A novel method for endothelial cell isolation

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Abstract. The present study aimed to develop a quick and efficient method for purification of newborn endothelial cells from tumor tissues. Fresh tissues were separated from C57BL/6 mice bearing tumors derived from mouse lung cancer Lewis cells, fully minced and divided into two parts. One part was subjected to collagenase type I digestion with a vortex to form a single-cell suspension, while another part was digested but without a vortex. Then, the $CD105^+$ cells were isolated using anti-CD105 antibody-coated Dynabeads. The isolated $CD105^+$ cells were grown in culture medium and examined for the surface expression of CD105 by a fluorescence-activated cell sorter (FACS). The uptake of acetylated LDL and the ability to maintain capillary tube-like structure formation in the $CD105^+$ cells were also examined by Dil-Ac-LDL uptake assay and tube formation assay. The expression of tumor newborn endothelial cells ($CD105^+$) was tested in Lewis xenografts by immunohistochemistry. The number of cells which were obtained by the digestion process with a vortex was $5.70\pm0.23x10^4$ much higher than the number without a vortex ($0.32\pm0.04x10^4$) ($P<0.01$). The purity of $CD105^+$ cell digestion with a vortex was significantly higher than that without a vortex. Dil-Ac-LDL uptake assay and tube formation assay confirmed that the $CD105^+$ cells digested with a vortex exhibited typical functions of endothelial cells. In conclusion, the $CD105^+$ cells isolated by the new method had high purity and displayed features of vascular endothelial cells. The modified method provides $CD105^+$ cells with superior conditions for mechanistic research on the development of vessel-based disease.

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Introduction

Angiogenesis plays an essential role in promoting tumor growth. Tumor development beyond 1-2 mm is dependent on the formation of a functional blood supply system for nutrient delivery (1-3). Based on previous studies involving intact established cell lines or vessels, the blood vessels of tumors and those of normal tissues differ in regards to permeability, composition of the basement membrane, extracellular matrix and cellular composition. When compared to normal blood vessels, tumor vessels are tortuous, exhibit poorly organizational characteristics, high permeability and are inclined to leak allowing macromolecules of the tumor microenvironment into blood circulation (4-6). The study of the mechanism of the development of tumor blood vessels plays a major role in tumor diagnosis and therapy. However, endothelial cells (ECs) comprise only 1-2% of the total amount of tumor tissues, and they are embedded in matrix components and tightly surrounded by various other cell types (7). Therefore, it is particularly difficult to isolate endothelial cells from tumor tissues.

In recent years, purification of ECs for culture and molecular profiling has gained more and more interest, and different techniques have been employed (8-10). However, these techniques are all prone to obtain a mixed sample with unwanted cells. This study reports a new purification method by which to obtain numerous endothelial cells in superior conditions.

Materials and methods

Cells and animals. C57BL/6 mice were obtained from Vital River Company (Beijing, China) and were housed and cared for in accordance with the Federation of European Laboratory Animal Science Association guidelines, and all protocols were approved by the Animal Ethics Committee of Guangxi Medical University (Nanning, Guangxi, China). Mouse lung carcinoma cells (Lewis) ($1x10^6$) were injected into the right flank of mice. Tumors were excised for study 45 days after injection.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were utilized. Dewaxed sections of Hep1-6 xenografts were blocked with 3% hydrogen peroxide and 10% normal serum from the secondary antibody species, and then incubated at 4˚C with the primary antibody mCD105 (Abcam, Cambridge, UK) overnight. This was then followed by biotin labeled secondary antibody for 30 min and horseradish peroxidase-conjugated ultrastreptavidin-labeling reagent for 30 min. Color was developed with 3,3'-diaminobenzidine (DAB) solution.

Endothelial cell isolation. Tumors were removed and placed in cold PBS solution with 50 U/ml heparin. Peripheral and necrotic tissues were excised and the remaining tumor was minced using a scalpel. Dissociation of 0.1x0.1x0.1 cm^3 minced tissue was performed in an enzyme cocktail of 10 mg collagenase type I, 20 ml Dulbecco's modified Eagle's medium (DMEM) and 2 ml FBS at room temperature for 60 min of constant mixing with a vortex. The cell suspension was passed through 80 mesh strainer, washed with PBS solution, and then the cells were resuspended in 100 μl buffer [PBS (pH 7.2), 0.5% FBS, 2 nM ethylenediaminetetraacetic acid]. Single cells were magnetically labeled with anti-CD105 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4˚C for 30 min and applied to the prepared MS Column (Miltenyi Biotec, Bergisch Gladbach). $CD105^-$ cells were collected in the flow-through of the column, while $CD105^+$ cells bound to the beads were flushed out by applying the plunger supplied with the column. Sorted $CD105^+$ cells were plated into 6-well plates and cultured in endothelial cell medium (ScienCell, USA).

Fluorescence-activated cell sorting (FACS). For flow cytometry, the cells were stained at the concentration of $1x10^6$ cells/90 μl buffer and 10 μl phycoerythrin-conjugated anti-CD105 (eBioscience, San Diego, CA, USA) at 4˚C for 25 min before FACS analysis. All data were analyzed by EXPO32 software.

Tube formation assay. To analyze the capillary-like tube formation ability of $CD105^+$ cells, 50 μl/well of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was laid into 96-well plates to solidify. Cells were seeded into 96-well plates. After 12 h, the tube formation was assessed with microscopy.

Dil-Ac-LDL uptake assay. $CD105^+$ cells were plated into 6-well plates at $5x10^4$ cells/dish. At 75% confluency, the culture medium was replaced with serum-free DMEM for 24 h, followed by incubation with 2 μg/ml Dil-ac-LDL for 5 h at 37˚C in 5% CO_2. Then, the cells were washed and fixed with 4% paraformaldehyde at 4˚C for 30 min, followed by DAPI staining for 3 min. The Dil-ac-LDL uptake was assessed using microscopy.

Statistical analysis. Data are expressed as mean ± SEM. The significance of differences between groups was assessed by a t-test. All analyses were performed with GraphPad Prism program version 5 (GraphPad Software, La Jolla, CA, USA). A P-value of <0.05 was considered to indicate a statistically significant result.

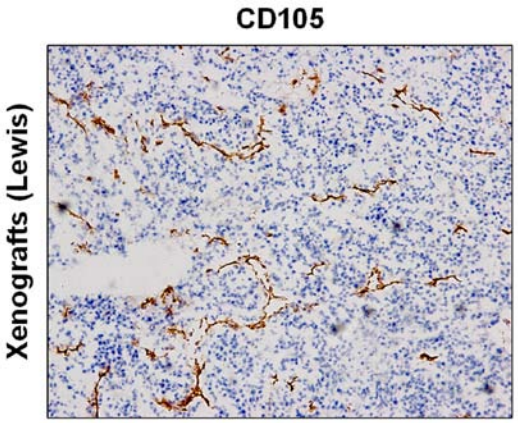

Figure 1. Immunohistochemistry of $CD105^+$ endothelial cells (ECs) (original magnification, x100).

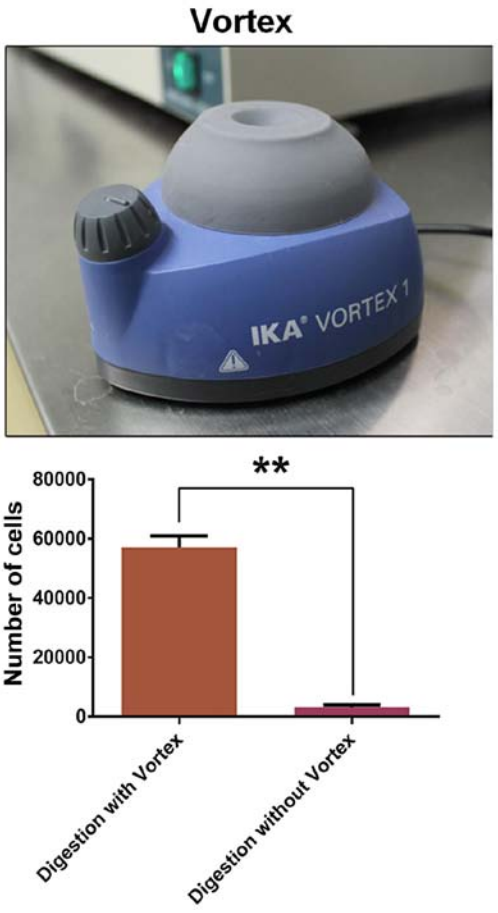

Figure 2. The cell number after digestion was determined. Data are presented as the mean ± SEM (n=3). $^{**}P<0.01$.

Results

CD105 expression of vascular endothelial cells in tumor tissues. Immunohistochemistry revealed high expression of CD105 in the tumor tissue (Fig. 1).

Enrichment and purity of $CD105^+$ cells. After magnetic separation, the number of $CD105^+$ cells digested with a

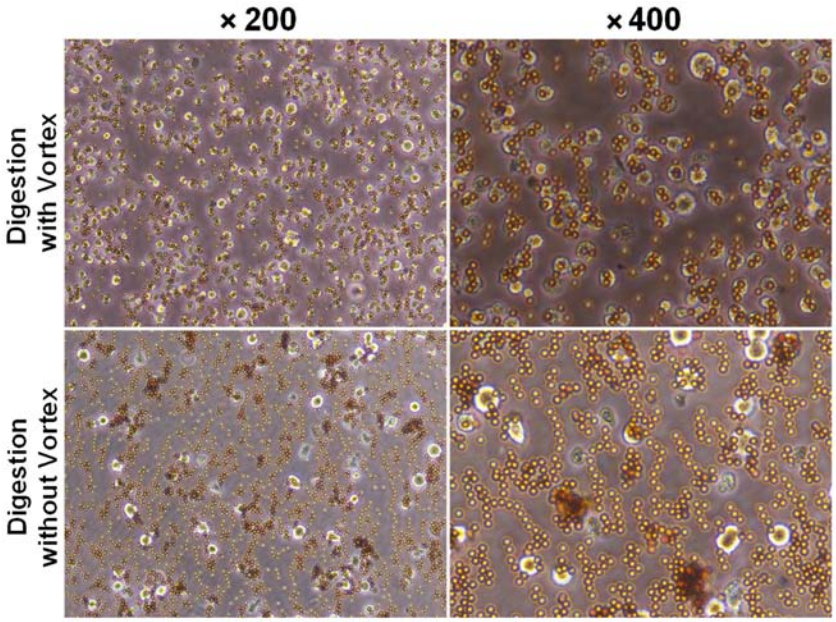

Figure 3. Cells combined with microbeads.

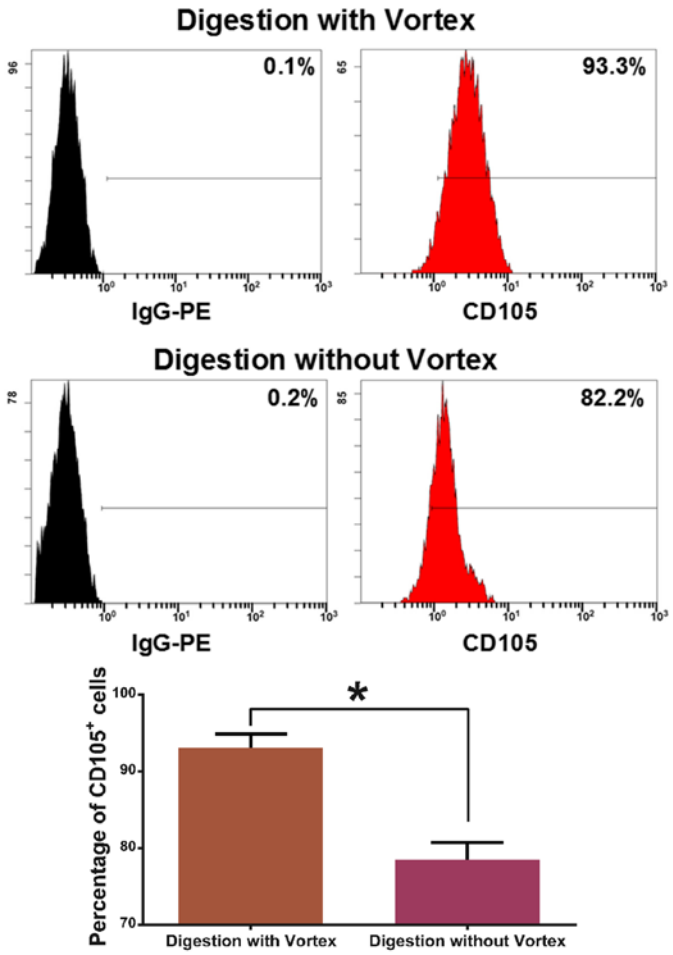

Figure 4. Flow cytometry results of the CD105 subpopulation in cells isolated from tumor tissues. Data are presented as the means ± SEM (n=3). *P<0.05.

vortex ($5.7\pm0.23x10^4$) was much more than the number without a vortex ($0.32\pm0.04x10^4$) (Fig. 2). After magnetic cell separation (MACS), we showed that $CD105^+$ cells combined with microbeads as detected under a microscope (Fig. 3). The

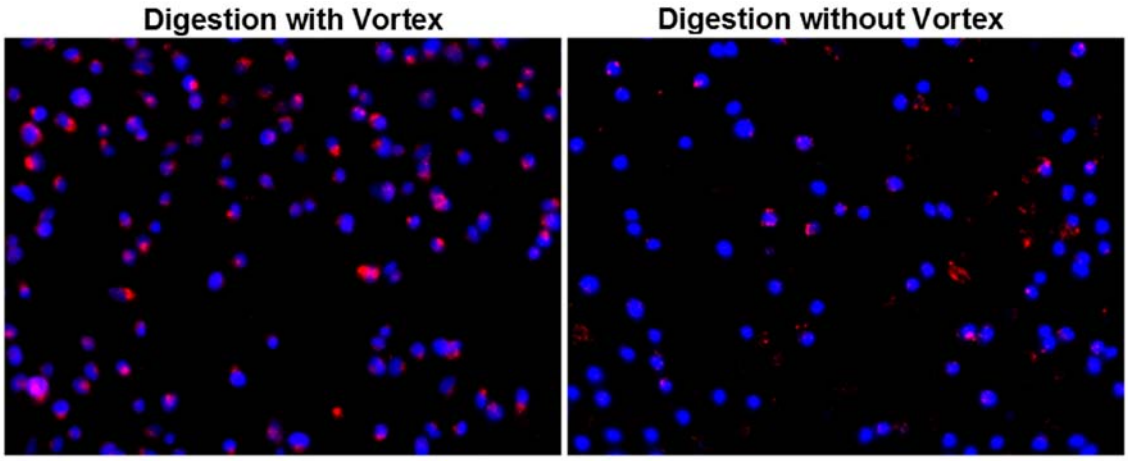

Figure 5. Dil-Ac-LDL assay of the CD105$^+$ cells (fluorescence microscopy at x200 magnification).

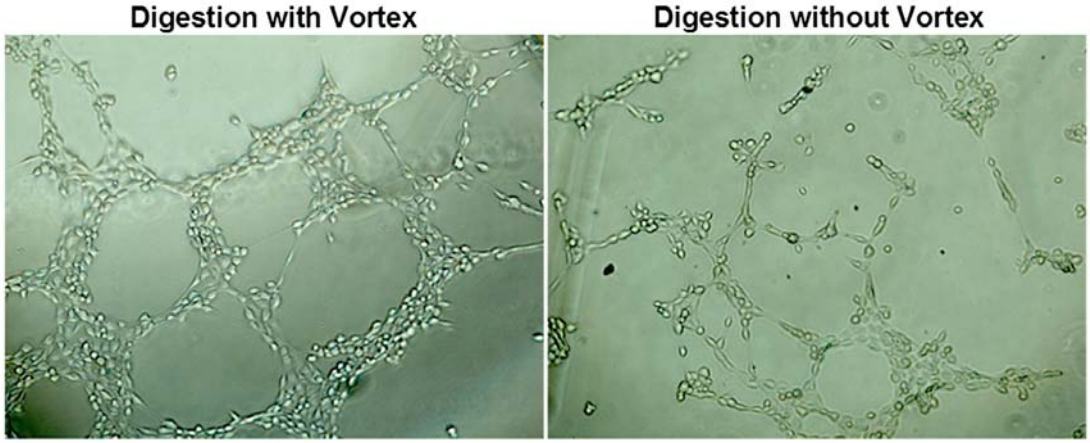

Figure 6. Tube formation assay of CD105$^+$ cells digested with a vortex and digested without a vortex (original magnification, x100).

purity of the CD105$^+$ cells was 93.07±1.7% as established by digestion with a vortex, while the purity was 78.53±2.2% as established by digestion without a vortex (Fig. 4).

Detection of CD105$^+$ endothelial cell activity. Dil-ac-LDL uptake assay showed that CD105$^+$ cells which were digested without a vortex exhibited red fluorescence, and CD105$^+$ cells which were digested with a vortex exhibited stronger fluorescence (Fig. 5). Cells which were digested without a vortex and seeded onto Matrigel formed capillary-like tube structures within 12 h, while cells which were digested with a vortex formed more capillary-like tube structures (Fig. 6).

Discussion

In the process of tumor growth, tumor angiogenesis and apoptosis have emerged as important aspects. Morover, pro-angiogenic factors are always dominant during the process of tumor development. It is well known that during tumor angiogenesis, endothelial cells undergo cellular and molecular changes that accompany the phenotypic appearance of angiogenic vessels (11,12).

Angiogenesis is necessary for tumor growth and metastasis (13,14). CD105 is an important marker in angiogenesis but is also essential for the proliferation of endothelial cells and the stimulation of the active phase of angiogenesis (15-17).

In view of the known heterogeneity of endothelial cells, it would appear logical to study endothelial cells derived from tumor tissues when studying the mechanisms of tumor progression (18-20). Even though methods have been described for the isolation of endothelial cells, the efficiency and purity of sorting have not been described.

In the present study, we described a method to isolate endothelial cells, a rare cell population found in tumor tissue, through digestion with a vortex. The subsequent isolation of endothelial cells was achieved using anti-CD105 antibody-coated microbeads. This purification technique produces isolated cells with a purity in excess of 93%, and a higher number of CD105$^+$ cells than that produced by digestion without a vortex. These cells express surface markers consistent with their endothelial cell origin, and maintain the ability of capillary tube-like structure formation and the uptake of acetylated LDL. This technology has a significantly important role in tumor angiogenesis research.

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