Inhibitory effect of dimeric β peptide on the recurrence and metastasis of hepatocellular carcinoma in vitro and in mice

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Abstract

AIM: To block the adhesion of tumor cells to the extracellular matrix, and prevent tumor metastasis and recurrence, the dimer of the β peptide (DLYLMDLSYSMK-GDLYLMDSL YSMK, β2) was designed and synthesized and its anti-adhesion and anti-invasion effects on hepatocellular carcinoma cells were assessed. Additionally, its influence on the metastasis and recurrence of mouse hepatocellular carcinoma was measured.

METHODS: The anti-adhesion effect of β2 on the highly metastatic hepatocellular carcinoma cell line HCCLM6 cells and fibronectin (FN) was assayed by the MTT assay. The inhibition of invasion of HCCLM6 cells by β2 was observed using a Transwell (modified Boyden chamber) and matrigel. Using the hepatocellular carcinoma metastasis model and LCI-D20 nude mice, the influence of β2 on the metastasis and recurrence of hepatocellular carcinoma after early resection was investigated.

RESULTS: HCCLM6 cells co-incubated with 100 µmol/L, 50 µmol/L, 20 µmol/L, or 10 µmol/L β2 for 3 h showed an obvious decrease in adhesion to FN. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. Additionally, HCCLM6 cells cultured with 100 µmol/L β2 had a dramatic decrease in cell invasion. β2 was also observed to inhibit the incisal edge recurrence and the distant metastasis of nude mice hepatocellular carcinoma after early resection (P < 0.05).

CONCLUSION: The β2 peptide can specifically block the adhesion and invasion of HCCLM6 cells, and can inhibit HCC recurrence and metastasis of LCI-D20 model post-hepatectomy in vivo. Thus, β2 should be further studied as a new anti-tumor drug.

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Key words: β peptide; Hepatocellular carcinoma; Anti-adhesion; Invasion; Metastasis; Recurrence

INTRODUCTION

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC) and the prevention of postoperative metastasis, the 5-year postoperative recurrence rate of HCC is still very high[9-12]. Many efforts have been made to develop a more efficient treatment to inhibit and prevent tumor metastasis, as the recurrence and metastasis of HCC is still a large problem in clinical practice. It is well known that the metastatic process is very complex, including tumor cells dissociating from the primary locus, invading the surrounding tissue, entering and extravasating from the circulation, and growing in distant organs[13-16]. During this process, cell adhesion is one of the most important events[16]. Many studies have been focused on the synthesized anti-adhesion peptides[6-8]. However, the application of these short peptides is limited due to their short half-life and high dosage required. To prolong the peptide's half-life, the polymer and a derivative of synthesized peptides were designed[9-11]. The anti-tumor metastasis effect of the repeat sequence of synthesized peptides was stronger than that of non-repeat peptides[12,13].

Integrins are a family of adhesion molecules located on cells and in the extracellular matrix. The expression level of integrins is related closely to a cell's migration ability[14,15]. The anti-adhesion peptide β (DLYLMDL YSMK, β1) was designed by Liu et al[8], according to the conserved sequence of the integrin α and β unit. This peptide can block the
interaction between tumor cells and the extracellular matrix and can also inhibit intrahepatic and pulmonary metastases after carcinosectomy in a nude mouse model with human HCC of high metastatic potential (LCI-D20)\textsuperscript{[17-21]}. On the basis of these studies, here we have designed and synthesized the dimeric peptide β (β2). The effects of β2 on the adhesion of human liver cancer cell line HCCLM6 cells to fibronectin (FN), the invasion of HCCLM6 cells to reconstituted basement membrane, as well as liver cancer recurrence and metastasis after hepatectomy in a nude mouse model were investigated.

**MATERIALS AND METHODS**

**Cell culture**

The highly metastatic hepatocellular carcinoma cell line HCCLM6, initially established and preserved by the Liver Cancer Institute, Fudan University, was cultured in Dulbecco’s modified eagle’s medium (DMEM, Gibco, UK), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and grown at 37°C under an atmosphere of 5% CO\textsubscript{2}. The medium was replenished every three days to maintain cell growth.

**Coating the 96 well high bind microplate with FN**

Ten μg/mL FN (Sigma, USA) solution (containing 10 μg/mL FN, 20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl\textsubscript{2}, 1 mmol/L CaCl\textsubscript{2}, 1 mmol/L MnCl\textsubscript{2}) was added to a 96-well high bind microplate (Corning, USA) (100 μL per well), and allowed to incubate at 4°C overnight. The plate was then incubated with blocking buffer (10 mmol/L Hepes, pH 7.4, 140 mmol/L NaCl, 5.4 mmol/L KCl, 5.56 mmol/L glucose, 3% BSA, 1 mmol/L MgCl\textsubscript{2}, 2 mmol/L CaCl\textsubscript{2}, 1 mmol/L MnCl\textsubscript{2}) at 37°C for 2 h and air dried for further use.

**Cell adhesion assay**

β2 peptide was designed in our laboratory using the sequence DLYYLMDLSYSMKGGDLYYLMDLSYSM. The peptide was synthesized by Shanghai Sangon Bioengineering Company. 100 μL of a HCCLM6 suspension (2 × 10\textsuperscript{5}/mL) was plated in each well of an FN coated 96-well high bind microplate. 100 μL DMEM medium containing β2 at a concentration of 200 μmol/L, 100 μmol/L, 40 μmol/L or 20 μmol/L was added to the cells concomitantly. The same volume of cell culture medium in place of β2 was added to the control group. 200 μL of cell culture medium only was added in the plate for the blank group. The assay was conducted in quintuplicate for each sample. After incubation for 3 h at 37°C, under an atmosphere of 5% CO\textsubscript{2}, the unattached cells were gently washed away with HANKS buffer. The attached cell number in each well was measured by MTT. The inhibition rate of β2 on cell adhesion to FN was calculated with the following equation: Cell adhesion inhibitory rate = (average OD of control well-average OD of β2-treated well)/average OD of control well-average OD of blank well) × 100%.

**MTT assay**

The number of attached cells in each well was examined by the MTT assay, as previously described\textsuperscript{[22]}, and quantified by a micro-titer plate reader (Amersham, USA). Briefly, after incubation for 3 h at 37°C in 5% CO\textsubscript{2}, the unattached cells were removed by gentle washing with HANKS buffer. 100 μL DMEM and 20 μL MTT (5 mg/mL) (Sigma, USA) were added to each well. After incubation at 37°C for 4 h, the medium was discarded. 200 μL of 0.04 mol/L hydrochloric acid in isopropanol was added to each well. The amount of MTT formazan product, which reflects the number of cells adhering to FN, was determined by measuring absorbance with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

**Invasion assay**

Invasion assays were performed as described previously\textsuperscript{[23]}. Briefly, the upper portion of Transwell chambers (Corning, USA) were coated with 75 μL of Matrigel (BD, USA) diluted 1:10 in serum-free DMEM and incubated at 37°C for 2 h. The supernatants of HCCLM6 cells containing DMEM with 10% FCS were harvested after the cells had grown to confluence, and after adding FN at a final concentration of 5 μg/mL, resulting in conditioned medium. The trypsinized cells were harvested and diluted to a 2 × 10\textsuperscript{4}/mL cell suspension with serum-free DMEM. 100 μL of the cell suspension and 100 μL of 200 μmol/L β2 peptides in serum-free DMEM or serum-free DMEM only as a control were added in the upper chambers. Concurrently, 600 μL of conditioned medium was added to the bottom chamber of the Transwell plate. After incubation at 37°C for 48 h under a 5% CO\textsubscript{2} atmosphere, the non-invading cells and the gel were gently removed from the upper chamber with cotton-tipped swabs. Cells were rinsed with PBS, and the cells on the filters were fixed with Formaldehyde and stained in Giemsa staining solution for 30 min. The number of invaded cells on the filters was counted in 5 randomly selected high-powered (× 200) fields per filter under a microscope (Leica, Switzerland). Invasion inhibitory rate was expressed as the following equation: Invasion inhibitory rate = [1 - (invaded cell number in β2 chamber/invaded cell number in control chamber)] × 100%.

**Animal model and treatment**

Twelve 5-wk-old male nude mice (BALB/cA) weighing 17-20 g were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The nude mouse model of human hepatocellular carcinoma with high metastatic potential (LCI-D20), which was established in Zhongshan Hospital Liver Cancer Institute, Fudan University, was used in this study. A tumor block of LCI-D20 nude mice human liver cancer metastasis model was implanted into the left lobe of the nude mouse liver as described previously\textsuperscript{[24]}. Briefly, a left upper abdominal transverse incision was made under anesthesia; the left lobe of the liver was exposed and a part of the liver surface was mechanically injured with scissors. Next, a tumor block of 0.2 cm × 0.2 cm × 0.2 cm was fixed within the liver tissue. After the operation, mice were kept in laminar-flow cabinets under specific-pathogen-free conditions and given free access to mouse chow. Liver cancer early resection
RESULTS

The inhibitory effect of β2 on the adhesion of HCCLM6 cells to FN

The inhibitory effect of β2 on the adhesion of HCCLM6 cells to FN is shown in Figure 1. HCCLM6 cells co-incubated with 100 μmol/L, 50 μmol/L, 20 μmol/L and 10 μmol/L β2 for 3 h led to an obvious decrease in cellular adhesion. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. This observation indicates that β2 is able to inhibit the adhesion of HCCLM6 cells to FN, and thus β2 might obstruct the invasion of HCC cells to paratumor liver parenchyma.

The inhibitory effect of β2 on the invasion ability of HCCLM6 cells

After incubation with 100 μmol/L β2, the number of invaded HCCLM6 cells was decreased. The inhibitory rate was 36.8% (Table 1). Thus, β2 might block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation in vivo.

The influence of β2 on the intrahepatic recurrence of the LCI-D20 model after early resection

On the 10th d post-tumor-implantation, LCI-D20 tumors were resected, and β2 or the same volume of saline was subcutaneously injected. On day 55, mice were sacrificed to check for intrahepatic recurrence. The recurrent tumor was located around the incisal margins. Compared with the control group, the weight of the intrahepatic recurrent tumor of the β2 group was markedly decreased and statistically significant. There were 4 (4/6) mice with intrahepatic recurrent tumor in the β2 group, while there were 6 (6/6) mice with an intrahepatic recurrent tumor in the control group (Figure 1 and Table 2). These results indicate that β2 have inhibitory effects on tumor recurrence in the incisal margin.

DISCUSSION

The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. Blocking the interaction between tumor cell adhesion molecules and their ligands is a major target in the prevention of cancer metastasis. Many studies have focused on the synthesized anti-adhesion peptides. One such peptide is RGD, derived from the common conserved sequence of the main matrix
proteins such as fibronectin, collagen and fibrinogen. A second peptide is YIGSR\(^{[31]}\), which originated from the basement membrane protein laminin. The third peptide is EILDV\(^{[32]}\), which stemmed from the core sequence of fibronectin. The application of these short peptides was limited due to their short half-life, the ease with which they are degraded and the requirement for a high dosage. To prolong the peptides’ half-life, the polymer and derivative of synthesized peptides were designed. The anti-tumor metastatic effect of repeat sequence of synthesized peptides was stronger compared to non-repeat peptides. The more times the sequence is repeated, the stronger the anti-metastasis effect is.

FN is an important cell adhesion molecule in the extracellular matrix. It mediates cell adhesion and migration, and plays a significant role in tumor invasion and metastasis. Assaying FN adhesion to tumor cells is a method commonly used for studying tumor cell metastasis. In this study, the extracellular matrix was simulated by coating cell culture plates with FN, after which the inhibitory effects of \( \beta_2 \) peptide on FN adhesion to liver cancer cells were investigated. The results demonstrated that after co-culturing the peptides with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of \( \beta_2 \) peptide on FN adhesion to tumor cells was observed.

Tumor cells must penetrate the basement membrane for invasion of the basement membrane for at least three times during metastasis; i.e. dislodging from the original site, entering blood circulation, and migrating from blood flow into remote sites. Matrigel, used as a basement membrane matrix, is produced from mouse Engelbreth-Holm-Swarm sarcoma rich in extracellular matrix protein. The artificial basement membrane is plated on a Millipore filter in Transwell culture chambers, and forms a membrane structure similar to natural basement membrane. Invasive, metastatic tumor cells can penetrate the membrane under the induction of chemotactics, simulating tumor cells’ invasion of the basement membrane in vivo. The results indicated that \( \beta_2 \) exerted significant inhibitory effects on the invasion of HCCLM6 cells.

Metastasis and recurrence of liver cancer is a major determinant for the prognosis and long-term survival of liver cancer patients. Polypeptide therapy is a newly developed treatment for tumors\(^{[33]}\), but its clinical application is restricted by the degradation of these peptides. \( \beta \) peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisal margins.

The \( \beta \) peptide blocked tumor cell adhesion to FN through two possible mechanisms. First, the \( \beta \) peptide took up the integrin binding site competently through binding to the RGD sequence of the matrix protein. Next, the \( \beta \) peptide also interacted with integrin because the \( \beta \) peptide was designed according to the conserved sequence of the integrin \( \alpha \) and \( \beta \) unit.

Taken together, these cell and animal studies demonstrated that the \( \beta_2 \) peptide can prevent and treat liver cancer adhesion and metastasis and recurrence. Therefore, the \( \beta \) peptide is worthy of further investigation, as it is a potential drug for blocking tumor metastasis and recurrence.

### Table 3 The lung metastasis in liver cancer nude mouse models after early resection

| Number  | The total number of metastatic nodes in lung | The number of mice with lung metastatic nodes |
|---------|---------------------------------------------|---------------------------------------------|
| Control group | 6  | 30  | 4 |
| \( \beta_2 \) group | 6  | 11\(^{a}\) | 2 |

\(^{a}\) \( P < 0.05 \) vs control group.

### COMMENTS

#### Background

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC), metastasis and recurrence remain the main obstacles for HCC patients gaining a better outcome and long-term survival. It is well known that during the metastatic process, cell adhesion is one of the most important events. The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. So, blocking the interaction between tumor cell adhesion molecules and their ligands has become a major target in prevention cancer metastasis.

#### Research frontiers

To prevent tumor metastasis and recurrence through inhibiting the adhesion of tumor cells, many studies have focused on the synthesized anti-adhesion peptides such as RGD, YIGSR and EILDV. These peptides are derived from the common conserved sequence of the main matrix proteins such as fibronectin, collagen, fibrinogen and laminin. Liu et al designed a new anti-adhesion peptide \( \beta \) (DLYLYMDLSYSMK, \( \beta_1 \)) according to the conserved sequence of the \( \alpha \) and \( \beta \) unit of integrins. These peptides can inhibit the adhesion of tumor cells and cancer metastasis and recurrence. But their application is limited due to the short half-life and high dosage required.

#### Innovations and breakthroughs

On the basis of Liu’s study, to prolong the peptide’s half-life, the dimmer of \( \beta \) peptide (DLYLYMDLSYSMKGGDLYYLMDLSYSMK, \( \beta_2 \)) was designed and synthesized and the anti-adhesion and anti-invasion effect of it on hepatocellular carcinoma cells, as well as it’s influence to the metastasis and recurrence of mouse hepatocellular carcinoma were measured. The result showed that \( \beta_2 \) can inhibit the adhesion of HCCLM6 cells to FN in dose-effect manner. And the number of invaded HCCLM6 cells was decreased when incubated together with \( 100 \) \( \mu \)mol/L \( \beta_2 \). Compared with the control group, the weight of the intrahepatic recurrent tumor and the number of metastatic nodes in lung of the \( \beta_2 \) group were markedly decreased.

#### Applications

\( \beta_2 \) might obstruct the invasion of HCC cells to paratumor liver parenchyma and block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation in vivo. In addition, \( \beta_2 \) have inhibitory effects on tumor recurrence in the incisal margin and a significant preventive and therapeutic effect on the metastasis of liver cancer. Taken together, these cell and animal studies demonstrated that the \( \beta_2 \) peptide can prevent and treat liver cancer adhesion, metastasis and recurrence.

#### Peer review

On the basis of previous work, the \( \beta_2 \) peptide (DLYLYMDLSYSMKGGDLYYLMDLSYSMK, \( \beta_2 \)) was designed and synthesized. After co-culturing with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of \( \beta_2 \) peptide on FN adhesion to tumor cells was observed. And also \( \beta_2 \) showed significant inhibitory effects on the invasion of HCCLM6 cells. Furthermore, \( \beta_2 \) peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisal margins. These results indicate that \( \beta_2 \) have a significant preventive and therapeutic effect on the metastasis of liver cancer.

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