Circulating tumor cells: capture with a micromachined device

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ABSTRACT

The isolation and analysis of circulating tumor cells from blood is the subject of intense research. Tests to detect metastasis are available, but progress has been hampered by the lack of tumor-specific markers and predictable DNA abnormalities. CTCs from solid tumors can be separated from normal hematopoietic cells based on size and other inherent physical and biological properties. The main challenge in this endeavor is the small number of available cells of interest, 1-2 per ml in human whole blood. A micromachined device for fractioning whole blood using physical methods to enrich and/or isolate rare cell types from peripheral circulation has been designed. It has four segments of microfluidic channels, each consisting of a 2-D array of columns. The gap between the columns progressively narrows across the device. Current devices have channels ranging in width from 20μm down to 5μm, and in depth from 20μm down to 5μm. When healthy adult blood, spiked with cells from each of eight cancer cell lines tested was loaded into the device, all cancerous cells were retained in a well-defined area of the device, while blood cells migrated to the output reservoir. Use of these devices will advance non-invasive methods used to monitor patients, stage disease, and assess treatment. Furthermore, insights into metastasis will be gained.

Keywords: biochips, cell sorting, circulating tumor cells, cancer.

1 BACKGROUND

Circulating tumor cells (CTCs) in the peripheral blood are the result of metastasis from a primary tumor; metastasis accounts for the majority of cancer deaths. The presence of these cells is the reason for the recurrence of malignancies even after surgical resection has left a tumor-free margin. For example, half of stage I to III breast cancers will recur after the primary tumor has been removed by surgery [1], due to the early dissociation of cancer cells from the original tumor, which cannot be detected by conventional staging techniques [2].

The detection and analysis of CTCs, or micrometastases, are important for an understanding of the process of metastasis, for disease screening and staging, for predicting prognosis, for monitoring patients during and after therapy, and for improving therapy design.

The main challenge in detecting CTCs is their low frequency. In bone marrow, one or two cancer cells can be found per million cells, whereas they are much rarer in the peripheral circulation, where there may only be one or two cells per milliliter of whole blood, or approximately 500-1000-fold less per number of cells than in bone marrow. As a result, the bone marrow has been favored for staging [1,3].

Three main techniques have been used to detect tumor cells in bone marrow: immunocytochemistry (ICC), immunofluorescence, and polymerase chain reaction (PCR) [2], of which ICC is the most widely utilized. ICC relies on ectopic expression of epithelial (e.g., cytokeratins) or tumor markers to detect metastasis. Flow cytometry exploits differences in cell size and granularity, and uses a fluorescence-labeled monoclonal antibody (mAb) to sort cells so as to provide a purified, or at least enriched, tumor cell population for further study. Molecular methods such as reverse transcription-PCR (RT-PCR) can be used to examine tumor cell-specific gene expression.

Due to the rarity of these cells, detection techniques are usually preceded by an enrichment step such as immunomagnetic separation (IMS or magnetic cell sorting), which increases detection rates up to 10-fold [4,5].

Despite some successes, none of these methods has proven ideal to detect circulating cancer cells in a patient's blood or bone marrow. ICC and immunofluorescence are labor-intensive, particularly when integrated with an enrichment technique. These techniques also require preliminary studies to identify cell surface targets and are only capable of assessing the presence and quantity of tumor cells; neither technique collects viable cells for downstream analysis. The application of FACS and magnetic cell sorting is limited by the availability of specific antibodies, the long preparation times, and the requirement for skilled technicians and expensive equipment. Thus, while these techniques have been in development for over a decade, they remain elusive to many laboratories, limiting test applicability and importantly, availability. While molecular techniques using RT-PCR are very sensitive, very few disease-specific mutations are known for circulating cancer cells from solid tumors, and the high sensitivity associated with these methods can prove to be disadvantageous if the target is also expressed in normal cells [6].

It is known that malignant tumor cells differ from benign tumor cells in structure, growth rate, invasiveness, and ability to metastasize. Cancer cells undergo modifications in their morphology, mainly increasing the size of the nucleus before they become invasive, i.e., in dysplasia and carcinoma *in situ*. The nucleus of a

dysplastic cell can be up to four times as large as that of a non-dysplastic cell. Nuclear changes such as an increase in size, deformation, and a change in internal organization are among the most universal criteria for detection of malignancy [7]. The changes in physical and morphological characteristics described above have been exploited in this study, to create a microfabricated separation device.

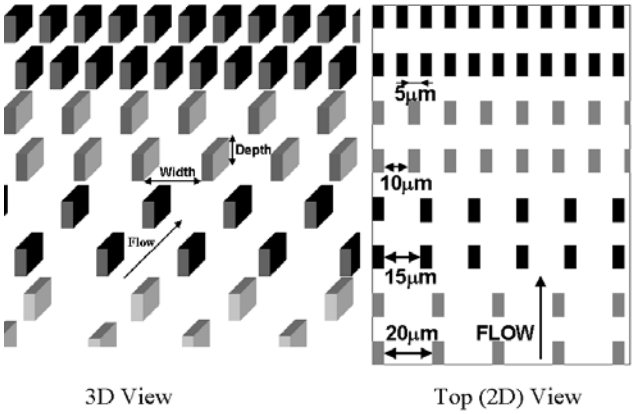

Figure 1: Schematic showing the device's progressively smaller channel gap widths and the flow direction; channel depth is constant over the entire device.

2 MATERIALS AND METHODS

The device has four segments of successively narrower channels along the flow axis; these have 20, 15, 10, and 5μm spacing (figure 1). The channel design was laid out on computer-aided design software, and fabricated on 4" wafers using standard micromachining techniques as described in [8]. All channels have a fixed depth, since the whole wafer is etched at once. Several silicon masters were fabricated by this method, with depths of 20, 15, 10, or 5μm. After fabrication, each silicon wafer had over a million channels covering a 30mm wide by 60mm long area, with one reservoir on each end. Each reservoir was 30mm by 10mm.

Final devices were molded using polymers such as polydimethylsiloxane (PDMS) (Sylgard-Dow Corning or GE RTV615- General Electric), polystyrene (Sigma-Aldrich), and/or polyurethane (Smooth-On Inc., Easton, IL); these are non-toxic to cells, transparent, handle fluids easily, and can create a high-quality replica of any feature on the wafer down to the sub-micron level.

For this study, devices were either molded in PDMS and sealed with polystyrene tops, or else were molded in polyurethane and sealed with polyurethane tops as described in [8].

Five neuroblastoma cell lines (SK-N-MC, SK-N-AS, SK-N-SH, SH-SY5Y, and BE(2)-M17) were obtained from ATCC (Manassas, VA) and three adult epithelial cancer lines (MDA231, breast; SW620, colon; and HEK293, kidney) were a gift from Dr. Erasmus Schneider of the Wadsworth Center; cell lines were cultured under standard conditions. Blood samples were obtained from a healthy donor. Cultured cells were stained with the nucleic acid stain SYTO Red (Molecular Probes, Eugene, OR) prior to spiking whole blood by incubating with 0.5μL SYTO Red per 750,000 cells at room temperature for 10 min.

Devices were wetted with 2% (v/v) tetra (ethylene glycol)-dimethyl-ether solution (Sigma Aldrich) in Eagle's MEM and were drawn through the device under vacuum (20 inch Hg), with little effect on cell viability. Cells in medium (≅750,000 cells per mL) only, or whole blood spiked with cultured cells and diluted 1:10 (v/v) in medium (≅75,000 cultured cells per mL) was introduced after wetting. All experiments were performed under a fluorescence microscope equipped with a digital video camera for visualization and recording.

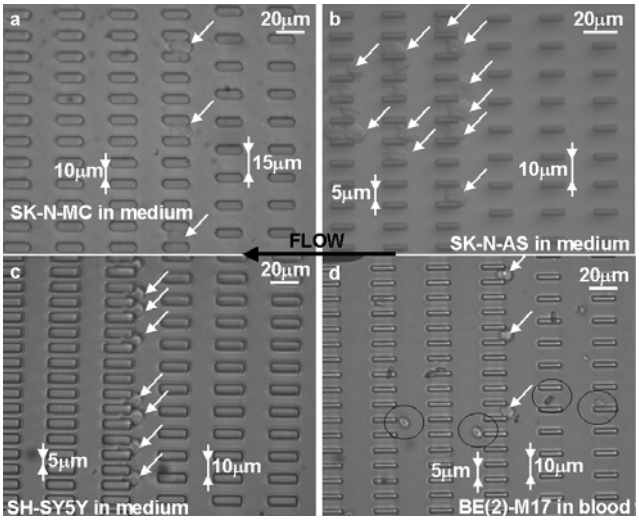

Figure 2: (a) SK-N-MC (shown by arrows) cells retained at the start of the 10μm wide by 20μm deep channels; (b) SK-N-AS and (c) SH-SY5Y cells retained at the 5μm wide by 10μm deep channels; (d) BE(2)-M17 cells, when mixed with blood, (shown by arrows) retained at the start of 5μm wide by 10μm deep channels while blood cells (shown in circles) moved freely to the output reservoir.

3 RESULTS

The 5μm channel depth permitted all cell types from healthy human whole blood to traverse the entire device without resistance [8]. When this device was used to isolate SK-N-MC cells (Table 1), some neuroblastoma cells adhered to the channel walls far upstream due to the roughness of the column walls and the shallowness of the channels. A 20μm deep device was cast to enhance the flow and minimize the possibility of inappropriate adherence of cells upstream. When the 20μm deep device was used, cultured SK-N-MC human neuroblastoma cells in medium

were retained consistently in the 10µm wide channels (Figure 2a).

Four additional neuroblastoma cell lines were tested: SK-N-AS, SK-N-SH, SH-SY5Y, and BE(2)-M17; these cell types were each applied in medium to the device. SK-N-AS cells were retained at the 5µm gap in 20µm deep devices. However, a few cells were able to deform and pass through more than the first few rows in this gap segment. Some variability in size and behavior is expected, and the goal is to find the dimensions and flow conditions that produce retention of cells at the first row (or the first few rows) while allowing all of the blood cells to pass through. Retention of cells at the transition point between one segment and the next improves the sensitivity of the device and minimizes the area of the device that must be visually inspected. A change to a 10µm deep device, proved to be sufficient to retain the SK-N-AS cells at the first row of the 10µm to 5µm interface (Figure 2b). In addition, the SK-N-SH (results not shown), SH-SY5Y (Figure 2c) and BE(2)-M17 cells (Figure 2d) lined up at reproducible gap widths (see Table 2).

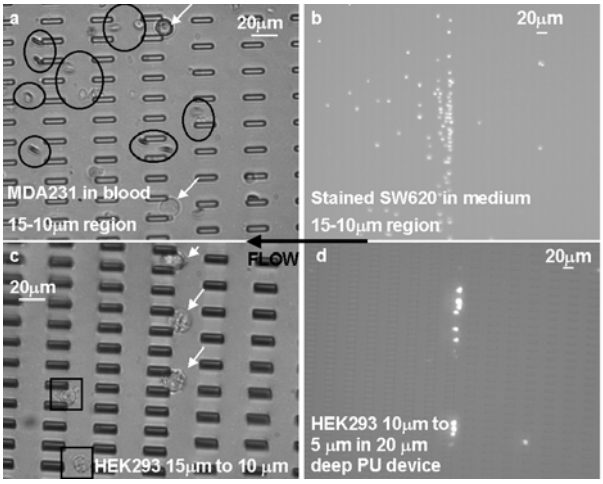

Figure 3: (a) MDA231 cells, when mixed with blood, (shown by arrows) were retained at the start of 10µm wide by 20µm deep channels while blood cells (shown in circles) flowed freely to the output reservoir. (b) Stained SW620 cells retained at the 10µm wide by 20µm deep channels. (c) Larger population of HEK293 cells retained at the start of 10µm wide by 20µm deep channels while the smaller population (shown in squares) migrated through this segment and were not retained until the 5µm wide by 20µm deep channels (panel d).

To determine the utility of this device for other cell types, we investigated the behaviors of three additional cell lines from tumors of epithelial origin namely: MDA231, SW620, and HEK293 cells (n≥3 experiments per line) in addition to the neuroblastoma cells (neuroblasts) used earlier. Breast and colon cancer cells behaved similarly and were retained at the beginning of the 10µm wide by 20µm deep channel segment. Figure 3a shows results for breast cancer cells mixed with whole blood and Figure 3b shows the results for the stained (SYTO Red) colon cancer cells. Cells from a given line were retained repeatedly at a particular gap width when they are either tested in medium only or mixed with whole blood. In contrast with the breast and colon cancer cell line, HEK293 cells demonstrated more variability in size. We observed larger cells retained at the beginning of the 10µm wide by 20µm deep channel segment (Figure 3c), while smaller cells migrated through this segment and were retained at the 5µm wide by 20µm deep channels segment; results from stained cells are shown in Figure 3d. The latter behavior might be more representative of what we would find in a clinical sample. However, these data demonstrate that the device is capable of retaining any cancer cell that is larger and less deformable than blood cells.

Cell Line	Tissue Origin
SK-N-MC	Brain neuroblast
SK-N-AS	Brain neuroblast
SK-N-SH	Brain neuroblast
SH-SY5Y	Brain neuroblast
BE(2)-M17	Brain neuroblast
MDA231	Breast epithelial
SW620	Colon epithelial
HEK293	Kidney epithelial

Table 1: Panel of Cell Lines Used in this Study.

4 DISCUSSION AND CONCLUSIONS

The lack of a simple and reliable method to detect CTCs has delayed the field of cancer research from addressing questions about cell specific changes during metastasis, gene expression, and the design and monitoring of individualized treatment. We have designed and constructed a microfabricated device for the isolation of cancer cells from peripheral blood, as a new tool for isolating CTCs. The device exploits differences in size, structure, and deformability between normal and cancer cells as a method of separation. This approach requires no prior information about cell surface markers or DNA. A simple dilution of the whole blood only is required before the sample is loaded onto the device. Currently, experiments run for 1 hr/mL of whole blood without human intervention; experimental times will be reduced with future design changes. All cancer cell lines tested to date were retained in a well-defined area of the device, while normal peripheral blood cells traversed the device unimpeded. The use of a transparent polymer permits easy quantitation of the retained cells under a microscope. By choosing the proper channel dimensions, we have been able to isolate

admixed cancer cells from blood, mimicking metastatic cells in circulation.

The next goal of our studies will be to determine the analytical limits of this device, using limiting dilution to validate its performance in model systems and clinical samples.

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Sample	Device Depth	Area cells retained
SK-N-MC	20µm	10µm wide channels
SK-N-AS	10µm	5µm wide channels
SK-N-SH	10µm	5µm wide channels
SH-SY5Y	10µm	5µm wide channels
BE(2)-M17	20µm	5µm wide channels
MDA231	20µm	10µm wide channels
SW620	20µm	10µm wide channels
HEK293	20µm	10µm wide channels
HEK293	20µm	5µm wide channels

Table 2: Summary of results.

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