ONCOLOGY

A novel technique for the enrichment of primary ovarian cancer cells

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OBJECTIVE: Primary cancer cells that are extracted from ovarian tumors can serve as an optimal substrate to study the biologic characteristics of ovarian cancer. We describe an efficient and effective method of enriching ovarian tumor cells from ascitic fluid using an immunomagnetic-based method.

STUDY DESIGN: Mononuclear cells were isolated from ascites specimens by Ficoll gradient separation. Epithelial ovarian cancer cells were labeled magnetically with monoclonal human epithelial antigen-125 that is conjugated to microbeads. After immunomagnetic separation, the purity of tumor cells before and after purification was quantified by cytologic analysis and confirmed by fluorescence-activated cell sorter analysis.

RESULTS: Peritoneal ascites specimens were obtained from 6 patients with ovarian cancer. The median age of our patients was 61.5 years (range, 46-79 years). Three patients had papillary serous carcinoma; 2 patients had clear cell carcinoma, and 1 patient had an undifferentiated adenocarcinoma. The mean tumor purity was only 22.8% ± 10% (range, 1%-60%) before separation. After enrichment, the purity improved to 82.3% ± 4.0% (range, 70%-90%). Our enrichment technique increased the tumor purity by 59.5% ± 8.4%. The mean percent yield after positive enrichment was 30.1% ± 14.5%.

CONCLUSION: The immunomagnetic cell separation technique is an efficient and effective method for isolating and purifying ovarian tumor cells from ascites. Results from experiments with fresh tumor cells rather than cancer cell lines may be more relevant for clinical application.

Key words: immunomagnetic cell separation, ovarian cancer, peritoneal ascites

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Ovarian cancer is the most lethal gynecologic malignancy and the fifth most common cause of cancer death among women in Western countries.[1] Although most patients initially respond well to conventional chemotherapy, most patients will experience relapse with refractory disease and ultimately die because of complications from disease progression. Consequently, there is a strong impetus to investigate the molecular characteristics of this lethal disease to develop more effective diagnostic and therapeutic strategies. To date, established ovarian cancer cell lines have served as a useful substrate to study this disease. However, immortalized cell lines undergo many manipulations during their development that may limit our ability to translate experimental results from cell lines to actual ovarian disease in the clinical setting. Thus, primary tumor cultures may provide a better substrate for in vitro and in vivo studies. However, the ineffective and cumbersome process of isolating and purifying tumor cells from patient specimens has limited our ability to use primary tumor cells for experimentation. In particular, the contamination of malignant cells with benign cells (such as leukocytes, erythrocytes, fibroblasts, mesothelial cells, and monocytes) frequently results in poor tumor cell yield.

Magnetic cell separation has provided an efficient method for the enrichment and purification of tumor cells. This process involves labeling tumor cells that are admixed within a heterogeneous cell suspension with a tumor-specific monoclonal antibody (mAb) that is coupled to a magnetic bead. The mixed tumor-cell suspension is then exposed to a magnetic column where the tagged tumor cells are

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TABLE 1
Patient characteristics

Case	Age (y)	Stage of disease	Site	Histologic finding
1	62	IIIC	Peritoneal	Adenocarcinoma
2	61	IIIC	Peritoneal	Serous
3	79	IIIC	Peritoneal	Serous
4	64	IIIC	Peritoneal	Clear cell
5	58	IIIC	Peritoneal	Serous
6	46	IIIC	Peritoneal	Clear cell

extracted and eluted from other cells.[2] Microbeads that are conjugated to mAbs against human epithelial antigen-125 (HEA-125) can be used to label tumor cells of epithelial origin for separation from other cell types. HEA-125, also known as EpCAM or Ber-EP4, provides an optimal antigenic target for the separation of malignant cells of epithelial origin because it is expressed on >80% epithelial tumor cells and does not cross react with mesothelial cells, fibroblasts, and other nonepithelial cells, which can often contaminate tumor cell purifications.[3,4] Most studies have shown that Ber-EP4 is present in most serous tumors without variation based on histologic cell type.[5-7] In this study, we sought to determine the ability of HEA-125 immunomagnetic microbeads to enrich epithelial ovarian tumor cells from ascitic fluids extracted from patients with ovarian cancer.

MATERIALS AND METHODS

Isolation of mononuclear cells from ascites

With Institutional Review Board approval, physicians in the division of gynecologic oncology at Stanford University obtained ascites specimens from 6 consecutive patients with ovarian cancer at the time of surgery. Approximately 500 mL of ascites was obtained from each patient. To prevent coagulation of cells, 1 mL of Endrate (Abbott Laboratories, Abbott Park, IL) per 100 mL ascites fluid was added. Mononuclear cells were isolated from 30 mL ascitic fluid specimen with the use of a Ficoll gradient separation. The ascitic fluid was then diluted in 2-4 volumes of phosphate-buffered saline solution (PBS; Gibco, Grand Island, NY) that contained 2 mmol/L EDTA (Sigma-Aldrich, St. Louis, MO) that was layered over 15 mL Ficoll-Paque (Amersham Biosciences AS, Uppsala, Sweden) and centrifuged at 2200 rpm for 30 minutes at 20°C. The interphase mononuclear layer was transferred to a fresh conical tube and washed twice with PBS and 2 mmol/L EDTA. The pellet was resuspended in a final volume of 300 μL per 5 $\times$ 10^7 cells in a labeling buffer (PBS with 0.5% bovine serum albumin and 2 mmol/L EDTA). A volume of 3-5 mL of tumor cell suspension was prepared for cytologic analysis, and 1 $\times$ 10^6 cells were prepared for analysis by flow cytometry.

Magnetic labeling and enrichment of tumor cells

After Ficoll gradient separation, the mononuclear cells were labeled with colloidal superparamagnetic microbeads that were conjugated with monoclonal mouse antihuman HEA-125 according to manufacturer's protocols (Miltenyi Biotech Bergisch, Gladbach, Germany). Briefly, 100 μl HEA-125-conjugated microbeads per 1 $\times$ 10^7 total cells was added to cells and incubated for 30 minutes at 6°C in 500 μL labeling buffer. After being washed and resuspended in 500 μL labeling buffer, the labeled cells were then enriched with the use of an automated magnetic-activated cell separation unit (Miltenyi Biotech Bergisch).

Analysis of tumor cell purity by flow cytometry

Mononuclear cells that were isolated from ascites fluid were analyzed by flow cytometry before and after epithelial cell enrichment. A total of 1 $\times$ 10^6 cells were washed with PBS and labeled with 5 μL anti-HEA mAb that was conjugated to fluorescein (Miltenyi Biotech Bergisch) and anti-CD45 mAb that was conjugated to phytoerythrin (Becton Dickinson, San Diego, CA). The reaction mixture was incubated for 20 minutes on ice in the dark. The cells were washed with PBS with 2% fetal calf serum (Sigma-Aldrich, St. Louis, MO) and 10 μl pyridinium iodide (Sigma-Aldrich) was added immediately before flow cytometry analysis. Two-color analysis was performed on the FACscan (Becton Dickinson). Data were analyzed on FlowJo software (Tree Star, Inc, San Carlos, CA).

Cytologic analysis of purified ovarian tumor cells

Cytologic analysis was performed on cells before and after purification to determine yield and purity. The ascitic fluid specimen was concentrated by centrifugation at 600*g* for 10 minutes. After the supernatant was decanted, the concentrated ascitic fluid specimen was vortexed to resuspend the cell pellet, then the cells were washed with 30 mL of CytoLyt solution. The washed specimen was added to a vial of PreservCyt solution and run on a ThinPrep2000 processor (Cyty Corporation, Marlborough, MA). A Papanicolaou stain was performed on the resulting slide. Blinded cytologic evaluation was performed by a cytopathologist (C.S.K.). Purity was calculated by a visual estimation of the number of tumor cells as a percentage of the total number of tumor cells per high-power field. The cytopathologist estimated the intraobserver reproducibility for the purity count to be $\pm$5%. Photomicrographs were taken at $\times$300 magnification with a Spot RT Color digital camera (Diagnostic Instruments, Inc, Sterling Heights, MI) attached to a microscope (Eclipse E1000M; Nikon, NikonUSA, Melville, NY).

RESULTS

Peritoneal ascites specimens were collected from 6 patients with stage IIIC disease at the time of surgery. The median age of patients was 61.5 years (range: 46-79 years). All 6 patients were white. There were 3 cases of serous carcinomas,