

行政院國家科學委員會專題研究計畫 期中進度報告

以基因分析評估腫瘤血管內皮細胞基因的表達:從鱗鼠到人類(2/3)

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一、計畫中文摘要

血管新生-從已存在的血管生長出來的微血管-對胚胎的發育、器官的形成、組織的重建和重建是經不可或缺的。血管新生已經被證實對腫瘤生長和轉移的發生和進展有所貢獻。抗血管新生被認為是抵抗腫瘤細胞抗藥性的一種新的癌症治療。然而許多關於腫瘤血管的問題仍無法回答。最近人類大腸直腸癌的腫瘤血管內皮細胞被發現表達一些和正常血管內皮細胞不相同的基因。看來在腫瘤組織中血管的內皮細胞和正常組織中血管的內皮細胞其性質並不相同。因此我們提出這個兩年度的計畫來回答一些有趣的問題。首先，在其他腫瘤中的血管內皮細胞其基因的表達是否和正常組織中血管的內皮細胞並不相同。其次，不同器官所產生的不同腫瘤的血管內皮細胞其基因的表達是否會相同。我們將利用在鼯鼠動物模式的各個細胞株以分示法來評估腫瘤血管內皮細胞基因表達的差異。接著我們將進一步評估在鼯鼠動物模式所觀察到的腫瘤血管內皮細胞基因表達的差異，是否也可以在人類腫瘤被觀察到。最後我們對所找到的基因做功能的分析。這些問題的回答將對了解不同器官的腫瘤血管新生和針對特異腫瘤的抑制血管新生這個新的治療方式具有決定性地衝擊。

二、計畫英文摘要

Angiogenesis, the outgrowth of new capillaries from pre-existing vessels, is essential for embryonic development, organ formation, tissue regeneration, and remodeling. Angiogenesis has been proved to contribute to the development and progression of tumor growth and metastasis. Anti-angiogenesis was regarded to be a new cancer therapy resistant to drug resistance of tumor cells. However, many basic questions about tumor vessels remain unanswered. Recently tumor endothelium of human colorectal cancer has been found to express some different genes than normal

endothelium does. It seems that endothelium that lines blood vessels in tumors qualitatively different from endothelium in vessels of normal tissue. So we propose this two-year projects to answer some interesting questions. First, if the gene expressions of tumor endothelium are different from those of normal endothelium in the other different tumors. Second, if the gene expressions of tumor endothelium are the same in different tumors from different organs. We will utilize the tumor cells lines in the murine model to evaluate the differences of gene expressions in the tumor endothelium by differential expression. Then we will further evaluate if the differences of gene expressions that we observed in murine model can also be observed in human tumors. Finally, we will try to evaluate the function of these observed genes from tumor endothelium. The answers to these questions critically impact the potential for understanding the tumor angiogenesis in different organ and for new therapeutic approaches to inhibit angiogenesis in a tumor-specific manner.

三、研究計畫之原由及目的：

Tumors require a blood supply for expansive growth (1-3), an observation that has stimulated a profusion of research on tumor angiogenesis. However, several basic questions about tumor vessels remain unanswered. For example, is endothelium that lines blood vessels in tumors qualitatively different from endothelium in vessels of normal tissue ? What is the relation of tumor angiogenesis to angiogenesis associated with wound healing or other physiological processes? The answers to these questions critically impact the potential for new therapeutic approaches to inhibit angiogenesis in a tumorspecific manner.

To determine if tumor-specific endothelial markers exist, we will compare gene expression profiles in endothelium derived from normal and tumor tissue.

Human colorectal cancer was chosen for these studies because it has a high

incidence, tends to grow slowly, and is often resistant to chemotherapeutic drugs. Importantly, the progressive growth of this tumor type appears to be angiogenesis-dependent (4).

Global analysis of gene expression in tumor and normal endothelium is difficult because (i) the endothelium is enmeshed in a complex tissue consisting of vessel wall components, stromal cells, and epithelial cells, and (ii) only a small fraction of the cells within these tissues are endothelial. Thus, we needed to develop methods for isolating highly purified endothelial cells (ECs) and for evaluating gene expression profiles from relatively few cells.

To overcome the first obstacle, we attempted to purify ECs from dispersed human colorectal tissue using CD31, an endothelial marker commonly used for this purpose (5–8). This resulted in a substantial enrichment of ECs but also in contamination of the preparations by hematopoietic cells, most likely due to expression of CD31 by macrophages (9). We therefore purified ECs from human tissues using P1H12, a recently described marker for ECs (10). Unlike CD31, CD34, and VE-cadherin, P1H12 specifically localized to ECs of all vessels including microvessels of normal and cancerous colorectal tissue (Fig. 1A). Our purification protocol also optimized the detachment of ECs from neighboring cells, leaving cell surface proteins intact, and included positive and negative affinity purifications using a mixture of antibodies (Fig. 1B). The ECs purified from normal colorectal mucosa and colorectal cancers were essentially free of epithelial and hematopoietic cells as judged by reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 1C) and subsequent gene expression analysis (see below).

To evaluate modification of the serial analysis of gene expression (SAGE) technique (11). SAGE associates individual mRNA transcripts with 14–base pair (bp) tags derived from a specific position near their 3' termini (12). The abundance of

each tag provides a quantitative measure of the transcript level present within the mRNA population studied. SAGE is not dependent on preexisting databases of expressed genes and therefore provides an unbiased view of gene expression profiles. This feature is particularly important in the analysis of cells that constitute only a small fraction of the tissue under study, as transcripts from these cells are unlikely to be well represented in extant expressed sequence tag (EST) databases. generated a SAGE library of ;96,000 N.T.

四、研究方法、進行步驟及執行進度：

Murine part

1. Tumor cell line

The production and maintenance of TC-1 cells has been described previously [Lin, 1996 #175]. HPV-16 E6, E7 and *ras* oncogene were used to transform primary lung epithelial cells of C57BL/6 mice. This tumorigenic cell line was named TC-1.

2. Isolation of tumor endothelial cells

C57BL/6 mice (five per group) were injected with 5×10^4 TC-1 tumor cells will be injected subcutaneously in the right hind leg or intravenously via tail vein. Mice will be were sacrificed. The subcutaneous tumors and lungs will be explanted on 28 days after tumor challenge.

After removal of lungs or subcutaneous tumors, these tumor tissues will be minced and will be digested with collagenase A and trypsin. The enzyme-treat tumor tissues will be filtered with 25 μm filters and Percoll gradient solution will be performed to get rid of RBC. The remaining cells were stained with VCAM-1 antibody, purified with anti-rat immunoglobulin G (IgG)-coupled magnetic beads.

3. Isolation of normal endothelial cells

The isolation of normal endothelial cells will be performed as the previous description of tumor endothelial cells. Lung tissues and aorta of the naive mice will be collected and the endothelial cells of the lungs and aortas will be further isolated as normal endothelial cells.

4. Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the National Taiwan University Hospital (Taipei, Taiwan) and bred in the animal facility of the National Taiwan University Hospital (Taipei, Taiwan). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Human part

1. Isolation of normal and tumor endothelial cells

Human gastric and hepatic tissues were obtained <30 min after surgical removal. Normal tissue was taken from regions 1.0 cm away from bulk tumor tissue that had clearly defined margins. For normal tissue, sheets of epithelial cells were removed with a glass slide after treatment with 5 mM dithiothreitol, then 10 mM EDTA, leaving an intact lamina propria for subsequent processing. After a 2-hour incubation in collagenase at 37°C, cells were filtered sequentially through 400-, 100-, 50-, and 25-mm mesh and centrifuged through a 30% preformed Percoll gradient to sediment red blood cells. Epithelial cells (Epithelial Fraction), which were found to nonspecifically bind magnetic beads, were removed with Dynabeads coupled to BerEP4. Subsequently, macrophages and other leukocytes (Hematopoietic Fraction) were removed with a mixture of beads coupled to anti-CD45, anti-CD14, and anti-CD64 (Dyna). The remaining cells were stained with P1H12 antibody, purified

with anti-mouse immunoglobulin G (IgG)–coupled magnetic beads.

五、結果與討論

Isolation and collection of tumor and normal endothelial cells from cancer patients

Human gastric tissues were obtained <30 min after surgical removal. Normal tissue was taken from regions 1.0 cm away from bulk tumor tissue that had clearly defined margins. For normal tissue, sheets of epithelial cells were removed with a glass slide after treatment with 5 mM dithiothreitol, then 10 mM EDTA, leaving an intact lamina propria for subsequent processing. After a 2-hour incubation in collagenase at 37°C, cells were filtered sequentially through 400-, 100-, 50-, and 25- μ m mesh and centrifuged through a 30% preformed Percoll gradient to sediment red blood cells. Epithelial cells (Epithelial Fraction), which were found to nonspecifically bind magnetic beads, were removed with Dynabeads coupled to BerEP4. Subsequently, macrophages and other leukocytes (Hematopoietic Fraction) were removed with a mixture of beads coupled to anti-CD45, anti-CD14, and anti-CD64 (Dyna). The remaining cells were stained with P1H12 antibody, purified with anti-mouse immunoglobulin G (IgG)–coupled magnetic beads.

Tumor and normal endothelial cells were collected from 12 gastric cancer patients. These isolated tumor and normal endothelial cells were frozen in -70°C refrigerator for further experiments. The identification and characterization of the magnetic beads-isolated cells were further stained with anti-CD34 antibody to clarify that these isolated cells were endothelial cells (Figure 1).

Isolation and collection of tumor and normal endothelial cells from murin tumor and normal tissues

C57BL/6 mice (five per group) were injected with 5×10^4 TC-1 tumor cells will be injected subcutaneously in the right hind leg or intravenously via tail vein. Mice will be sacrificed. The subcutaneous tumors and lungs will be explanted on 28 days after tumor challenge.

After removal of lungs or subcutaneous tumors, these tumor tissues will be minced and will be digested with collagenase A and trypsin. The enzyme-treated tumor tissues will be filtered with 25 μm filters and Percoll gradient solution will be performed to get rid of RBC. The remaining cells were stained with VCAM-1 antibody, purified with anti-rat immunoglobulin G (IgG)-coupled magnetic beads.

The isolation of normal endothelial cells will be performed as the previous description of tumor endothelial cells. Lung tissues and aorta of the naive mice will be collected and the endothelial cells of the lungs and aortas will be further isolated as normal endothelial cells. The identification and characterization of the magnetic beads-isolated endothelial cells were further stained with anti-CD34 antibody to clarify that these isolated cells were endothelial cells.

六、計畫成果自評

我們將人類及鱈鼠腫瘤中的血管內皮細胞分離出來。我們將進一步利用分示法分析腫瘤組織內血管內皮細胞基因表達與正常組織內血管內皮細胞基因表達的差異。接著尋找人類血管內皮細胞中與血管新生相關的基因，比較人類及鱈鼠調控血管新生基因的相同處和差異處。這將幫助我們了解調控腫瘤血管新生的基因的功能，對未來以抗腫瘤血管新生作為治療癌症提供新的治療方針。

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Figure Legends

Figure1. C57BL/6 mice (five per group) were injected with 5×10^4 TC-1 tumor cells will be injected intravenously via tail vein. Mice will be were sacrificed. on 28 days after tumor injection. After removal of lungs tumors, these tumor tissues will be minsed and will be digested with collagenase A and trypsin. The enzyme-treate tumor tissues will be filtered with 25 μm filters and Percoll gradient solution will be performed to get rid of RBC. The remaining cells were stained with VCAM-1 antibody, purified with anti-rat immunoglobulin G (IgG)-coupled magnetic beads. (A) Isolated cells from tumor tissues showed positive CD144 surface marker expression. (B) Isolated cells from spleen showed negative CD144 surface marker expression.

Figure 1A

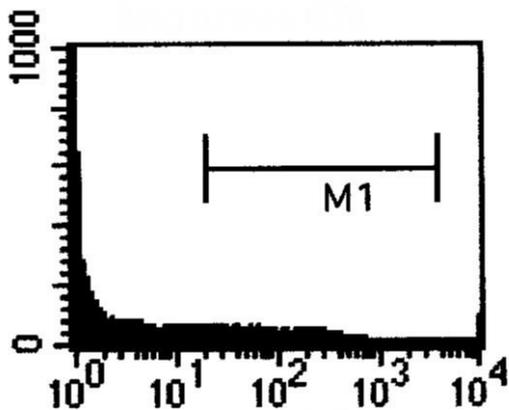


Figure 2

