Flow Cytometric Method for Determining Folate Receptor Expression on Ovarian Carcinoma Cells

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Abstract
The α-folate receptor (α-FR) is a folate transporter with restricted expression levels in normal tissues. It is over-expressed in several cancers, particularly epithelial carcinomas, including nonmucinous ovarian carcinoma. It offers a novel therapeutic target for selective imaging and cytotoxic agents. Measurement of the receptor could be a valuable tool in selecting patients more likely to respond to new drugs that target the α-FR, and monitoring them while on treatment. While tumor samples are often unavailable, a number of patients who relapse develop ascites, which are often rich in tumor cells. We have therefore developed a triple antibody flow cytometric method to assess α-FR expression on tumor cells from ascites. An antibody to BerEP4, an epithelial cell marker expressed on >90% ovarian cancers, labeled with fluorescein, and an α-FR antibody labeled with antimouse phycoerythrin have been used to label tumor cells, with a CD45-phycoerythrin-cyanine5 antibody used to exclude white blood cells from the analysis. The method was optimized using human carcinoma cell lines (JEG-3, IGROV-1, and KB cells). Calibrated beads were used to quantify the number of antibodies bound per cell. The triple antibody protocol successfully measured α-FR expression levels in cell lines spiked with blood. Tumor cells were obtained from ascites in 25 patients with relapsed ovarian cancer. In each case sufficient cells were harvested to identify an epithelial cell population to estimate the number of binding sites/cell. All the samples contained a single population of BerEP4, α-FR positive cells between $5 \times 10^3$ and $5 \times 10^5$ antibody binding sites/cell. The method can be used to determine the number of anti-α-FR antibodies bound per epithelial cell in ascites from patients with ovarian carcinoma. The results obtained were reproducible and the method could be applied to specimens that had been stored at $-80^\circ$C.

Key terms
folate receptor; ovarian carcinoma; flow cytometry

Drugs that target folate metabolism have been widely and successfully used in chemotherapy. Most of these drugs are predominantly transported into the cell via the reduced folate carrier (RFC). The RFC is expressed in many normal tissues and this limits the use of antifolate drugs because of unwanted side effects.

The α-folate receptor (α-FR) is a folate transporter that is expressed at low levels on the apical surface of several normal epithelial tissues, particularly glandular cells (1). It is over-expressed in several cancers, especially some epithelial carcinomas. Notably ~90% of epithelial ovarian cancers over-express α-FR (2–5). Further study has shown that the level of over-expression is also related to histological sub-type, with mucinous cancers having low levels (6). A high level of α-FR expression in nonmucinous ovarian cancers has a positive correlation with high histological grade and stage, and there is a suggestion that it may correlate with resistance to platinum therapy (5,7). Consequently, the α-FR offers a novel target for both selective imaging and therapeutic strategies including the development of cytotoxic agents such as BGC 945, an α-FR targeted thymidylate synthase inhibitor (8).

The levels of α-FR on normal and malignant human tissues have been estimated using a number of methods. mRNA has been extracted from fresh or formalin
embedded fixed material and amplified using the reverse transcriptase polymerase chain reaction. α-FR mRNA expression levels have been shown to correlate reasonably well with protein levels both in vitro and in vivo, although small sample sizes and the use of different semiquantitative methods have lead to results that are difficult to interpret (6,9). A radioligand binding assay that assesses FR levels in homogenized fresh tissue using 3H-folic acid has also been described (1). The method does not identify the site of receptor expression (e.g., apical vs. basolateral membrane), but, by requiring ligand binding, partially assesses receptor functionality. However, both these methods measure the total amount of α-FR in the sample and cannot take into account the dilution of the sample by normal cells. Cytometric methods have the advantage that the tumor cells can be identified and measurement is restricted to the cells of interest. Immunohistochemical analysis has been extensively used but is limited to fresh tissue as no antibody that binds to α-FR in paraffin embedded tissue is commercially available. A flow cytometric assay also has the advantage that the amount of receptor per cells can be more accurately quantified.

Although a sample from primary ovarian tumors can be accessed, biopsy material from patients who have relapsed is seldom available in ovarian cancer. However, many patients develop peritoneal ascites that are often rich in tumor cells.

Access to fluid samples from patients with ovarian cancer was available within our institution. The number of tumor cells in ascites is variable and samples are often heavily contaminated with blood. To measure the α-FR expression levels of these tumor cells, a flow cytometric assay was developed and optimized with the aim of quantifying the α-FR expression of ascitic tumor cells isolated from patients with ovarian cancer. The method has been successfully applied to three human tumor cell lines and to ascitic cells from 25 patients with ovarian carcinoma. This method may be useful in identifying a cohort of patients more likely to respond to α-FR targeted therapy.

MATERIALS AND METHODS

Cells

Three cancer cell lines, IGROV-1 (ovarian carcinoma), JEG-3 (choriocarcinoma), and KB (nasopharyngeal carcinoma) were cultured in folate-free Roswell Park Memorial Institute (RPMI 1640) medium (Sigma-Aldrich, Gillingham, Dorset, United Kingdom) supplemented with 20 nM leucovorin, and 10% heat inactivated dialyzed fetal calf serum (Introgen, Paisley, United Kingdom) in a 5% CO₂ incubator at 37°C. For flow cytometry, single cell suspensions were obtained using a 0.53 mM EDTA solution.

Peripheral blood was obtained from healthy donors.

Ascites samples were collected from patients with ovarian cancer; 5,000 units of heparin per liter of fluid was added to prevent coagulation. Samples were either processed immediately or stored in 10% DMSO/RPMI medium at −80°C.

Reagents

An α-FR specific monoclonal mouse antibody [LK26; (Ref. 3)] was purchased from Signet Pathology Systems MA (cat. no. 02026). FITC-BerEP4 (epithelial membrane antigen), PE-Cy5-CD45 (pan-leucocyte marker), and goat-anti-mouse R-phycocerythrin antibody (RPE) antibodies were purchased from Dako (cat. nos. F0860, C7099, and R0480, respectively) as the isotype control IgGs. Normal mouse serum was purchased from Hardensrea (P-0133D).

Cellquant calibrator beads (ref. no. 7208) were purchased from Biocytex, Marseille, France. The beads contained calibrated numbers of mouse IgG molecules on their surface.

The incubation and washing buffer was PBS with 2% foetal calf serum.

Cell Staining and Analysis

All incubations with antibody were on ice for 30 min. Antibody concentrations were determined in preliminary experiments using the cell lines, KB and JEG-3, for the LK26 and Ber-EP4 antibodies, respectively. The concentration of LK26 was selected to be just sufficient to achieve saturation. Red cells were removed by incubation in 0.15 M ammonium chloride solution for 15 min at room temperature, followed by washing in PBS. Incubation with either LK26 or an isotype control and subsequently secondary goat-anti-mouse-Ig-RPE was followed by mouse serum (to block unreacted sites on the antimouse-Ig). After further washing, the samples were incubated with BerEP4-FITC and CD45-PE-Cy5 antibodies. They were then washed and fixed in 0.5% paraformaldehyde prior to analysis on a Beckman-Coulter FCS500 bench top flow cytometer. Data were analyzed off-line using WinMDI (http://facs.scripps.edu/software.html) and figures prepared using FCS Express (DeNovo Software).

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RESULTS

Cell Lines

Two hundred microliters of peripheral blood was added to 0.5 × 10⁶ cells from each of the three cell lines, IGROV-1, JEG-3, and KB, and the cells stained using the triple antibody protocol described in Materials and Methods (Fig. 1). The cultured cells, which showed strong autofluorescence, could be clearly distinguished from the blood cells. The results were compared with the data obtained using cells without added blood and the antibody, LK26, alone. The two sets of results showed close concordance. The measurements were repeated several times giving an average of 1.52 × 10⁶ ± 0.77, 0.71 × 10⁶ ± 0.21, and 0.14 × 10⁶ ± 0.024 antibodies bound per KB, IGROV-1, and JEG-3 cell, respectively (Fig. 2).

The flow cytometric data also showed a strong positive relationship with α-FR surface expression levels measured by a 3H-folic acid radio-ligand binding assay, expression levels estimated by the two methods being within 4-fold of each other (Fig. 2).
Ascites

The method was then applied to a series of 25 ascites samples from patients who had relapsed ovarian carcinoma. In all cases, a population of Ber-EP4 positive cells, which expressed z-FR, could be identified (Fig. 3). In some patients, the small number of tumor cells made cytological identification difficult but by flow cytometry the BerEP4, z-FR positive cells could be identified, and the number of anti-z-FR antibodies bound quantified (Fig. 4). In the sample shown, the BerEP4 positive cells comprised only 0.1% of the total number of cells in the sample.

In five of these samples, one aliquot was processed without prior storage and a second analyzed after storage at -70°C in medium/DMSO for between 4 and 6 months. The number of binding sites detected was not appreciably affected by storage (Fig. 5A).

In all of the 25 samples processed, a single BerEP4 positive cell population was observed with a distribution of z-FR positive cells similar to that observed in the cell lines. There was no evidence for a secondary subpopulation with differing z-FR expression, indicating that there was homogenous z-FR expression on these ovarian cancer samples. The number of anti-z-FR antibodies bound per cell ranged from $5 \times 10^3$ to $5 \times 10^5$ (Fig. 6A). With this limited number of patients, there was no evidence that the number of binding sites related to tumor grade (Fig. 6B).

In 3 patients, after drainage of the ascites, further ascites developed within 3 weeks, before any further treatment had been undertaken. A comparison of the two samples showed that the number of binding sites/cell showed no apparent difference (Fig. 5B).

**DISCUSSION**

The method described can be used to measure the number of anti-z-FR antibodies binding to BerEP4 positive cells in
ascites from patients with ovarian carcinoma. This number should relate closely to the number of receptors, since each antibody molecule can only bind between one and two receptors. The method can be applied to cells that have been frozen, which enhances its utility since a patient sample may not be available until late in the day. A major advantage to the flow cytometric method is that it could be used on ascites with only small numbers of tumor cells present as this tumor cell

Figure 3. Ascites from patients with ovarian carcinoma labeled with BerEP4-FITC, FR-PE, and CD45-PC5. The final histogram of FR-PE was gated on large cells expressing BerEP-4. A histogram of the Cellquant beads incubated with the FR-PE antibody is also shown. Patient with a high percentage of BerEP4 cells.

Figure 4. As shown in Figure 3; patient with relatively few BerEP4 positive cells.
population can be recognized; other quantitative methods, such as radioligand binding, are not representative in circumstances when there are too few tumor cells relative to all the cells in the sample. The disadvantage of the present flow cytometric method is that it only measures the presence of the receptor protein and not whether it is functional. This drawback might be overcome by using a fluorescently labeled ligand in place of the LK26 antibody.

The BerEP4 antibody was selected as the epithelial marker because it has been reported that it is highly selective for ovarian carcinoma cells, and is negative in reactive mesothelial cells and in mesotheliosis (10–12). It also stained the ovarian carcinoma cell lines more strongly than an antibody to epithelial membrane antigen (EMA) (data not shown). There is a possibility that a poorly differentiated tumor might express BerEP4 too weakly to be detected, in which case this method would not be appropriate. However, BerEP4 positive cells were detected in all 25 of the samples analyzed in this study. Only a single population of z-FR expressing, BerEP4 positive cells was detected in these samples, consistent with homogeneous expression of z-FR. Conversely, the z-FR positive cells all expressed BerEP4, which suggested that, in these 25 samples at least, there was no interference from inappropriate staining of mesothelial cells. This method could also be used to measure z-FR expression levels on tumor cells in peritoneal or pleural fluid associated with other cancers such as lung adenocarcinomas that often also express BerEP4, or adapted for use in other cancers such as malignant mesothelioma.

The folate receptor targeted drug, BGC 945, is about to enter clinical trials. In the subset of patients who develop ascites, we intend to use this method in correlative studies to investigate the relationship between z-FR expression of ascitic

Figure 5. (A) Comparison of the number of z-FR antibody binding sites of the BerEP4 positive cells in 5 ascites samples processed either fresh or after DMSO storage for 4-6 months. (B) Comparison of the number of z-FR antibody binding sites of the BerEP4 positive cells in paired ascites samples collected from 3 patients in whom ascites reoccurred within a month.

Figure 6. (A) The number of z-FR antibody binding sites of the BerEP4 positive cells in 25 ascites samples from patients with ovarian cancer (●) and 3 cell lines (●). IC is average level for the isotype control, which is representative of IC levels for all cell lines and clinical samples. (B) Ascites from poorly (●) and moderately (●) differentiated tumors.
cells and z-FR expression of tumor tissue (by IHC where possible), and ultimately whether these levels correlate with sensitivity to the drug.

LITERATURE CITED


