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• ORIGINAL RESEARCH •

Molecular mechanism about lymphogenous metastasis of hepatocarcinoma cells in mice

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Abstract

AIM **To investigate the correlation between lymphogenous metastasis and matrix metalloproteinases (MMPs) activity and the expression of Fas ligand of tumor cells in lymph nodes.**

METHODES **Fifty-six inbred 615-mice were equally divided into 2 groups and inoculated with Hca-F and Hca-P cells. Their lymph node metastatic rates were examined. Growth fraction of lymphocytes in host lymph nodes was detected by flow cytometry. The Hca-F and Hca-P cells were cultured with extract of lymph node, liver or spleen. The quantity of MMPs in these supernatants was examined by zymographic analysis. The expression of Fas ligand, PCNA, Bcl-2 protein of Hca-F and Hca-P cells in the mice were examined by immunohistochemistry. The apoptosis signals of macrophages in lymph nodes were observed with *in situ* DNA fragmentation.**

RESULTS **On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes was as follows: in the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, while in Hca-P cells, the peak appeared on the 7th day post inoculation and then kept at a high level. With the extract of lymph node, the quantity of the MMP-9 activity increased (P<0.01) and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with the extracts of the liver and spleen. The expression of Fas Ligand of Hca-F cells was stronger than that of Hca-P cells (P<0.01). The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area were the same as that of Hca-P cells. *In situ* DNA fragmentation showed that the positive signals of macrophages were around Hca-F cells.**

CONCLUSION **Secretion of MMPs which was associated with metastatic ability of Hca-F and Hca-P tumor cells depends on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from being killed by host lymphocytes.**

Subject headings liver neoplasms/pathology; lymphatic metastasis; tumor cells, cultured; metalloproteinases/secretion

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INTRODUCTION

Metastasis is the most lethal attribute of a cancer[1,2]. Metastasis is a complex process which is made up of several steps[3]. Lymph nodes are often the first organ to develop metastasis[4,5]. Whether lymph nodes or other sites first develop metastases remain poorly understood. Lymph node metastases form a bridgehead for further metastatic spread. But its molecular mechanism is still unclear because of its complicated course. Anchorage on lymph node and escape from being killed by host immune cells in lymph node were two important steps. A mouse hepatocarcinoma cell line (Hca-F) with high lymphogenous metastatic potential and its syngeneic cell line (Hca-P) with low one were separated from hepatocarcinoma (HCC)[6] in mice. Matrix metalloproteinases (MMPs)[7] are a class of proteinases with variable substrate such as collagen, fibronectin and are related to the invasion-metastasis of hepatocellular cancer (HCC)[8]. Therefore, MMPs can be used as a marker of tumor cell infiltration in lymph node. The potential of tumor cells to induce apoptosis of host immune cells is to escape from being killed by immune cells. In this study, we detected the differences in the MMPs productions of Hca-F from Hca-P cells under various conditions, and different potentials of the carcinoma cells with different lymphogenous potentials to inhibit host immune reaction.

MATERIALS AND METHODS

Animals, cell lines and flow cytometry

Fifty-six inbred 615-mice maintained in our laboratory were equally divided into two groups. The Hca-F and Hca-P tumor cell lines preserved in our laboratory were inoculated at 2×10^6 in 28 mice subcutaneously in each group. On the 7th, 14th, 21st, and 28th day post-inoculation, two mice from each group were killed, and their lymphocytes were collected and detected for growth fraction with flow cytometry. The process of flow cytometry is as follows[9]: the lymph nodes were minced and centrifuged at 3000×g, and the supernatant was discarded. After repeated washing, cells were suspended in PBS. The lymphocytes at 10^5/100μL suspension were stained for 30 minutes by Propidum Iodide. Flow cytometry

was performed on a FACScan cytometer with LYSYII software. The fluorescence of 10^4 cells was analyzed for each sample. The other 40 mice were terminated on the 28th day post-inoculation, and their lymph nodes were H.E. stained and examined under microscope by paraffin sections. Therefore, the lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

Cell culture and zymographic analysis

The Hca-F and Hca-P cells cultured were put into different wells at 5×10^5, and then added 50mg extract of lymph node, liver or spleen respectively. The RPMI 1640 medium without fetal calf serum was placed into each well up to 1mL. The wells containing only Hca-F or Hca-P cells, and RPMI 1640 medium added only extracts of lymph node, liver or spleen up to 1mL served as controls. These cells were cultured at 37℃ for 24h. The supernatant of cultured cells was collected by centrifugation at 3000×*g*. MMP-2 and MMP-9 and their active type, and MMP-8 contained in supernatants of Hca-F and Hca-P with or without extracts of lymph node, liver or spleen were detected by zymographic analysis, according to the method described by Fridman *et al*[10]. The density and area of each band were measured using QuantiScan Software (Biosoft, USA).

Immunohistochemistry

The expressions of Fas-L (Santa Cruz, USA), proliferating cell nuclear antigen (Santa Cruz, USA, PCNA) and Bcl-2 (Santa Cruz, USA) protein in the tumor cells of inoculated area of Hca-F and Hca-P tumor cells, and metastatic tumor cells of lymph nodes of Hca-F were detected by standard immunohistochemistry[11]. The semiquantitative estimation of cancer cells stained was classified into 4 categories by assessing the percentage of stained tumor cells: 0, <2%; 1, 2%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75% cells.

In situ DNA fragmentation

We examined DNA fragmentation of the tumor cells of inoculated area of Hca-F and Hca-P cells, and metastatic tumor cells of Hca-F cells in lymph node, by the method of Zhu *et al*[12]. After desparaffin, these slides were pre-treated with $20mg\cdot L^{-1}$ proteinase K for 30 minutes, and then incubated with terminal deoxynucleotidy transferase and fluorescein labeled dUTP containing nucleotide mixture (TUNEL reaction mixture, *in situ* Cell Death Detection Kit/ POD, Boehringer Mannheim, Germany) in a humid atmosphere at 37℃ for 30 minutes. Each experiment set up by TUNEL reaction mixture without terminal transferase served as negative control.

RESULTS

Lymph node metastatic rate and flow cytometry

On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes from lymph nodes of mice transplanted with Hca-F and Hca-P tumor cells was examined using flow cytometry (Figure 1). For the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, and for the Hca-P cells, the peak appeared on the 7th day post-inoculation and then kept at the high level.

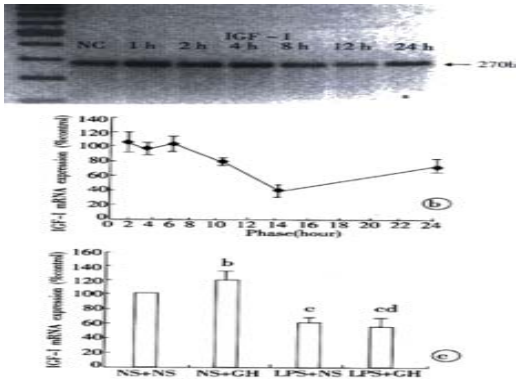

Figure 1 Changes of growth fraction of lmphocytes (G_2+S/G_1+G_2 +S+M) in draining lymph nodes of Hca-F/Hca-P burden mice.

Zymographic analysis

Both Hca-F and Hca-P cells produced a small account of MMP-9, but did not produce MMP-2 and MMP-8 (Table 1, Figure 2). Both Hca-F and Hca-P cells with extract of lymph node produced higher amounts of MMP-9 than Hca-F and Hca-P cells without extract of lymph node ($P<0.01$), and produced active MMP-9 and MMP-2. However, the Hca-F cells produce much greater quantities of activity of MMP-9, active MMP-9 and MMP-2 than Hca-P cells ($P<0.05$). The extract of lymph node did not contain any MMPs (Table 1, Figure 2). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of liver (Hca-F: 1767, Hca-P: 1564). The extract of liver contained activity of MMP-8 (1837). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of spleen (Hca-F: 2036, Hca-P: 1993). The extract of spleen contained the same quantity of activity of MMP-8 (1784). Therefore, we think that both Hca-F and Hca-P cells in the environment of liver and spleen did not produce activity of MMP-8 (Figures 3, 4).

Table 1 Activities of MMPs secreted from Hca-F and Hca-P cells under different conditions ($\bar{x}\pm s$)

Condition	MMP-2	Active MMP-2	MMP-9	Active MMP-9
Hca-F				
RPMI1640 medium	0	0	1256±157	0
Medium with lymph node extract	7364±2001	2009±901	12403±894	7297±1657
Hca-P				
RPMI1640 medium	0	0	2642±385	0
Medium with lymph node extract	2997±1990	1237±905	9086±686	3914±1253
Lmyph node extract	0	0	0	0

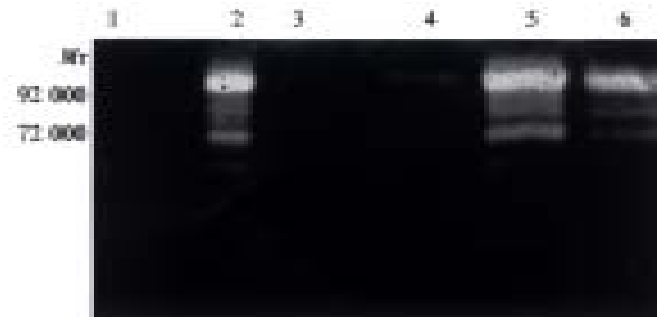

Figure 2 MMPs activity of Hca-F and Hca-P cells in RPMI 1640 with or without lymph node extract using zymographic analysis.
1. lymph node extract; 2. Type-four collagenase; 3. Hca-F cells; 4. Hca-P cells; 5. Hca-F cells in lymph node extract; 6. Hca-P cells in lymph node extract.

Figure 3 MMPs activity of Hca-F and Hca-P cells in liver extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in liver extract;
3. Hca-P cells in liver extract; 4. Liver extract.

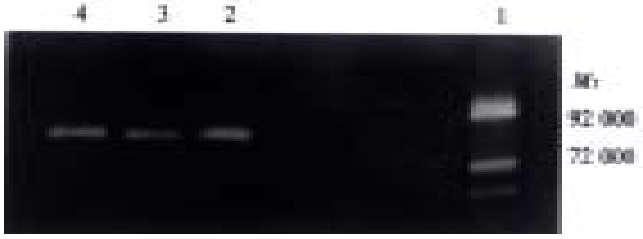

Figure 4 MMPs activity of Hca-F and Hca-P cells in spleen extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in spleen extract;
3. Hca-P cells in spleen extract; 4. Spleen extract.

Table 2 Analysis of Fas-L expression in Hca-F and Hca-P cells

Tumor	Grade				
	0	1	2	3	4
Primary tumor of Hca-F	0	0	0	8	12
Primary tumor of Hca-P	2	10	8	0	0
Metastatic tumor of Hca-F	0	5	9	6	0

Immunohistochemistry

The expression of Fas ligand protein of Hca-F cells was significantly higher than that of Hca-P cells ($P<0.01$, Table 2, Figure 5). The expressions of PCNA and Bcl-2 protein of Hca-F cells were as strong as those of Hca-P cells.

In situ DNA fragmentation

Few positive Hca-F and Hca-P cells were observed. Positive signals appeared in the macrophages around Hca-F cells (Figure 6).

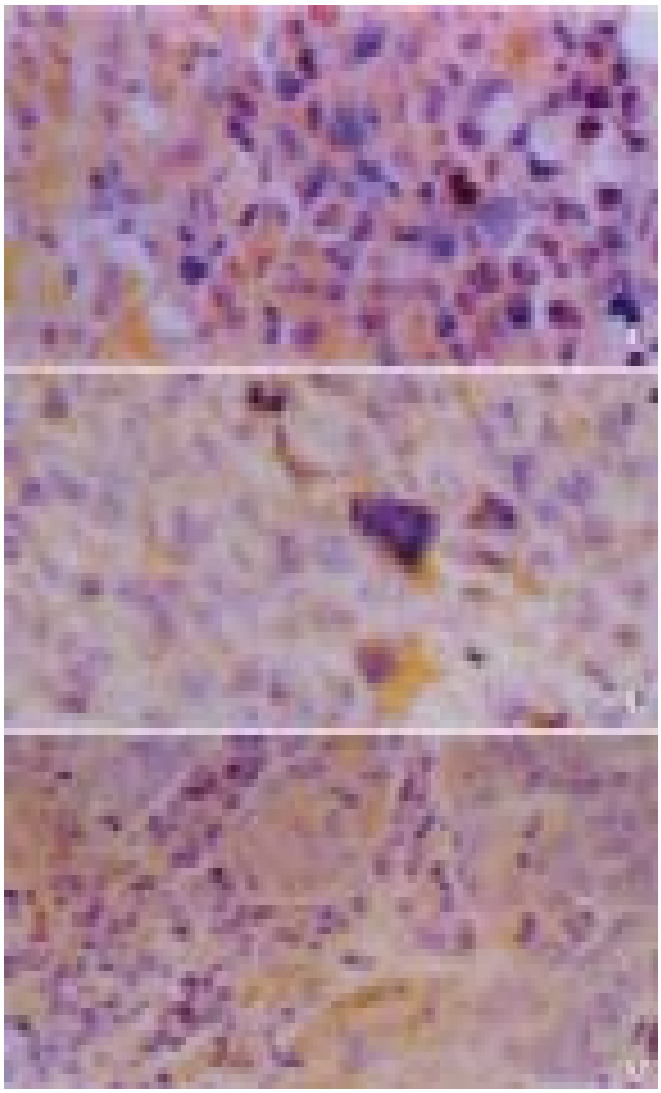

Figure 5 Fas-L expressed in Hca-F and Hca-P cells.
A: Fas-L expressed in Hca-F cells; B: in Hca-P cells;
C: in metastatic Hca-F cells.

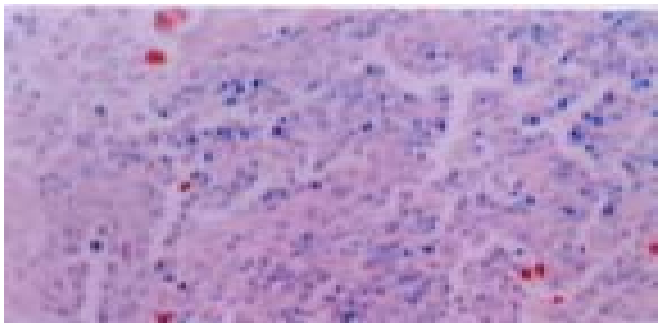

Figure 6 TUNEL of metastatic lymph node of Hca-F cells.

DISCUSSION

As early as a hundred years ago, the fact that tumor cells had organ-specific metastasis had attracted scientists' attention. Recent major discoveries concerning invasion and metastasis are identification of certain molecular mechanisms leading to organ-selective metastatization[13]. Cancer cell gene expression is regulated by interactions of tumor cells with host microenvironment, both in primary and secondary

lesions[14,15]. Whether the anchorage of carcinoma cells in lymph nodes is influenced by the specific environment of the lymph nodes remains unclear. Hca-F and Hca-P tumor cells have the potentials of specific lymphogenous metastasis. The matrix metalloproteinases[16] (MMPs) are a family of proteolytic enzymes, and the importance of MMPs in the processes of tumor invasion is now widely acknowledged in gastrointestinal cancer[17], breast cancer[18], colorectal cancer[19], and melanoma[20]. Inhibit the activity of MMPs can reduce the metastatic potential of cancer cells[21]. MMps digest collagen-containing structural barriers that cancer cells must pass in the step of cancer cell's anchoring to lymph node[22]. In this paper when the extract of lymph node was added, the quantity of the MMP-9 activity increased and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with extracts of liver and spleen. These results indicated that the secretion of MMPs of these tumor cells depend on the lymph node environment. In the environment with extract of lymph node, Hca-F cells with high lymphogenous metastatic potential produced much more MMPs than Hca-P cells with low one. So we can conjecture that Hca-F cells with high lymphogenous metastatic potential can easily receive the signal from lymph node and then start to infiltrate in lymph node to form metastatic focus.

The macrometastases were more proliferative than dormant micrometastases[23]. PCNA[24] functions as a cofactor of DNA-polymerase and is an important mark for evaluating the proliferation of colon cancer[25,26], gastric adenocarcinoma[27], lung cancer[28], ovarian cancer[29], thyroid carcinoma[30], and large intestine polyps[31]. We can use PCNA as an index of cellular proliferative status[32]. Bcl-2 proteins can extend cell survival by suppressing apoptosis[33] and are up-regulated in squamous cell carcinoma[34], breast cancer[35], lung cancer[36]. Bcl-2 proteins may promote metastasis in breast cancer[37] and melanoma[38]. The expressions of PCNA and Bcl-2 proteins could reflect accurately the status of cancer cells's growth. The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area are the same as those of Hca-P cells. Therefore, the proliferating ability of Hca-F was equal to Hca-P, although their lymph node metastatic potentials were different.

A metastatic tumor in lymph node may form as long as the tumor cells escape the killing of lymphocytes. Fas/Apo-1, together with its protein-binding partner (Fas ligand), is a key regulator of programmed cell death and induces apoptosis when it binds FasL[39-42]. In this study, we found that growth fraction of lymphocytes in host lymph nodes was lower with Hca-F cells stimulation than with Hca-P cells. The result suggests that Hca-F cells may inhibit the growth and function of lymphocytes in lymph nodes. Tumor cell survives only by evasion of the immune system[43]. The Fas/FasL system is involved in the induction of apoptosis and mediates T-cell cytotoxicyty[44]. The expression of Fas ligand in many cancers plays an important role in establishing immunologically privileged environments that allow tumors to escape the host's immune surveillance, such as in esophageal carcinomas[45,46], lung cancer[47], melanoma[48], gastric carcinoma[49], intrahepatic cholangiocellular carcinoma[50] and promotes these cancers' metastasis. The expression of Fas ligand protein of Hca-F cells was stronger than that of Hca-P cells. Hca-F cells also produced Fos ligand in lymph node. Macrophages in lymph node are one of important antigen-presenting cells, and Fas ligand in tumor cells can combine with Fas in the membrane of macrophages to induce apoptosis and decrease their function. Because lymphocytes can not receive the signals from the macrophages, tumor cells in lymph nodes can escape the suppression of lymphocytes, then accomplish the metastatic process in the lymph node.

In a word, secretion of MMPs, which was associated with metastatic ability, of Hca-F and Hca-P tumor cells depend on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with a high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from the killing of host lymphocytes and shape up metastatic focus in lymph nodes.

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