoxia (Fig. 6A3). Hypoxia apparently induced a lot more HIF-1α compared with normoxia, and HIF-1α mainly translocated into nucleus under hypoxia (Fig. 6A1). KBP treatment reduced HIF-1α proteins both under normoxia and hypoxia (Fig. 6A2). Consistent with the result of HIF-1α, KBP reduced VEGF expression both under normoxia and hypoxia (Fig. 6B). These results suggested that down-regulation of VEGF by KBP in SGC-7901 cells might be through inhibition of HIF-1α.

Discussion

Gastric carcinoma is still one of the most frequently diagnosed cancers and the second most common cause of death from cancer worldwide (26). Despite significant advance in treatment, the overall 5-year survival remains ~20% (27, 28). Because of the discouraging outcome, there is a vigorous search for new agent and strategy. In this study, we showed for the first time that KBP inhibited growth and metastasis of gastric carcinoma by reducing VEGF production and angiogenesis.

Our previous study has shown that KBP has antiangiogenic activity, and intravitreal injection of KBP inhibits retinal neovascularization in a rat model (5). In the present study, we showed that KBP treatment significantly inhibited growth of gastric carcinoma by antiangiogenesis. After i.p. injection of KBP, an average of 61.4% suppression of primary tumor growth was observed in heterotopic tumor. Biological properties of implanted tumor depend on the proper organ microenvironment. The orthotopic model...
provides reproducible and reliable methodology for the study of organ-specific determinants of the biology and therapy of cancers (22, 29) and allows us to accurately determine the in vivo efficacy of KBP. Therefore, we further established orthotopic transplantation models. KBP also inhibited orthotopic transplanted tumor growth, and an average of 52.3% suppression of primary tumor growth was observed. Immunostaining with CD34 antibody showed that microvascular density in tumor tissues treated with KBP were markedly decreased, suggesting that KBP inhibited tumor angiogenesis. A previous study also showed that KBP gene delivery markedly inhibited human breast tumor xenograft growth by antiangiogenesis (4). It was found that KBP did not affect proliferation and apoptosis of SGC-7901 cells even at high dose in this study, indicating that KBP has no direct anticancer property by targeting cancer cells. These data showed that KBP was a potent angiogenic inhibitor and suppressed tumor growth by antiangiogenesis.

Comparing with the effect of KBP on angiogenesis, the mechanism underlying the antiangiogenic activity is not well understood. Our previous study showed that the antiangiogenic activity of KBP was not through inhibiting kallikrein activity or kinin production (5). Here, we showed that treatment with KBP resulted in growth inhibition and apoptosis of HUVECs in a dose-dependent manner, consistent with previous studies (4, 5). KBP has been shown to significantly inhibit proliferation, adhesion, and migration of endothelial cells induced by VEGF or basic fibroblast growth factor, and attenuated VEGF and basic fibroblast growth factor–induced increase in capillary density in s.c. implanted Matrigel plugs (4). KBP also blocked VEGF binding to endothelial cells (5). KBP is a heparin-binding protein as VEGF and basic fibroblast growth factor (30); the major heparin-binding domain was identified in the region between the H helix and C2 sheet of KBP (31). These findings suggested that KBP could inhibit angiogenesis by competing with some growth factors (e.g., VEGF and basic fibroblast growth factor), binding to endothelial cells, and attenuating endothelial cells response to growth factors, and resulted in endothelial cells growth inhibition and apoptosis.

VEGF is the most potent and specific angiogenic stimulator and secreted by almost all solid tumor cells.
(32). Our previous study showed that KBP treatment resulted in decrease of VEGF expression and release in retinal capillary endothelial cells. Intravitreal injection of 25 µg KBP reduced retinal VEGF concentrations to ~35% (5). However, the effect of KBP on the expression and release of VEGF in tumor cells has not been investigated. In this study, we showed for the first time that KBP reduced production of VEGF in SGC-7901 tumor cells in vitro and in vivo. Western blot analysis showed that expression of VEGF in cultured SGC-7901 cells and tumor tissues treated with KBP was markedly decreased, and the release of VEGF in conditioned medium of SGC-7901 cells was also reduced, as examined by ELISA. Down-regulation of VEGF mRNA by KBP in SGC-7901 cells was confirmed by reverse transcription-PCR, thus suggesting the regulation at the transcriptional level.

Therefore, HIF-1α, a crucial transcriptional factor for VEGF expression, was examined in SGC-7901 cells treated by KBP. Hypoxia is inevitable during tumor progression and subsequently promotes tumor progression by stimulating angiogenesis, invasion, and metastasis (33). In response to hypoxia, cells exhibit adaptive responses and the key factor involved in the adaptive response is HIF-1α. HIF-1α is a crucial upstream transcriptional factor of VEGF expression, especially in tumors (34). Under hypoxic condition, HIF-1α expression increases as a result of decreased ubiquitination and degradation. Then, HIF-1α translocates into the nucleus, binds to the hypoxic response element in the gene promoter, and activates transcription of downstream genes, including VEGF. A previous study has shown that expression of HIF-1α is closely associated with VEGF expression, angiogenesis, and outcome of gastric carcinoma (35). Inhibition of HIF-1α reduced VEGF expression and angiogenesis in gastric carcinoma, and suppressed tumor growth by antiangiogenesis (36, 37). In the present study, it was found that hypoxia apparently induced a lot more HIF-1α compared with normoxia and HIF-1α protein mainly translocated into nucleus under hypoxia (Fig. 6A1). KBP treatment reduced HIF-1α proteins in SGC-7901 cells (Fig. 6A and B); thus, fewer HIF-1α in the presence of KBP led to fewer HIF-1α in the nucleus, suggesting that KBP could inhibit HIF-1α nuclear translocation. Consistent with the result of HIF-1α, KBP significantly reduced VEGF expression in SGC-7901 gastric carcinoma cells and xenografted tissues. These results suggested that KBP might suppress gastric carcinoma through down-regulation of VEGF in tumor cells via inhibition of HIF-1α. The exact mechanism for the effect of KBP on HIF-1α is uncertain. KBP might directly inhibit HIF-1α expression or indirectly affect HIF-1α degradation via VHL. The details need to be further investigated.

VEGF expression significantly correlated with tumor angiogenesis, progression, and prognosis of gastric carcinoma (38, 39). Antiangiogenesis therapy has shown to effectively inhibit tumor growth and metastasis of gastric carcinoma (40, 41), and VEGF has become a very important target for tumor therapy (42, 43). In the present study, we found that KBP reduced VEGF production of gastric carcinoma in vivo and in vitro, and i.p. injection of KBP resulted in growth inhibition of gastric carcinoma xenografts. Moreover, our preliminary data suggested that KBP might have inhibitory effect on metastasis of gastric carcinoma, although the final conclusion needs to be further evaluated by enlargement amount of samples. These results suggested that KBP might be a promising candidate of antiangiogenic reagent for gastric carcinoma.
KBP had some advantages over cytotoxic chemotherapy. KBP inhibited growth and induced apoptosis of HUVECs, but had no direct effect on proliferation and apoptosis of tumor cells. A previous study also showed that KBP did not influence growth of pericytes or Müller cells (5), suggesting that KBP specifically targeted endothelial cells. Vascular endothelial cells are genetically stable cells with low mutation rate, and hence agents targeting endothelial cells are less likely to induce drug resistance (44). The immature characteristics of tumor vasculature are quite different from normal quiescent vasculature; therefore, antiangiogenic agents targeting tumor vasculature have little toxicity (45). Intravitreal and i.p. injection of KBP did not cause any detectable inflammatory response or toxicity, suggesting that systemic administration with KBP was safe. In addition, KBP has shown a protective effect during acute phase inflammation and increase the survival rate of mice after endotoxin shock (46). Moreover, KBP could be produced with a high yield in E. coli as a soluble protein with antiangiogenic activity (5).

Taken together, we showed for the first time that KBP inhibited growth of gastric carcinoma by antiangiogenesis. Down-regulation of VEGF expression and release in tumor cells through inhibiting HIF-1α, thus attenuating the paracrine effect of VEGF on endothelial cell proliferation and vascular permeability in tumor tissues, may represent a novel mechanism for the antiangiogenic and antitumor activity of KBP.

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