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Quantitation of circulating tumor cells in blood samples from ovarian and prostate cancer patients using tumor-specific fluorescent ligands

Wei He[1], **Sumith A. Kularatne**[1], **Kimberly R. Kalli**[2], **Franklyn G. Prendergast**[3], **Robert J. Amato**[4], **George G. Klee**[5], **Lynn C. Hartmann**[6], and **Philip S. Low**[1,*]

[1]Department of Chemistry, Purdue University, West Lafayette, IN

[2]Department of Oncology, Mayo Clinic College of Medicine, Rochester, MN

[3]Department of Pharmacology, Mayo Clinic College of Medicine, Rochester, MN

[4]The Methodist Hospital Research Institute, Houston, TX

[5]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN

[6]Division of Medical Oncology, Mayo Clinic, Rochester, MN

Abstract

Quantitation of circulating tumor cells (CTCs) can provide information on the stage of a malignancy, onset of disease progression and response to therapy. In an effort to more accurately quantitate CTCs, we have synthesized fluorescent conjugates of 2 high-affinity tumor-specific ligands (folate-AlexaFluor 488 and DUPA-FITC) that bind tumor cells >20-fold more efficiently than fluorescent antibodies. Here we determine whether these tumor-specific dyes can be exploited for quantitation of CTCs in peripheral blood samples from cancer patients. A CTC-enriched fraction was isolated from the peripheral blood of ovarian and prostate cancer patients by an optimized density gradient centrifugation protocol and labeled with the aforementioned fluorescent ligands. CTCs were then quantitated by flow cytometry. CTCs were detected in 18 of 20 ovarian cancer patients (mean 222 CTCs/ml; median 15 CTCs/ml; maximum 3,118 CTCs/ml), whereas CTC numbers in 16 gender-matched normal volunteers were negligible (mean 0.4 CTCs/ml; median 0.3 CTCs/ml; maximum 1.5 CTCs/ ml; $p < 0.001$, χ^2). CTCs were also detected in 10 of 13 prostate cancer patients (mean 26 CTCs/ml, median 14 CTCs/ml, maximum 94 CTCs/ml) but not in 18 gender-matched healthy donors (mean 0.8 CTCs/ml, median 1, maximum 3 CTC/ml; $p < 0.0026$, χ^2). Tumor-specific fluorescent antibodies were much less efficient in quantitating CTCs because of their lower CTC labeling efficiency. Use of tumor-specific fluorescent ligands to label CTCs in peripheral blood can provide a simple, accurate and sensitive method for determining the number of cancer cells circulating in the bloodstream.

Keywords

circulating tumor cell; quantitation of tumor burden; fluorescent conjugates of tumor-specific ligands; ovarian cancer diagnosis; prostate cancer diagnosis

*__Correspondence to:__ Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47906, USA. Fax: 765−494−5272. E-mail: plow@purdue.edu.

Published studies estimate that growing tumors shed anywhere from 10^5 to 3×10^6 CTCs/day/g malignant tissue.[1-4] Thus, the measurement of circulating tumor cell (CTC) numbers in peripheral blood constitutes one of the most sensitive methods for assessing residual malignant disease.[5-10] Indeed, recent clinical studies demonstrate that CTC analyses can predict outcome in multiple cancers, including cancers of the breast, prostate and colorectal tissues.[11-13] Consequently, increased effort is being focused on improving methods for detecting CTCs in blood samples from cancer patients.[5,14]

Established protocols for CTC detection rely on either the polymerase chain reaction (PCR) or flow cytometry to assess the presence of malignant cells. In PCR methods, freshly isolated blood samples are screened for the presence of multiple cancer-specific transcripts.[15,16] While highly sensitive, such PCR methods require considerable time and yield largely qualitative answers. Although more advanced PCR techniques such as real-time PCR can provide reasonably quantitative results, their interpretation can also be confused by nonspecific amplification of normal sequences closely related to cancer genes[17] and low level expression of target cancer genes in noncancerous cells.[17-22] In contrast, flow cytometry methods have the advantage of being faster, simpler to perform and more quantitative, but they also suffer from problems associated with cancer specificity. Thus, most flow cytometry methods rely on antibodies that recognize not only malignant cells, but also some healthy cells that express malignant markers (*e.g.*, epithelial cell antigens such as cytokeratin). Further, in cases where marker expression is weak or masked, the same assays can lead to false negative results because of failure to detect masked malignant cells present in the sample.[18,19] Therefore, a CTC detection method with the rapidity and ease of flow cytometry but the specificity and sensitivity of PCR could find utility in the clinic.

Recent immunohistochemical analyses demonstrate that many human carcinomas overexpress a receptor for the vitamin folic acid (*e.g.*, 90% of ovarian and endometrial cancers, 75% of kidney cancer, 78% of nonsmall cell lung cancer, etc.[23-28]). In contrast, most normal tissues either lack folate receptors (FR) or express FR at a site that is inaccessible to the circulatory system. For example, an average ovarian cancer cell will have 1−3 million FR, whereas an average FR-expressing normal cell will have <50,000 FR. More importantly for CTC analysis in blood samples, no non-malignant blood cells except a subpopulation of activated monocytes express a functional FR. In the specific case of the aforementioned subpopulation of monocytes, the number of FR/cell is ~100,000; *i.e.*, between 10 and 30 times fewer FR/cell than ovarian cancer cells. This ~20-fold difference in FR expression between ovarian cancer cells and monocytes allows facile differentiation of the 2 cell types by flow cytometry. Because fluorescent folate conjugates bind CTCs *in vitro* with low nanomolar affinity ($K_D = \sim 1$ nM),[29,30] and since these fluorescent ligands label cancer cells ~100-fold more intensely than tumor-specific antibodies,[31] we decided to explore whether folate conjugates might provide the enhanced sensitivity and specificity needed for flow cytometric quantitation of CTCs. In this report, we describe the use of both folic acid and a prostate-specific ligand in the development of a highly accurate CTC assay. After optimizing the assay, we proceed to demonstrate its utility in detecting CTCs in fresh peripheral blood samples from a number of ovarian and prostate cancer patients.

Material and methods

Patients

All experimental procedures including blood sampling, processing and analysis were performed under the auspices of Clinic Institutional Review Board-approved protocols at the Mayo Clinic and Purdue University. Eight milliliters of peripheral blood were withdrawn into anticoagulant tubes from normal volunteers or suspected ovarian or prostate cancer patients. Ovarian cancer patient age and tumor histology/pathology are summarized in Table I.

Peripheral blood samples were collected from 18 gender-matched normal volunteers for the prostate cancer CTC analysis and from 16 gender-matched normal volunteers for the ovarian cancer CTC analysis. These latter samples were assumed to be cancer-free and were used as negative controls.

Reagents

Ficoll-Paque™ was purchased from Amersham (Piscataway, NJ). RosetteSep™ Human Circulating Epithelial Tumor Cells Enrichment Cocktail was purchased from StemCell Technologies (Vancouver, Canada). A23187, ammonium chloride lysis buffer, Histopaque™ 1077, 1083 and 1119 were from Sigma (Milwaukee, WI). Oncoquick® kit and LeucoSep tube were purchased from Greiner Bio-One (Frickenhausen, Germany). Folate-Alexa-Fluor 488 and DUPA-FITC (The synthesis and characterization of DUPA-FITC is described in the manuscript by Kularatne SA, Wang K, Santhapuram HR, He W, and Low PS. Prostate-Specific Membrane Antigen (PSMA)-Targeted Imaging and Therapy of Prostate Cancer with a High Affinity PSMA-Targeting Ligand, unpublished data.) were synthesized as reported elsewhere.[31] AlexaFluor 647 conjugated anti-CD45 antibody was from Serotec (Raleigh, NC). Monoclonal and polyclonal anti-FR antibodies were prepared according to the literature elsewhere.[31] Sterile PBS containing 2% fetal bovine serum serves as the washing buffer (PBSF) for use in the CTC enrichment methods described below.

Selection of optimal CTC enrichment technique

To determine the optimal method for enrichment of CTCs from peripheral blood, 10^4 human nasopharyngeal cancer cells (KB, an FR^+ cancer cell line) were labeled with DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chloroben-zenesulfo-nate), a lipophilic dye that enables tracking of labeled cells in heterogeneous suspensions, and added to 2 ml aliquots of whole blood from healthy donors. After conducting the enrichment procedures described below, the efficiency of KB cell recovery was determined by flow cytometry. Nine different isolation procedures were compared. They are as follows: (*i*) *Ficoll-Paque:* whole blood samples were diluted with an equal volume of PBSF and mononuclear cells were separated by Ficoll density centrifugation at 1,000*g* for 20 min, as described by the manufacturer; (*ii*) *A23187 treatment plus Ficoll-Paque:* blood samples were treated with 1 μM A23187 for 30 min on ice to shrink erythrocytes and then separated by the above Ficoll procedure; (*iii*) *RosetteSep-Ficoll:* blood samples were treated with RosetteSep™ (50 μl/ml) for 20 min at RT followed by Ficoll separation, as described above; (*iv*) *Ammonium chloride lysis:* erythrocytes were hemolyzed by addition of NH_4Cl lysis buffer (v/v, 25:1) followed by cell pelleting twice, after which nonhemolyzed cells were collected and washed in PBSF at 200*g* for 15 min; (*v–vii*) *Histopaque:* blood samples were diluted with an equal volume of PBSF, and mononuclear cells were separated by Histopaque™ 1077, 1083 or 1119 density gradient centrifugation according to the manufacturer's procedures; (*viii*) *OncoQuick:* blood samples were layered onto OncoQuick separation medium and cell isolation was performed according to the instructions provided except the method was adapted for use with 2 ml total blood volume; (*ix*) *LeucoSep tube with Ficoll:* blood samples were processed according to LeucoSep instructions. To further improve the RosetteSep-Ficoll-based protocol, centrifugation speed, centrifugation time, dilution factor and pH of the incubation buffer were all individually optimized for maximal CTC enrichment and labeling.

Specificity and labeling efficiency studies

Whole blood samples from 3 healthy donors were spiked with PSMA-expressing prostate cancer cells (LNCaP) that had been previously labeled with DiD to allow their tracking. CTC-enriched fractions were then isolated from the above blood samples by the RosetteSep-Ficoll protocol and labeled with 1 μM DUPA-FITC, a fluorescein-derivatized ligand that binds with

~8 nM affinity to prostate-specific membrane antigen.[1] The cell suspension was then analyzed by flow cytometry for the presence of both DiD and DUPA-FITC. DUPA-FITC labeling specificity was defined as: $\mathrm{LS}=\frac{\text{No. of DUPA}^+/\text{DiD}^+ \text{ cells}}{\text{No. of DiD}^+ \text{ cells}}$ and was evaluated by flow cytometry.

For comparison of the labeling efficiencies of KB cells with (*i*) folate-dye conjugates, (*ii*) polyclonal anti-FR antibodies or (*iii*) monoclonal anti-FR antibodies, cultured KB cells were incubated for 30 min at RT with folate-FITC, a polyclonal affinity purified rabbit anti-FR antibody (PU17), or a monoclonal anti-FR antibody (mAb343), respectively. Primary antibody-labeled cells were then treated with FITC-conjugated secondary anti-IgG antibodies for 30 min at RT before analysis by flow cytometry. In some experiments, primary polyclonal anti-FR antibodies (PU17) were directly labeled with FITC, obviating the need for secondary antibody labeling. Unlabeled KB cells and KB cells incubated with 10 μM free folic acid prior to addition of 100 nM folate-FITC (to block all available FRs) served as negative controls. All samples were analyzed on a Beckman Coulter FC500 flow cytometer using CellQuest software.

Clinical evaluations

To validate this methodology, we conducted a double-blind study on patients with metastatic ovarian and prostate cancers. Twenty-three suspected ovarian cancer patients and 13 prostate cancer patients were treated with folate-AlexaFluor 488 or DUPAFITC conjugates, respectively. Similarly treated healthy donors served as negative controls for each cancer type. All blood samples were prepared using the RosetteSep-Ficoll protocol and examined by flow cytometry. A background threshold of 1.5 CTCs/ml for ovarian cancer patients and 3 CTCs/ml for prostate cancer patients was set to define the difference between CTC positive and CTC negative samples. Based on this cutoff, CTC levels were statistically analyzed by a chi-square test with a confidence level of 95%.

Results

Specificity studies

Previous studies have shown that folate conjugates label CTCs in peripheral blood with a specificity of 99%.[31] To determine the specificity of our novel DUPA conjugate for prostate cancer cells in similar blood samples, whole blood collected in anticoagulant from 3 healthy donors was spiked with PSMA-expressing prostate cancer cells (LNCaP) that had been labeled with DiD to allow their tracking. After collecting a CTC-rich fraction (see Methods), DUPA-FITC was added to the suspension to label LNCaP cells and the suspension was analyzed by flow cytometry. By measuring the fraction of DiD-labeled cells that were also DUPA-FITC positive, it was determined that LNCaP cells could be labeled with DUPA-FITC in whole blood with 93.8% ± 1.7% efficiency (data not shown). More importantly, no FITC-positive cells were detected in any blood samples lacking cancer cells, confirming that DUPAFITC exhibits no nonspecific affinity for normal blood cells. These data indicate that DUPA conjugates, like folate conjugates, can be used to specifically label cancer cells in whole blood samples.

Because most current methods for CTC quantitation rely on labeling with fluorescent antibodies, it was important to compare the labeling efficiency of a low molecular weight ligand-dye conjugate (*e.g.*, folate-FITC) with those of various tumor-specific antibodies. For this purpose, KB cells were incubated *in vitro* with: (*i*) folate-FITC, (*ii*) affinity-purified polyclonal anti-FR antibody labeled with FITC (PU17-FITC), (*iii*) a monoclonal anti-FR antibody (mAb343) followed by FITC-labeled goat anti-mouse IgG or (*iv*) affinity-purified polyclonal anti-FR antibody (PU17) followed by FITC-labeled rat anti-rabbit IgG. After allowing time for each agent to bind, labeling intensities were compared by flow cytometry. The data in Figure 1 demonstrate that despite the signal enhancement inherent in multi-antibody labeling (“piggybacking”) methods, the fluorescence intensity of folate-FITC incubated cells

was still >20-fold greater than that of any antibody-based method. When considered with the undesired tendency of antibodies to bind immune cells with Fc receptors, these data argue that high affinity, low molecular weight, tumor-specific ligands can serve as optimal reagents for CTC detection.

Selection of optimal CTC enrichment technique

Initial efforts to quantitate CTCs with low molecular weight ligand conjugates were performed on unfractionated whole blood samples. Although these methods were highly successful, the enormous preponderance of erythrocytes in such samples required long periods of flow cytometer time to count even small volumes of blood. Therefore, to significantly reduce flow cytometer usage, a method was needed for enrichment of CTCs from peripheral blood. To identify such a method, 9 different enrichment procedures were compared and their efficiencies of CTC isolation were quantitated on the same blood sample. As summarized in Table II, the RosetteSep-Ficoll procedure exhibited the highest recovery efficiency (62.5%), followed by the simple Ficoll (42.3%) protocol. Ammonium chloride lysis showed the lowest detection efficiency (5.3%), perhaps because the high concentrations of folic acid released from lysed erythrocytes[32] competed with fluorescent folate conjugates for binding to CTCs. Other methods yielded recovery efficiencies ranging from 17.2 to 31.9% (Table II).

To demonstrate that the RosetteSep-Ficoll protocol can also be used for enrichment of CTCs originating from ovarian or prostate cancers, we performed the recovery efficiency study on human ovarian and prostate cancer cell lines (IGROV and LNCaP, respectively). Both cell lines exhibited similar recovery efficiencies to KB cells (IGROV cells: 62%; LNCaP: 59% and KB: 62%). These data suggest that the efficiency of the separation protocol is independent of cancer cell type.

CTC quantitation in clinical samples

Using the above procedures, CTCs were detected in blood samples from 18 of 20 patients with metastatic epithelial ovarian cancer (mean 254 CTCs/ml; median 26 CTCs/ml; maximum 3,118 CTCs/ml), whereas CTC counts in 16 normal volunteers were negligible (mean 0.4 CTCs/ml; median 0.3 CTCs/ml; maximum 1.5 CTCs/ml) (Fig. 2*a*). Further, microscopic analysis of the fluorescent objects detected in healthy blood samples revealed that they were either dead cells with entrapped dye or amorphous fluorescent particles. Using 1.5 CTCs/ml as the threshold to distinguish healthy from malignant blood samples, chi-square analysis of the data in Table III demonstrated that CTC levels were significantly higher in cancer patients than healthy volunteers ($p < 0.001$, χ^2). More specifically, 100% of patients with serous tumors were CTC positive, while 67% of samples from patients with nonserous ovarian carcinoma were CTC positive. CTC levels for serous samples averaged 239 CTCs/ml, but only 2 CTCs/ml for nonserous samples. Among the 3 patients initially diagnosed with benign disease, only 1 patient with a mucinous cystadenoma and a hemorrhagic cyst exhibited measurable CTCs. As these cysts can contain malignant cells, further monitoring will be required to establish whether this diagnosis was a false positive.[33]

Analyses of prostate cancer patients using the DUPA-FITC conjugate yielded similar results. CTCs were detected in 10 of 13 peripheral blood samples from prostate cancer patients (mean 26 CTCs/ml, median 14 CTCs/ml, maximum 94 CTCs/ml), but absent from 18 healthy subjects (mean 0.8 CTCs/ml, median 1, maximum 3 CTC/ml) (Fig. 2*b*). As shown in Table III, prostate cancer patients also exhibited statistically higher CTC counts in their blood samples than healthy controls ($p < 0.002$, χ^2), assuming a threshold value of 3 CTC/ml. Although several subjects in the control group exhibited nonzero CTC counts, the difference in CTCs between prostate cancer patients and normal volunteers was nevertheless significant.

Discussion

We have described a method for quantitating CTCs in peripheral blood samples of suspected ovarian and prostate cancer patients. The method is based on a simple enrichment step followed by labeling of CTCs with high affinity, low molecular weight tumor-specific ligands. Studies with both normal blood samples spiked with tumor cells and cancer patient blood samples demonstrate that the method is sensitive, quantitative and tumor cell specific. Because of the wide availability of flow cytometers and the simplicity and rapidity of the test (multiple samples can be analyzed within 2 hr), the method could conceivably be applied in a variety of clinical settings.

Potential applications of any *ex vivo* CTC detection protocol would likely require repeated patient blood sampling. These include continued monitoring of a patient's response to therapy and screening for disease recurrence after surgery/chemotherapy. Because cancer patients are often anemic, both as a consequence of their disease burden and their cytotoxic therapy, it is important that repeated CTC assays consume as little blood as possible. The assay described above requires only 2 ml whole blood per sample and therefore can be performed at reasonable intervals without significantly aggravating the anemia that might be present.

Most *in vitro* diagnostic tests for cancer suffer from potential false positive results that derive from expression of tumor antigens/markers on normal cells. For example, cytokeratins,[12] generic markers for epithelial CTCs, are also found on healthy epithelial cells released into circulation as well as on a subpopulation of granulocytes.[17-19] Further, CA125, a serum marker used for monitoring ovarian cancer burden, can yield false positive results during normal ovulation,[34] endometriosis,[35,36] emergence of benign ovarian cysts,[37,38] during the first trimester of pregnancy[34,38] and as a consequence of several types of pelvic inflammation.[38-40] For postmenopausal women over the age of 45, the latter group of complications can cause a false positive rate as high as 80%.[41,42] Similar aberrations in PSA levels have also been observed to produce false positive diagnoses for prostate cancer.[43,44] In contrast, FR-expressing cells are essentially absent from the bloodstream of normal individuals. In fact, only an activated subpopulation of monocytes expresses FR, and FR levels on these cells are ~20-fold lower than the average FR level on ovarian cancer cells (manuscript in preparation). In those rare cases where “CTCs” have been detected in healthy samples, they were invariably identified as dead cells or nonbiologic fluorescent particles and represented fewer than 1.5 CTCs/ml. Indeed, continuous screening for CTCs in the superficial vasculature of live tumor-free mice by intravital vivo flow cytometry has revealed no CTCs in the animal's entire blood volume.[31]

Finally, because hematopoietic cancer cells lack epithelial markers, antiepithelial cell antibodies cannot be used to quantitate such cancer cells in circulation. Current flow cytometry methods for leukemia CTC analyses, therefore, employ a different combination of antibodies to distinguish malignant cells from healthy cells in whole blood samples.[45,46] Because such antibody panels vary for different leukemias, a full set of such panels must be used for quantitation of unknown leukemias, rendering the assays cumbersome and expensive.[46,47] In contrast, FR can serve as a single marker for identifying CTCs derived from myeloid leukemias, because these leukemias have been shown to overexpress FR (>70% in acute myeloid leukemia, 100% chronic myeloid leukemia[48-50]). Thus, in addition to applications in solid tumor diagnoses, use of folate conjugates could find utility in monitoring the disease status of some leukemias.

In summary, our study demonstrates that flow cytometry coupled with high affinity, tumor-specific, fluorescent ligands can be used to quantitate CTCs in blood samples from ovarian and prostate cancer patients. While the methodology must still be validated in prospective

studies focused on specific applications such as (*i*) detection of cancer recurrence, (*ii*) monitoring of disease progression and (*iii*) assessment of response to therapy, the assay nevertheless shows potential for increasing the repertoire of tools available to oncologists for improving management of malignant diseases.

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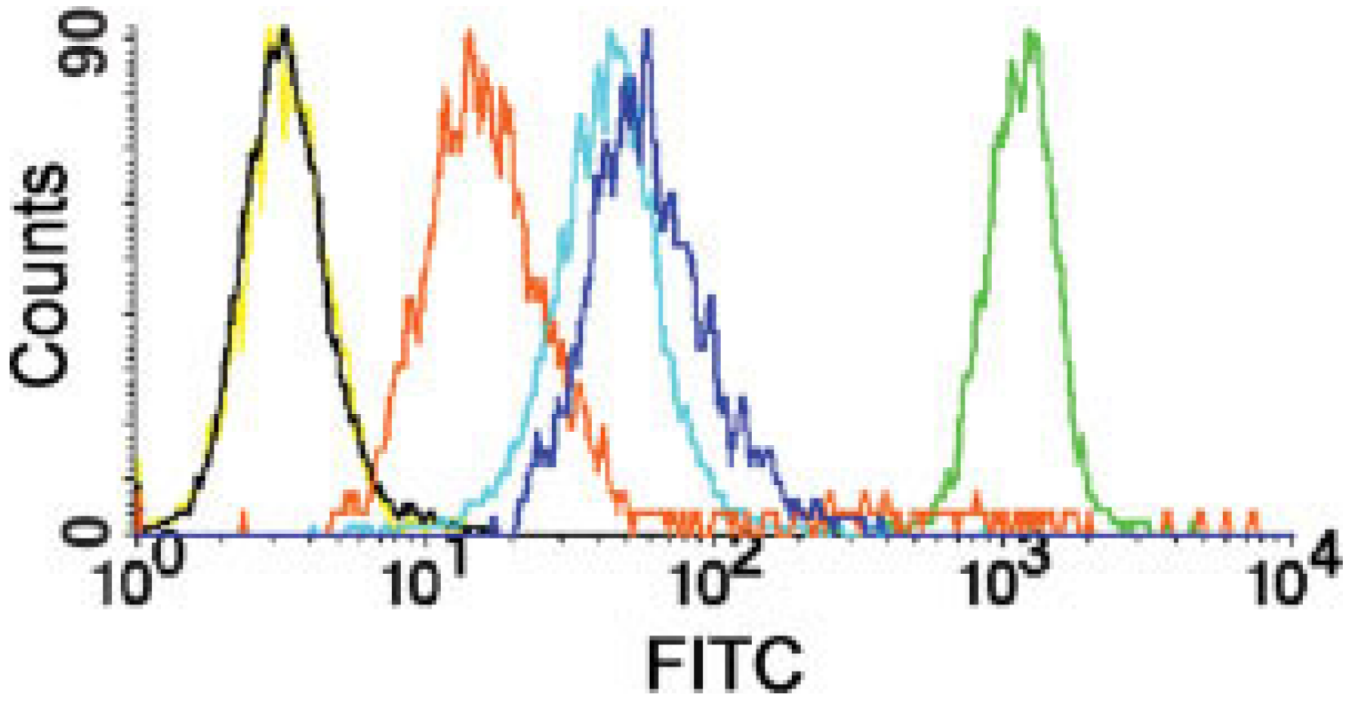

Figure 1.

Comparison of KB cell labeling intensity with folate-FITC *versus* various anti-FR antibodies. KB cells were labeled with (*i*) folate-FITC (green), (*ii*) affinity-purified polyclonal anti-FR antibody directly labeled with FITC (PU17-FITC, orange), (*iii*) monoclonal anti-FR antibody (mAb343, cyan) followed by FITC-labeled goat anti-mouse IgG or (*iv*) affinity-purified polyclonal anti-FR antibody (PU17, navy) followed by FITC-labeled rat anti-rabbit IgG prior to analysis by flow cytometry. Unlabeled KB cells (black) and KB cells incubated with 10 μM folic acid plus 100 nM folate-FITC (yellow, totally competed) served as negative controls.

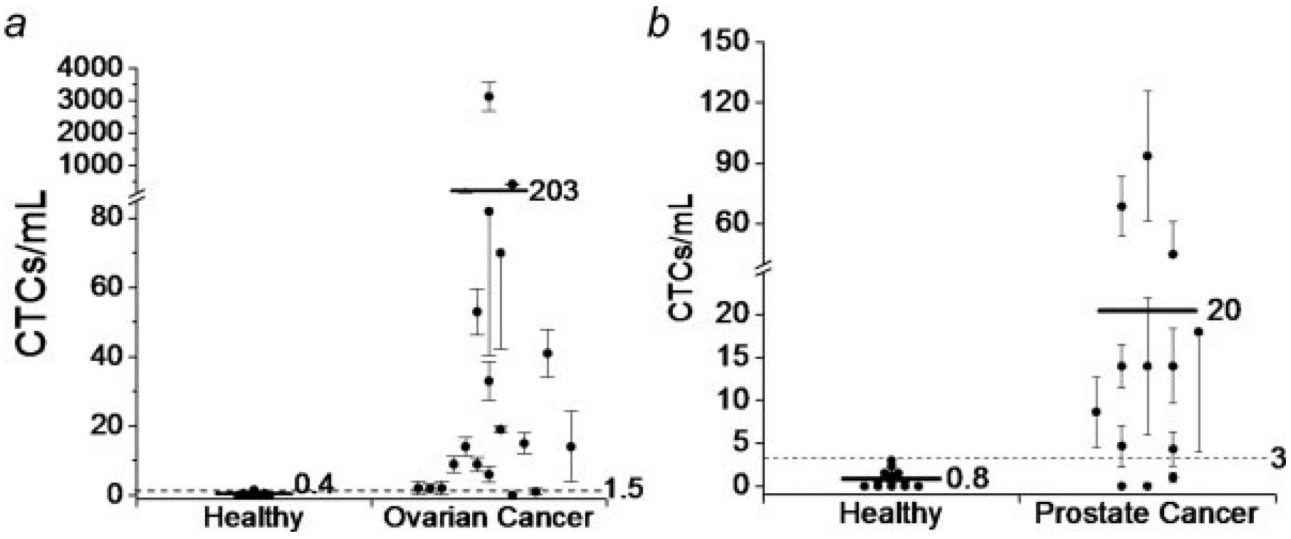

Figure 2.

CTC quantitation in blood samples from ovarian and prostate cancer patients. Dots in (*a*) and (*b*) represent mean values from multiple independent measurements of CTCs in blood samples from individual healthy subjects, or ovarian or prostate cancer patients. Bars in (*a*) and (*b*) indicate the average number of CTCs in the collection of blood samples from healthy donors, or ovarian or prostate cancer patients. Threshold values of 1.5 CTCs/ml for ovarian cancer samples and 3 CTC/ml for prostate cancer samples are indicated by dashed lines in (*a*) and (*b*).

TABLE I

PATIENT CHARACTERISTICS IN OVARIAN CANCER STUDY

Characteristics	Number of patients	%
Number of patients[1]	23	100
FIGO stage		
I	2	9
II	1	4
III	17	74
Benign	3	13
Histology		
Ovarian cancers	20	87
Serous ovarian	13	57
Serous primary peritoneal	4	17
Endometrioid	2	9
Borderline malignant	1	4
Benign tumors	3	13
Mucinous cystadenoma	1	4
Serous cystadenoma	2	9

[1] Patients were diagnosed ovarian cancer at the age range of 49−85 and the median age of 62.

TABLE II

SUMMARY OF RECOVERY EFFICIENCIES OF 9 ENRICHMENT METHODS

Method no.	Method name	The efficiency of recovery (%)			
		Exp. 1	Exp. 2	Exp. 3	Mean ± SD
1	Ficoll	45.1	39.5	42.2	42.3 ± 2.8
2	A23187 treatment plus Ficoll	33.9	31.5	30.2	31.9 ± 1.9
3	RosetteSep-Ficoll	61.3	64.2	62.0	62.5 ± 1.5
4	Ammonium chloride lysis	5.0	5.2	5.6	5.3 ± 0.3
5	Histopaque 1077	24.3	25.1	27.2	25.5 ± 1.5
6	Histopaque 1083	27.5	26.9	28.1	27.5 ± 0.6
7	Histopaque 1199	17.2	18.1	16.3	17.2 ± 0.9
8	OncoQuick	20.0	22.4	21.9	21.4 ± 1.3
9	LeucoSep with Ficoll	30.0	33.2	32.1	31.8 ± 1.6

TABLE III

SUMMARY OF CLINICAL EVALUATIONS

	Malignant		Benign		Healthy	
	n	**%**	***n***	**%**	***n***	**%**
Ovarian cancer study[1]	20	87	3	13	16	100
CTC+	18	90	1	33	0	0
CTC−	2	10	2	67	16	100
Prostate cancer study[2]	13	100	–	–	18	100
CTC+	10	77	–	–	0	0
CTC−	3	23	–	–	18	100

[1]The threshold value was set at 1.5 CTCs/ml for ovarian cancer study.

[2]The threshold value was set at 3.0 CTCs/ml for prostate cancer study.