Inhibitory effects of transfection of arresten gene on liver metastasis from colorectal cancer in nude mice

Miao-Yun Long,* Hong-Hao Li, Jun-Yao Xu, Dong-Min Lai and Zhen-Hong Weng

Department of General Surgery; The Second Affiliated Hospital of Sun Yat-sen University; Guangzhou, Guangdong P.R. China

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Background and Objective: Liver metastasis is the most common cause of death due to colorectal cancer. Above 50% colorectal cancer patients have liver metastasis. This study was to investigate the effect of transfection of arresten gene on liver metastasis from human colorectal cancer (LoVo) xenografts in nude mice.

Methods: The eukaryotic expression plasmid pSecTag2-arresten was transfected into human colorectal cancer cell line LoVo using Lipofectamine 2000. Cells were divided into pSecTag2-arresten group, pSecTag2 group and control group. Expressions of arresten at mRNA and protein levels were detected by RT-PCR and Western blot, respectively. The effect of arresten on proliferation of LoVo cells was measured using MTT assay. LoVo cells transfected with pSecTag2-arresten were implanted into nude mice to investigate the effect of arresten on hepatic metastasis of colorectal cancer. Microvessel density (MVD) of xenograft tumors was assessed using immunohistochemistry with FVIIIRag monoclonal antibody.

Results: Arresten was successfully transfected and expressed in LoVo cells. Inhibition of cell proliferation did not differ significantly in all three groups (p > 0.05). The metastatic rate was lower in pSecTag2-arresten group [(25.1 ± 2.1)%] than in pSecTag2 group [(87.1 ± 1.2)%] or control group [(87.1 ± 1.5)%] in LoVo cells (p < 0.05). The number of xenograft tumors and MVD were higher in pSecTag2-arresten group [(24.5 ± 0.5) and (15.3 ± 3.5)] than in pSecTag2 group [(19.6 ± 2.5) and (42.2 ± 2.6)] or in control group [(20.4 ± 2.5) and (45.6 ± 5.1)] in nude mice. Conclusion: Arresten can inhibit hepatic metastasis from colorectal cancer, which may be through its inhibition on tumor angiogenesis.

Early in 1971, Folkman1 firstly identified tumor angiogenic factor (TAF). He postulated that inhibition of tumor angiogenesis may inhibit growth, metastasis and recurrence of tumors effectively. This hypothesis has been verified widely. It is shown that the solid tumor demands new blood vessels to expand when its size exceeds 1mm³, otherwise the growth would stop and the tumor would remain in a dormant state with a size of 2–3 mm³, and with the cell number of around 1 x 10⁸; the tumor could remain in situ for several months or even several years without metastasis.²,³ As soon as tumor cells gain an angiogenic phenotype, angiogenesis is induced. Then a new network of blood vessels may start to form, which would promote the rapid growth of tumor and the metastatic potential.⁴ Therefore inhibition of tumor angiogenesis, blocking the supply of nutrients to tumor cells may effectively block malignant behaviors of tumors.

Arresten, a potent angiogenic inhibitor discovered by Colorado et al.,⁵ is a polypeptide fragment in the NCI domain of the carboxyl terminal of the human collagen IV α1 chain, with a molecular weight of 26 ku. Colorado et al.⁵ have revealed that the angiogenesis-inhibiting effect and stability of arresten are significantly stronger than those of endothelin, therefore arresten may be used as an inhibitor of tumor angiogenesis. In this research, we introduced arresten gene into human colorectal cancer LoVo cells, and inoculated nude mice with these cells to establish a model of hepatic metastasis from colorectal cancer, thus to investigate the effect of arresten on metastasis of cancer.

Materials and Methods

Materials. Human eukaryotic expression plasmid pSecTag2-arresten was gifted by Dr. Song ZF, Union Hospital of Tongji Medical School, Huazhong University of Science and Technology.⁶ LoVo cells were gifted by the General Surgery Laboratory, Union Hospital of Tongji Medical School, Huazhong University of Science and Technology. Twenty-four habl/c four-week-old nude mice, 18–22 g each, were purchased from the Department of Laboratory Animals, Hubei Center of Disease Prevention and Control (certificate No. SCXK 2007–0005). Lipofectamine 2000 and Zeocin were purchased from Invitrogen Co., Ltd. One-step RT-PCR kit was purchased from TAKARA Biotechnology (Dalian) Co., Ltd. Primers were synthesized by Sangon company and the PCR marker was a product of Tianwei Biotech Co., Ltd. The antibody against human arresten, HRP-labeled goat against rabbit IgG and FVIIIRag
polyclonal antibody were purchased from Wuhan Boster Biological Technology Co., Ltd. MTT was the product of US Gibco Co., Ltd.

Methods. Plasmid transfection Lipofectamine 2000 was used to transfect plasmids into LoVo cells. Cells were divided into three groups: pSecTag2-arresten transfected group, pSecTag2 transfected group, and control group. Transfection was performed according to the instructions provided by the manufacture. In brief, LoVo cells were digested two day before transfection, and inoculated onto 24-well plates, (1–3) x 10⁵ cell/well. Transfection was performed when the cell confluence reached 90%. The plasmid (1 μg) and 2 μg of Lipofectamine 2000 were dissolved in 50 μL culture medium, separately. After incubation for 5 min, the plasmid and Lipofectamine 2000 were mixed for 20 min, and added into LoVo cells cultured in OPTI-DMEM. The medium was replaced by F12 medium supplemented with serum 6 h later. After 24 h transfection, cells on the plate were dispersed and cultured at the ratio of 1:10; after 48 h, Zeocin, at an initial concentration of 130 μg/mL, was added to screen positive clones. When a positive clone appeared, the concentration of Zeocin was adjusted to 100 μg/mL and sustained for 15 days.

RT-PCR. Primers were designed by Primer primer 5 software and synthesized by Sangon company. The sequences of arresten were adjusted to 100 clones. When a positive clone appeared, the concentration of Zeocin was adjusted to 100 μg/mL and sustained for 15 days.

Inhibitory effects of transfection of arresten gene on liver metastasis from colorectal cancer in nude mice. Twenty-four mice were randomized into three groups, eight mice in each group. Cells (5 x 10⁶) at the logarithmic phase of growth were collected from three groups, and suspended in 0.1 mL of culture medium for further use. Nude mice were anesthetized using 2.5% sodium pentobarbital (40 mg/kg) subcutaneously under sterile conditions. An incision of 2.0–2.5 cm long was made in the left abdomen to gain access to the abdominal cavity. The lieniogastric ligament was ligatured and cut off, so that the spleen was fully exposed to the outside of the abdominal cavity. A needle was inserted into the spleen to a depth of 1.0 cm or deeper from the upper pole of the spleen. Cells from various groups were injected into the spleen. The spleen was ligatured and excised 5 min after withdrawal of the needle, and the abdomen was closed.

Observation of the growth of tumors in the abdominal cavity. After inoculation, mice were given food and water freely. Observations on their body weight, mental state, and food intake were made every day. All nude mice were euthanized at day 21 by dislocating the cervical spine. The abdominal cavity was then dissected to allow for inspection for metastatic tumors. Tumor nodes on the liver surface were counted visually and those on the section of the liver were counted under optical microscopy. Livers of nude mice were excised and fixed in 10% neutral formalin, and embedded in wax. Tissue slices (4 μm thick) were prepared; six coronal sections of the liver were taken, and the distance between two consecutive sections was 2 mm. The same node appearing on different sections was counted as one node. The total number of nodes was calculated as those observed visually and those observed under optical microscopy. Cases without detectable nodes were regarded as negative for hepatic metastasis. Tumors were dissected and weighed.

Microvessel density determined by immunohistochemistry. Microvessel density (MVD) of tumor tissues was determined using FVIIIRag polyclonal antibody. Immunohistochemistry was performed according to the specifications of the two-step immunoassay kit produced by Wuhan BOSTER. The primary antibody was replaced by PBS as negative control, and a known positive slice was taken as positive control. The microvessels were counted according to the method reported by Weidner at al.7 as follows: firstly, a slice from their the neighboring tumor cells, or cells of connective tissue; immature endothelial cells, which were yellow-brown and distinctive was observed under 100x magnification to find the area with the densest blood vessels in tumors; then the area was observed under 400x magnification to find cell clusters formed by endothelial cells or immature endothelial cells, which were yellow-brown and distinctive from their the neighboring tumor cells, or cells of connective tissue; such a cell cluster was regarded as “a blood vessel”, or the branch of the cluster was also counted as a blood vessel if it was not connected to other structures; all blood vessels with a muscular layer and noticeable vessel cavities were excluded. The numbers of microvessels in three fields of view was recorded and the average value was taken as the MVD of the sample.

Statistics. Unifactorial variance analysis was adopted to compare the quantitative data among groups. The rank sum test was adopted to compare ranked data among groups. p < 0.05 was regarded as statistically different. Statistical software SPSS 11.0 was used to perform analyses and to plot the curve of cell proliferation.
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Results

Identification of arresten gene by RT-PCR. RT-PCR results revealed a specific band of 711 bp in pSecTag2-arresten-transfected group, whereas no corresponding bands appeared in the control group and pSecTag2-transfected group (Fig. 1). This verified that exogenous arresten gene had been successfully transfected into LoVo cells.

Expression of arresten protein. Results of Western blot showed that there was no expression of arresten protein in the control group and pSecTag-transfected group; while the expression of arresten protein in pSecTag2-arresten-transfected group was strongly positive (Fig. 2).

Inhibition of arresten on LoVo cell proliferation. The inhibition rates of arresten on cell proliferation in these three groups were similar (p > 0.05) (Fig. 3).

Hepatic metastasis in three groups. Multiple white-gray small nodes were observed on the liver surface of nude mice. The liver gradually became smaller, more brittle and harder. Although no tumor node was found on the liver surface of some tumors, sesame-like small metastatic foci were found on the coronal section of the livers. The normal liver is red and soft, and no metastatic nodes were revealed by either visual observation or under optical microscopy. The rate of hepatic metastasis was (25.1 ± 2.1)%, (87.1 ± 1.2)% and (87.1 ± 1.5)%, respectively, in the pSecTag2-arresten-transfected group, pSecTag2-transfected group, and the control group. The rate of hepatic metastasis and the number of metastatic tumor nodes were both significantly lower in the pSecTag2-arresten-transfected group than in the other two groups (t = 18.31, p < 0.05; t = 17.59, p < 0.05).

Results of hepatic metastasis from colorectal cancer. Tumors grew slowly in the pSecTag2-arresten-transfected group. The tumor weight was (0.6 ± 0.1)g, (1.4 ± 0.2)g and (1.5 ± 0.1)g in the pSecTag2-arresten-transfected group, pSecTag2-transfected group, and the control group, respectively. The difference between the pSecTag2-transfected and control groups was not statistically significant (p > 0.05), while differences between the pSecTag2-arresten-transfected group and the other two groups were significant (t = 17.24, p < 0.05).

MVD in tumor tissues. FVIIIIRag staining was located within endothelial cells of microvessels in the tumor tissue. MVD of tumors was significantly lower in pSecTag2-arresten-transfected group (15.3 ± 3.5) than in pSecTag2-transfected group (42.2 ± 2.6) (t = 21.47, p < 0.05) and the control group (45.6 ± 5.1) (t = 19.53, p < 0.05). The difference between the pSecTag2-transfected group and the control group was not statistically significant (t = 7.71, p > 0.05) (Fig. 4).

Discussion

Colorectal cancer is one of the most common malignant tumors. Hepatic metastasis, which occurs in over 50% of patients with colorectal cancer, is the most common metastasis and the most common cause of death.8 Due to the poor efficacy of conventional surgical treatment, new treatments are needed to improve the survival rate of late-stage colorectal cancer.

The metastasis of tumors is a multi-step process with multiple mechanisms. Research has proven that inhibition of tumor angiogenesis is an effective approach to inhibit tumor metastasis. Colorado et al.5 find the inhibitory effect of arresten on tumor angiogenesis. In addition, arresten protein is more stable and stronger than endostatin in inhibiting angiogenesis.5

In this research, we successfully introduced arresten gene into LoVo cells, and established an experimental model of hepatic metastasis from colorectal cancer. We found that arresten did not affect the proliferation of LoVo cells, whereas it effectively inhibited hepatic metastasis from colorectal cancer in vivo. Previous researches reported that arresten might inhibit the proliferation of endothelial...
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cells.\textsuperscript{9,10} We found the tranfection of arresten lowered the MVD and tumor weight of metastatic tumors, two indexes which were closely correlated. Moreover, the metastatic rate and number were significantly lower in the group with arresten gene than in the other two groups without the tranfection of arresten. We propose that arresten exerts it inhibitory effect on the growth of metastatic tumors through inhibiting tumor angiogenesis.

In summary, arresten could inhibit the growth of metastatic tumors by inhibiting tumor angiogenesis. Therefore arresten may be used as a new agent to inhibit metastatic tumors.

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References