Experimental Study of the Prophylactic and Therapeutic Effects of Venin on Metastasis and Recurrence of Liver Cancer

Jingjing Sun¹
Zhiyong Wu¹
Xinda Zhou²
Yinkun Liu²

¹ Department of General Surgery, Renji Hospital, Shanghai Second Medical University, Shanghai 200127, China.
² Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China.

OBJECTIVE To study the inhibitory effect of venin on adhesion and invasive ability of SMMC–7721 cells and to examine the prophylactic and therapeutic effect of venin on liver cancer metastasis and recurrence after hepatectomy.

METHODS The blocking effect of venin on the intercellular adhesive molecule (ICAM–1) of 7721 cells was analyzed by immunofluorescence flow cytometry. The influence of venin on the invasive ability of 7721 cells was observed by cell–migration experimentation and detachment of 7721 cells attached to fibronectin (FN), and the influence of venin on adhesion of 7721 cells to FN by the MTT method. 7721 cells to 7721 cells. 7721 cells to lymphocytes, and 7721 cells to endothelial cells by a cellular adhesion test. The preventive and therapeutic effect of venin on metastasis and recurrence of a liver cancer model was observed in nude mice after hepatectomy.

RESULTS The expression of ICAM–1 in the venin–treated group was significantly lower than that in the untreated group. Venin could not inhibit the invasive ability of 7721 cells, and could not exfoliate the 7721 cells adhered to FN. It could inhibit the adhesion between 7721 cells and 7721 cells, and between 7721 and endothelial cells, but could not inhibit the adhesion between 7721 and lymphocytes. The nude mice treated with venin had less intrahepatic or extrahepatic metastases and recurrences after hepatectomy.

CONCLUSION Venin can inhibit the adhesive ability of SMMC–7721 cells and can also prevent and treat the metastasis and recurrence of liver cancer in nude mice after hepatectomy.

KEYWORDS: liver cancer, metastasis, adhesion molecule, anti–adhesion, venin.

Although immunotherapy, traditional Chinese herbal treatment and preventive transhepatic artery chemotherapy embolism (TACE) have been used to prevent and treat metastasis and recurrence postoperation, liver cancer remains a disease with a poor prognosis, the 5-year recurrence rate after surgery is still as high as to 61.5%, even that of small hepatic cancer is up to 43.5%. At present, metastasis and recurrence are still the key points influencing the prognosis and long-term survival rate of patients with liver cancer. It is imperative that new treatment modalities be investigated. In recent years,
attention has been paid to the fact that tumor cell's high adhesiveness is related to its high metastatic ability. Anti-coagulant medication has an antineoplastic effect. The influence of venin on the ability of adhesion and metastasis of SMMC-7721 cells was observed in this research.

MATERIALS AND METHODS

Materials
The SMMC-7721 cell strain of liver cancer was taken from a model of high metastasis using a nude mice-human liver cancer from the Second Military Medical University and Liver Cancer Institute of Zhongshan Hospital affiliated to Shanghai Medical University. Murine anti-human ICAM-1 monoclonal antibody was from R&D Systems, goat anti-mouse double antibody was from BD Biosciences, bovine fibronectin (FN) was from Sigma Co., MTT was from ESRVA, imported and repackaged, ^H-TdR from Shanghai Isotope Co., M199 culture fluid and RPMI-1640 culture fluid were from Gibco Co., A LS-6500 liquid scintillation counter and FACS caliber flow-cytometer have from the Beckman Co. and 3350UV enzyme labeling instrument from the BioRad Co.

Venin was purchased from Shenzhen Changbaishan Pharmaceutical Factory, (92) Yue Wei Yao Zhun Zi No. K-034. Since nude mice have an excessively effective blood coagulation system, a dose of venin 20 times that used for patients per kilogram body weight or of unit volume blood was taken as the dose in animal and cell experiments in this study.

Methods

A flow-cytometer was used to detect the ICAM-1 expression on 7721 cell membrane surfaces
The 7721 cell strain cultured in the RPMI-1640 cell culture fluid containing \( 5 \times 10^{-4} \) U/ml venin and 10% calf serum was the venin-treated group, and that cultured in equal volume of normal saline was the control group; both specimens were cultured for 72 hrs. Then both 7721 cells were treated with 0.01% EDTA to prepare cell suspensions. After PBS washing, mouse anti-human monoclonal antibody ICAM-1, 30 \( \mu l \) (0.33 mg/ml) was added. Incubation at 4°C for 30 min, 20\( \mu l \) of 1:40 diluted goat anti-mouse fluorescein isothiocyanate (FITC) antibody was added and incubated at 4°C for 30 min, then the scintillation value was detected by FACS Calibur flow cytometry.

The invasion and adhesion experiment of 7721 cell to FN
The cell suspension was added into six-well cell culture plates coated with FN. When the cells grew up over the plate, a 2 mm wide cell-free strip was scraped out at the bottom of the plate. The cell culture fluid was replaced with new fluid, discarding the suspended cells, then venin at \( 5 \times 10^{-4} \) U/ml was added into the culture wells, and an equal volume of normal saline into that of the control group. At the 12th, 24th and 36th hour of cell growth, the width of cell-free strip was detected and adhesion of cells on the wall observed.

Quantitative determination of FN-7721 cell adhesion by the MTT method
A suspension of 7721 cells at \( 1 \times 10^6 \) /ml was added into a 96-well cell culture plate coated with FN, 100 \( \mu l \) per well. At the same time 100 \( \mu l \) of RPMI-1640 culture fluid of calf serum was added. Venin culture fluid \( 5 \times 10^{-4} \) U/ml was added into culture wells of the venin group, normal saline of equal volume added into wells of the positive control and those without added cells were taken as a negative control group. Five multiple wells were designed in each experiment. After incubation under 5% CO\(_2\) at 37°C for 10, 20, and 40 min respectively, the suspension was discarded. Irrigation by Hank's solution for three times was done to remove the nonadhered cells. After adding 20 \( \mu l \) of MTT solution at a concentration of 5 mg/ml and incubation under 5% CO\(_2\) at 37°C for 4 hrs, the fluid was discarded, and 200 \( \mu l \) of 0.04N isopropanol hydrochloride added. The OD value of every well at 570 nm was determined in 3350UV enzyme labeling instrument with the reference wavelength at 630 nm. The OD value represented the number of adhesive
cells.

**Primary culture of endothelial cells**
A mixture of 0.25% pancreatin and 0.01% collagenase was injected into the umbilical vein of a fresh human umbilical cord, and incubated at 37°C for 20 min. The digestion mixture was extracted, centrifuged and discarded. After adding 20% inactivated bovine serum, 10% inactivated fetal ox serum and 700 mg/L M199 of endothelial cell growth factor crude extract to a suspension, the suspension was incubated at 37°C in 5% CO₂, replacing the suspension every other day. After fusion of the cells, and the digestion with the mixture of 0.02% EDTA and 0.25% pancreatin was completed, the fluid was diluted 1:3 and transferred to another bottle for regeneration.

**Separation of lymphocytes**
Human anticoagulant blood (3ml) was collected through the antecubital vein, and was added to Ficoll lymphocyte-separating fluid, from the middle layer of which lymphocytes were taken out after centrifugation. RPMI-1640 culture fluid containing 10% AB human serum was added and incubated at 37°C in 5% CO₂. The culture fluid was replaced every other day for 7 days in order to kill the erythrocytes in the mixture and to increase the purity of the lymphocytes. Then the culture fluid was replaced by a fluid containing ³H-TdR 5μCi/ml, and the cells were labeled for 48 hr.

**Labeling of 7721 cells**
After regeneration, the 7721 cells were incubated at 37°C in 5% CO₂ for 24 hr. The culture fluid was replaced by a fluid containing ³H-TdR 5μCi/ml and the cells, labeled for 36 hr. Then the cells were irrigated with Hank’s solution 3 times, and 0.24% pancreatin added to form a cell suspension for use.

**Experiment for cell adhesion**
For 7721 cell-7721 cell adhesion, the target cells were all 7721 cells, the cells to be detected were lymphocytes. The target cells were transferred to a 96-well plate to be cultured and fused. 100 μl of labeled cells (2.5 x 10⁶/ml) were added. For the venin group, venin was added at 5 x 10⁻⁶ U/ml. For the control group an equal amount of normal saline was added; those specimens without removal of non-adhered labeled cells were taken as the positive control, 3 similar wells for each specimen. The cells were discarded after culturing at 37°C for 20 min, 40 min, 60 min and 90 min, and the plate irrigated with Hank’s solution 3 times. On digestion by pancreatin, the cells were adhered to 49# cellulose acetate filter paper and dried, LS-6500 liquid scintillator was used to determine the scintillation value in cpm. Cell adhesion percentage= (adhesion cell cpm-blank cpm)/(total cell cpm-blank cpm).

**Effect of venin on metastasis and recurrence of LCI-D20 human liver cancer metastasis model in nude mice after liver cancer resection**
In the model, metastasis occurred at 19th day after implantation of liver cancer. In nude mice, liver cancer resections were performed on 16th day (before metastasis or "early") and 22th day (after metastasis or "late") after in-situ implantation of liver cancer 0.2 cm x 0.2cm x 0.2 cm. They were given a subcutaneous injection of venin, 1.6 x 10⁻⁶ U/mouse/day for 5 days. The mice were killed 35th day after implantation, both lungs were taken out, fixed in 10% formalin solution and embedded with paraffin. Ten continuous sections were made at 5 μm thickness at the interval of 50 μm for each lung. The number of metastasis in the 20 sections of both lungs was recorded. The intrahepatic metastasis nodes and incisional margin recurrence were also recorded by gross observation. Diameter of incisional recurrence focus=(maximal diameter of tumor + perpendicular diameter of tumor) * 2. Besides the remote metastatic foci in the lungs, metastases were also grossly found and recorded at the incision, in the mesentry, genital organs, hepatic hilum, lower pole of the kidney, splenic hilum, diaphragm, and upper margin of the pancreas.
RESULTS

Expression of ICAM-1 on 7721 cell membrane
The scintillation cpm was 1531.30 in the control group and 591.48 in the venin treatment group, showing a marked decrease of ICAM-1 in 7721 cell surface expression after venin treatment.

Morphologic observation of the 7721 cell invading ability
In the control group, the cell-free strip shrank gradually with time and finally disappeared. In the venin-treated group the cell-free strip shrank but was still clearly visible after 36 hr.

Quantitative determination of 7721 cell -FN adhesion by the MTT method
As shown in Table 1, 7721 cells adhered to FN were less in the venin group than in the control group, but only statistically different at the 20 min cultured group.

Table 1. Comparison of OD value of 7721 cells -FN adhesion (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>10 min.</th>
<th>20 min.</th>
<th>40 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>5</td>
<td>20.0±5.6</td>
<td>56.0±16.7</td>
<td>56.6±9.0</td>
</tr>
<tr>
<td>Venin</td>
<td>5</td>
<td>15.1±7.2</td>
<td>25.1±8.1</td>
<td>47.4±17.5</td>
</tr>
<tr>
<td>Negative control</td>
<td>5</td>
<td>4.9±2.3</td>
<td>5.4±1.2</td>
<td>8.0±1.6</td>
</tr>
</tbody>
</table>

*In comparison with positive control group P<0.05.

Adhesion between 7721 cells and other cells
As shown in Table 2, when cells were cultured for 20 min, 40 min, and 60 min, the adhesion rate among 7721 cells in the venin group were lower than that in the control.

Table 2. Adhesion rate of 7721 cells to 7721 cells at different times in different groups (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>20 min.</th>
<th>40 min.</th>
<th>60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>10.2±1.2</td>
<td>63.2±35.6</td>
<td>60.8±10.1</td>
</tr>
<tr>
<td>Venin</td>
<td>3</td>
<td>17.2±5.8</td>
<td>34.6±5.4</td>
<td>37.9±5.1</td>
</tr>
</tbody>
</table>

* In comparison with control group, P<0.05.

Effect of venin on metastasis and recurrence of LCl--D20 nude mice human liver cancer model posthepatectomy

Intrahepatic metastasis and recurrence
Normal nude mouse liver has 7 lobes. The number of liver lobes affected by metastatic foci in the venin treated group was less than that in the corresponding control group, as shown in Table 5. Venin might also inhibit recurrences at the incisional margin after early resection of the tumor.

Table 3. Adhesion rate of 7721 cells and endothelial cells at different times in different groups (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>20 min.</th>
<th>40 min.</th>
<th>60 min.</th>
<th>90 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>3.4±1.2</td>
<td>19.3±9.2</td>
<td>83.9±4.9</td>
<td>96.2±11.1</td>
</tr>
<tr>
<td>Venin</td>
<td>3</td>
<td>4.2±4.1</td>
<td>7.5±7.2</td>
<td>28.5±7.1</td>
<td>42.7±29.2</td>
</tr>
</tbody>
</table>

* In comparison with control group, P<0.05.

Table 4. Adhesion rates between 7721 cells and lymphocytes at different times in different groups (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>20 min.</th>
<th>40 min.</th>
<th>60 min.</th>
<th>90 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>14.2±6.9</td>
<td>44.2±13.2</td>
<td>42.6±34.9</td>
<td>43.6±11.5</td>
</tr>
<tr>
<td>Venin</td>
<td>3</td>
<td>17.2±7.6</td>
<td>45.3±29.5</td>
<td>35.6±4.2</td>
<td>68.8±38.1</td>
</tr>
</tbody>
</table>

* In comparison with control group, P<0.05.

The adhesion rates between 7721 cells and lymphocytes showed no significant differences between the two groups at any time, as shown in Table 4.
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Table 5. Intrahepatic metastasis and recurrence of liver cancer after tumor resection (2±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>Positive metastasis (mice)</th>
<th>No. of intrahepatic metastatic foci / mouse</th>
<th>No. of lobes affected / mouse</th>
<th>Diameter of incisional tumor margin (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, early</td>
<td>5</td>
<td>5</td>
<td>7.2±2.3</td>
<td>3.7±0.6</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Venin, early</td>
<td>5</td>
<td>2</td>
<td>2.0±1.4**</td>
<td>1.6±0.9**</td>
<td>0.5±0.3**</td>
</tr>
<tr>
<td>Control, late</td>
<td>5</td>
<td>5</td>
<td>8.6±8.2</td>
<td>4.6±1.5</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>Venin, late</td>
<td>5</td>
<td>5</td>
<td>2.2±1.3</td>
<td>2.4±0.5**</td>
<td>0.7±0.5</td>
</tr>
</tbody>
</table>

* * In comparison with control group P<0.01.

Table 6. Long distance metastases of liver cancer (2±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice</th>
<th>No. of metastatic foci in lung</th>
<th>No. of mouse—positive lung metastasis</th>
<th>No. of metastatic locations</th>
<th>No. of mice with metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, early</td>
<td>5</td>
<td>2.8±2.5</td>
<td>4</td>
<td>6.8±0.5</td>
<td>5</td>
</tr>
<tr>
<td>Venin, early</td>
<td>5</td>
<td>0.0±0.0</td>
<td>0</td>
<td>0.6±0.8**</td>
<td>2</td>
</tr>
<tr>
<td>Control, late</td>
<td>5</td>
<td>63.8±28.8</td>
<td>5</td>
<td>6.8±3.0</td>
<td>5</td>
</tr>
<tr>
<td>Venin, late</td>
<td>5</td>
<td>0.4±0.8</td>
<td>1</td>
<td>1.6±1.3**</td>
<td>3</td>
</tr>
</tbody>
</table>

* * In comparison with control group P<0.01.

Long distance metastases

The number of mice with metastases, lung metastatic nodes and metastatic-involved locations in the venin group were all less than those in control (Table 6).

DISCUSSION

During the process of tumor metastasis, the tumor cells adhere to various host cells such as endothelial cells, platelets and lymphocytes and/or to extracellular stroma and basal membrane. This adhesion enhances the survival, capturing and invading ability of the tumor cells, factors that are important for metastasis. High thrombin ability and various coagulation-promoting agents can be found in many neoplastic tissues. This condition along with fibrinolytic activity, leads to hypercoagulability of the blood of cancer patients, making tumor cells adhere easily to the vascular beds.

Experimental studies have shown when hypercoagulable mouse ascites liver cancer AH130 cells are injected into the caudal vein, tumor emboli can easily be found in small arteries and capillaries of target organs, with an increase of metastatic foci. The thromboplastin formed, in turn, causes endothelial cell injury, with secondary hyperfibriolysis and increased vascular permeability, promoting extravasation of cancer cells or metastasis. Injection of hypocoagulable cells resulted in lesser microthrombi in target organs, a mild degree of platelet agglutination and lesser target organ foci than those in the hypercoagulable group. Thus an important role of microthrombus formation caused by hypercoagulability in the metastasis of tumor cells has been demonstrated. Microthrombi might promote the adhesion between the tumor cells and blood vessels, favoring the extravasation of tumor cells, avoiding the negative effect of the immune function of the organism.

Some Arg-Gly-Asp(RGD)-containing disintegrins of snake venom such as eristostatin, contertrostatin and tranicanycin might inhibit pulmonary metastasis of experimental melanoma cells. High thrombin ability and various coagulation-promoting agents can be found in many neoplastic tissues. This condition along with fibrinolytic activity, leads to hypercoagulability of the blood of cancer patients, making tumor cells adhere easily to the vascular beds.

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contortrostatin, a homodimeric disintegrin isolated from venom of the Southern Copperhead snake, which possesses two RGD sites, inhibited migration and invasion, significantly altered Matrigel-induced tube formation of human umbilical vein endothelial cells (HUVEC), and also significantly inhibited angiogenesis in vivo. Swenson et al. demonstrated that intravenous liposomal delivery of contortrostatin leads to potent antiangiogenic activity in the orthotopic, xenograft human mammary tumor model and limits breast cancer progression. A venom metalloproteinase from Viperidae affects blood cell-cell interactions, thus offers a potential approach to inhibit the formation of tumor thrombi in blood vessels. Salmosin, a novel snake venom-derived disintegrin also containing the RGD sequence, was reported to be both antiangiogenic and antitumorigenic. Kim found plasmids encoding the salmosin gene were able to produce functionally active salmosin proteins in vitro, it remarkably inhibited proliferation of bovine capillary endothelial cells and effectively inhibited the migration of highly metastatic B16BL6 mouse melanoma cells. Administration of recombinant salmosin into mice bearing tumors can augment antitumor activity in vivo.

Venin capsules have been used in clinical treatment of advanced lung cancer with satisfactory results. We have also experience in using venin capsules in the prevention of postoperative recurrences of liver cancer in the out-patient department. Through combination of a venin preparation and GPIIb/IIIa on the platelet membrane, the integration of platelets and FN could be blocked. The venin we selected was a kind frequently used in the clinic to improve microcirculation, expecting to inhibit metastasis of liver cancer through the specific and non-specific anti-adhesion effects.

In our previous studies, the intercellular adhesion molecule-1 (ICAM-1) was found associated with metastasis and recurrence of liver cancer. In this recent study the expression of ICAM-1 on 7721 cell surface obviously decreased after venin treatment. However, there was no marked inhibitory effect of venin on the invading ability, though with the slight effect of promoting exfoliation of 7721 cells adhered to FN, and no effect to inhibit adhesion between 7721 cells and fibronectin. Therefore, venin had no obvious inhibiting effect on the invading movement of tumor cells in the extravascular tissue spaces. Being able to inhibit the adhesion between 7721-7721 cells and 7721-endothelial cells, venin could inhibit the formation of tumor emboli through adhesion among tumor cells and the implantation of tumor cells in capillaries, thus reducing the opportunities of migration of tumor cells out of the blood circulation. However, the mechanism of the promotion of adhesion among 7721 cells in a short action time is not clear yet, and further animal experiments would be necessary. Venin had no inhibitory effect on the adhesion between 7721-lymphocytes, so there would be no influence of the cytotoxic effects of lymphocytes on tumor cells due to the anti-adhesion effect. The different effects of venin on adhesion of 7721 cells to other cells may be due to the different functional adhesion receptors. The results of these experimental studies revealed that venin could inhibit the intrahepatic and extrahepatic metastases and recurrence of the nude mice-human liver cancer model after liver cancer resection, so the prophylactic and therapeutic effects of venin on metastasis and recurrence following early resection would be more satisfactory than resection in an advanced stage.

REFERENCES


