行政院國家科學委員會專題研究計畫　成果報告

醣化酵素在老鼠胰臟癌細胞轉移至肝臟的角色

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計畫主持人：田郁文

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行政院國家科學委員會補助專題研究計畫 ■ 成果報告
□期中進度報告

（計畫名稱）

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中文摘要

新生血管的形成對於腫瘤的成長是必要的(1)，而且在我們之前的實驗裏發現「與腫瘤細胞進入血管這一轉移步驟」唯一有關的因子也是新生血管的形成(2-5)。原發腫瘤會分泌許多血管生成促進因子使血管內膜細胞增生，增生的內膜細胞為了能長進腫瘤會分泌細胞間質金屬蛋白脢來消化腫瘤間質(6)。在這過程中早期的腫瘤細胞是極可能被動地被包進血管內腔而轉移出去(5)。Hoglund 他們藉由 microarray 的方法觀察到存在於在臨床上無遠處器官轉移的病人的骨髓的腫瘤細胞是屬於腫瘤發育史上早期的細胞(7)。由於這些潛伏的腫瘤細胞擁有相當少的染色體變異，因此極可能是來自早期的腫瘤，到了目標器官後由於懷環境(土壤)的改變，因此進一步的細胞基因的突變變緩慢而停滯在冬眠狀態。

腫瘤在冬眠時由於無新生血管的形成因而無法獲得足夠的氧氣與養分，因此我們推論潛伏在肝臟的腫瘤細胞如果有較多的醣分解(glycolysis)酵素會有較大的機會在無氧而有大量肝醣(glycogen)存在的肝臟存活下來，也應該會較容易長成轉移病灶。為了證實這個推論，我們預備把老鼠醣化酵素(enolase α)基因轉殖到胰臟腫瘤細胞測試這些強烈表現醣化酵素的腫瘤細胞是否更容易造成肝臟轉移。

英文摘要及關鍵詞　　(keywords) : angiogenesis, Intravasation, metastasis。

Angiogenesis is essential for the growth of primary tumor (1) and, as shown in our previous studies (2-5), is also the only significant intravasation-related metastatic factor. Tumor'll secrete or induce many angiogenic factors to induce neovascularizatioin. Endothelial cells stimulated by these angiogenic factors such as vascular endothelial growth factor, secrete matrix metalloproteinase-2 (Gelatinase A) which contributes to degradation of basement membrane in microvessel walls (6). This breakdown in the vascular basement membrane may facilitate the extravasation of endothelial cells during formation of neovascularization sprouts, as well as intravasation of tumor cells into lumen (6)). If tumor vessel formation is rapid and haphazard and endothelial proliferation is insufficient or endothelial junctions are unstable, cancer cells may be exposed to the lumen and passively enter the circulation during the angiogenic process (6). Thus, in contrast to the present view "metastasis marks the end in a sequence of genomic changes underlying the progression of an epithelial cell to a lethal cancer", these results imply that tumor cells may passively enter the circulation early during multistep tumorigenesis.

From the synoptic analysis of cytogenetic data from thousands of solid tumors, it was deduced that the number of cytogenetic imbalances per tumor reflects to some extent the biological age of the tumor (7). Hence, the relative small number of chromosomal aberrations in many latent disseminated cells suggests they left the primary site early and the further accumulation of imbalances may have been decelerated, perhaps by environmental constraints, which leads to the tumor dormancy.

Results of our studies (2-5), tumor dormancy (7), and cancer of unknown primary syndromes (8,9) all stress the importance of soil to develop established metastatic growth. Thus, we're going to focus further studies on metastatic steps after extravasation.

As previous described, tumor depends on angiogenesis to sustained growth. Once the tumor mass reaches a diameter of ~2 mm, establishment of new vascular system is essential for its survival. Until then, endurance in hypoxic condition is essential for its survival. In liver, there is a plenty storage of glycogen and these glycogen can be used as substrate of glycolysis in an anoxic condition. Theoretically, tumor cells with enzymes for glycolysis (such as enolase α) may have a

greater chance to survive in liver and remained in dormant state. To test this hypothesis, we'll try to increase the liver metastasis by injecting murine-enolase-overexpressing pancreatic cancer cells.

(三)報告內容：

1.前言:

Angiogenesis is essential for the growth of primary tumor and primary tumor may actually suppress the growth of metastatic lesions by secreting antiangiogenetic factors (1). This is consistent with the evolutionary view point that the primary tumor's main goal is to assure its own growth rather than establish secondary growth in other organ. As shown in our previous studies (2-5), angiogenesis is also the only significant intravasation-related metastatic factor. Promotion of primary tumor growth is greatly dependent on angiogenesis, which result from the net balance of positive and negative regulators of neovascularization. Endothelial cells stimulated by mitogens such as basic fibroblast growth factor or vascular endothelial growth factor, secrete matrix metalloproteinase-2 (Gelatinase A) which contributes to degradation of basement membrane in microvessel walls (6). This breakdown in the vascular basement membrane may facilitate the extravasation of endothelial cells during formation of neovascularization sprouts, as well as intravasation of tumor cells into lumen (10). If tumor vessel formation is rapid and haphazard and endothelial proliferation is insufficient or endothelial junctions are unstable, cancer cells may be exposed to the lumen and passively enter the circulation during the angiogenic process (10). Thus, in contrast to the present view "metastasis marks the end in a sequence of genomic changes underlying the progression of an epithelial cell to a lethal cancer", these results imply that tumor cells may passively enter the circulation early during multistep tumorigenesis.

Previous cytogenetic analyses have not verified the postulated gradual acquisition of genomic changes, because in situ carcinomas already display chromosomal aberrations very similar to invasive carcinomas (11, 12)). Likewise, similar genetic alterations were observed in primary tumors and synchronous regional lymph node metastasis (13, 14). In contrast, asynchronous distant metastases often differ extensively from the corresponding matched primary tumors (15). From the synoptic analysis of cytogenetic data from thousands of solid tumors, it was deduced that the number of cytogenetic imbalances per

tumor reflects to some extent the biological age of the tumor (7). Hence, the relative small number of chromosomal aberrations in many latent disseminated cells suggests they left the primary site early and the further accumulation of imbalances may have been decelerated, perhaps by environmental constraints, which leads to the tumor dormancy. This presumption is compatible with our studies that tumor cells may passively enter the circulation early during multistep tumorigenesis.

The facts that apparently chromosomal aberrations do not disclose the clonal relationship between the primary tumor and its seed also imply that tumor cells acquire them later at the distant anatomical site. Thus, the further development of dormant tumor cells to overt metastasis apparently is a matter of mutation and selection for growth within the new environment (target organ). Within the spectrum of clinical courses, there is a very distinct scenario of cancer of unknown primary (CUP). This syndrome comprises up to 7 % of hospitalized cancer patients who are first diagnosed with distant metastases, whereas their primary tumor remains unrecognized (8, 9). CUP therefore demonstrates that a large tumor extent apparently is not an absolute requirement for a systemic spreading to occur. Cancer of unknown primary syndrome may be the infrequent situation that an early disseminated cell acquires an advantageous mutation at a distant site and evolves faster than the progenitor cell at the primary site.

Results of our studies (2-5), tumor dormancy (7), and cancer of unknown primary syndromes (8, 9) all stress the importance of soil to develop established metastatic growth. Thus, we're going to focus further studies on metastatic steps after extravasation.

As previous described, tumor depends on angiogenesis to sustained growth. Once the tumor mass reaches a diameter of ~2 mm, establishment of new vascular system is essential for its survival. Until then, endurance in hypoxic condition is essential for its survival. In liver, there is a plenty storage of glycogen and these glycogen can be used as substrate of glycolysis in an anoxic condition. Theoretically, tumor cells with enzymes for glycolysis (such as enolase α) may have a greater chance to survive in liver and remained in dormant state. To test this hypothesis, we'll try to increase the liver metastasis by

injecting murine-enolase-overexpressing pancreatic cancer cells into spleen.

2. 研究目的:

To test the role of glycolytic enzyme such as enolase-α in the hepatic metastasis of pancreatic cancer.

3. 文獻探討:

The metastasis of cancer involves the accumulation of a series of genetic events. A wide variety of the genetic differences that may occur in the development of metastasis have been examined, using various approaches. However, most studies are highly focused and do not provide insight into global gene expression. Recently, cDNA microarray technology has been used successfully to clarify the genetic background of various tumors. cDNA microarray studies did revealed preferential expression of enolase-α in hepatic metastasis from colorectal cancer tissue than in paired primary colorectal cancer tissue. Quantitative RT-PCR also proved the increased expression of enolase-α in hepatic metastasis.

4. 研究方法:

Materials and Methods:

Cell Lines and animals

Mice pancreatic cancer cells CRL-2389 and LT/Sv mice will be used in the studies. All animals will be obtained from animal center of our hospital and will receive human care. The studies will be carried out in accordance with the Declaration of Helsinski and with the guide for the care and use of Laboratory Animals as adopted and promulgated by the National Institute of Health. CRL-2389 cell lines will be grown in DME/F12 (Nissui, Tokyo) containing 10% FCS (Biowhittaker).

Transfection

The expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands), containing enolase-α cDNA in an EcoRI site under the control of cytomegalovirus promoter will be used. As a control, we'll use an empty PcDNA3 vector (mock). Plasmids (5μg) will be transfected into CRL-2389 cells by using SuperFect (Quiagen, German) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells will be transferred to selection medium containing 500μg

G418/ml (Calbiochem, Germany) for 14 days. Surviving colonies from four to five dishes will be pooled together and characterized. In addition, 10 additional different cell clones will be subcloned and compared with pooled population cell lines.

RNA Isolation and Northern Blot Analysis

Total RNA will be extracted using a commercial available RNA extraction Kit in accordance with the recommendations of the manufacturer. Samples of total RNA (20μg) will be separated by electrophoresis, stained with ethidium bromide, and blotted on GeneScreen Plus nylon membrane (NEN Research Products, Boston, MA) using standard conditions. The PCR clones for enolase α and glyceraldehydes-3-phosphate dehydrogenase cDNA will be [^{32}P]dCTP labeled by the random priming method. Hybridization will be carried out using standard conditions and exposed to X-ray film. Intensities of hybridization signals will be quantified by densitometry.

Western Blot Analysis

Serum-free RPMI 1640 medium with 1nM will be conditioned by enolase α or vector-only (mock)-transfected KM12-HX cells for 24 h. Cells from conditioned dishes will be lysed with lysis buffer containing 150 m**M** NaCL, 10 m**M** Tris-HCL (PH 7.4), 1% Nonidet P-40, and 2 m**M** phenylmethylsulfonyl fluoride. The amount of protein will be measured by using a micro-bicinchoninic acid protein assay (Pierce, Rockford, IL). Heparin-binding proteins will be isolated from the conditioned medium. Proteins from cell lysate (20 μg) and corresponding heparin-bound proteins from conditioned medium will be separated by SDS-PAGE electrophoresis. Enolase α will be detected by using a 0.2 μg/ml concentration of goat anti-enolase α antibody (R & D systems) and a secondary antibody, horseradish peroxidase-labeled antigoat IgG, at a 1:2000 dilution. Protein bands will be visualized by using an enhanced chemiluminescence detection system. As a negative control, normal goat IgG (R&D systems) will be used instead of anti-enolase α, at the same concentration.

Experimental hepatic metastasis

The male LT / Sv mice will be maintained and will be used for experiments when

they are 6 weeks old. To produce experimental hepatic metastases, cultured mice pancreatic cancer CRL-2389 cells will be harvested by treatment with 0.02% EDTA and 0.05% trypsin. Viable cells will be injected into the spleen of anesthetized mice at doses of 50.0 X 10^5, 25.0 X 10^5, 15.0 X 10^5, 9.0 X 10^5, 6.0 X 10^5, 3.0 X 10^5, in 0.1 ml PBS. The mice will be sacrificed 6 weeks later and the number of hepatic tumor lesions will be determined. The number of nodules on the liver surface will be counted by eye and the mean size of the nodules will be also measured.

1. 結果與討論（含結論與建議）

We did successfully transfected the pancreatic cancer cell line CRL 2389 with an expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands), containing enolase-α cDNA in an EcoRI site under the control of cytomegalovirus promoter. Nothern blot and Western blot analyses did proved significantly increased expression of enolas-α in transfected cells compared to wild type CRL2389. However, in experimental hepatic metastasis, transfected cancer cells failed to produce more metastatic lesions when compared to wild type cancer cells. Besides, the tumor sizes of metastatic lesions were not significantly different. Thus, hepatic metastasis from pancreatic cancer cell lines CRL 2389 may controlled or influenced many other factors.

Recently, the mechanism of metastasis was shown to be initiated with VEGFR1-positive haematopoietic bone marrow progenitors forming pre-metastatic niche in target organ. Before tumor implantation, minimal β-gal$^+$ BMDCs or GFP$^+$BMDCs were found in the lung. By day 14 after tumor implantation, but before the arrival of tumor cells, the extravasation and cluster formation of β-gal$^+$ BMDCs were detected. Individual DsRed-tagged tumor cells, associated with pre-existing BMDC clusters, were visible by day 18. In mice given B16-melanoma-conditioned media showed that this conditioning alone mobilized BMDCs that were capable of forming a pre-metastatic nche. MCM increased the number of tumor cells in the lung one day after tumor injection compared with media alone. Four days after tumor injection, the frequency and size of the lung nodules were augmented by MCM. Co-localizatiion of DsRed-tagged tumor cells with GFP$^+$BMDC cluster was > 93%.

Therefore, factoers provided by the primary tumor induced BMDCs to enter the blood stream and mobilize to organ-specific pre-metastatic sites, and this migration precedes the arrival of tumor cells.

Sites of BMDC cluster are tumor-type specific. Recruited BMDCs consist of haematopoietic progenitors. Clusters induced by either tumor type expressed VEGFR1. Further characterization revealed that subsets of VEGFR1 BMDCs coexpressed the stem/progenitor cell antigens CD133, CD34, and CD117, suggesting that these cells may comprise phenotypically marked VEGFR1^{+}HPCs. Early VEGFR1^{+} bone marrow clusters lack expression of VEGFR2 and CD31. VEGFR2-positive circulating endothelial progenitor cell migrated to fully formed BMDCs, and coincided with the arrival of tumor cells. BMDC clusters also occur in a spontaneous tumor model. BMDCs wew also noted to be recruited to premetastatic human tissue. Anti-VEGRR1 antibody treatment eliminated the initiating clusters and completely prevented metastasis, whereas anti VEGFR2 antibody did not prevent the formation of VEGFR1^{+} clusters but limited metastatic progression.