Cancer Cell Separation from Blood Using Iron Oxide Nanoparticles

Introduction

When compared to the microbead-based magnetic separation, nanoparticles have much higher surface to volume ratio (100-1000 times higher), resulting higher binding capacity and efficiency which are favorable especially for tagged ligands that have low binding affinity for their receptors. Due to their smaller size and biocompatible surface modification, magnetic nanoparticles are very stable in whole blood and efficient for the separation of target cells in whole blood which has high viscosity, high cell density and high protein content. Here we demonstrate the separation of circulating cancer cells using an antibody conjugated iron oxide nanoparticles (IO-Ab) under a magnetic field gradient.

Materials:

Ocean's iron oxide particle SHP30 (-COOH surface groups, 30 nm in size), conjugation kit (ICK), prussian blue staining kit (IPS) and SuperMag™ separator; anti-HER2 Ab (Genentech, San Francisco, CA); HER2-overexpressing human breast cancer cell line SK-BR3; fresh whole blood (Biological Specialty Corporation, PA); CMFDA (Invitrogen, CA); acridine orange; Norgen protein extraction kit (Norgen Biotek Corp, Thorold, Canada).

Procedures:

1. **Conjugation of Ab to IO particles:** Conjugate Ab to SHP30 using ICK kit according to the instruction. The conjugates were purified by a SuperMag™ separator.
2. **Attached SK-BR3 cells tagging with IO-Ab:** Approximately $1x10^5$ SK-BR3 cells were seeded in each well of a 6-well cell culture plate. Right before IO-Ab addition, the cells were fixed with ice-cold 95% EtOH for 15 min. This was followed by addition of IO-Ab (as 0.5 mg Fe) or IO (as 0.5 mg Fe) nanoparticles in the binding buffer (20 mm Tris, 150 mm NaCl, 2 mm $CaCl_2$, 1 mm $MnCl_2$, 1 mm $MgCl_2$, 0.1% (wt/vol) BSA; pH 7.4). The wells were incubated at room temperature for 1 h with gentle shaking. The excess reagents were removed and the cells were washed three times with DPBS buffer. Subsequently, the cells were incubated with Prussian blue staining solution for 40 min at room temperature. The cells were washed twice with DPBS buffer and then placed under the microscope for inspection.
3. **TEM of SK-BR3 tagged with IO-Ab:** Floating SK0BR3 cells were tagged with IO-Ab before fixation for TEM. Briefly, 0.05 mL of IO-Ab nanoparticles at a concentration expressed as 2 mg Fe/mL was added to 1 mL SK-BR3 cell suspension (containing 10^5 cells) and mixed gently at room temperature for 1 h to form IO-Ab tagged cells. The mixture was placed on a SuperMag™ separator for 1 h to allow magnetic isolation of the IO-Ab tagged cells. The supernatant was gently withdrawn with a pipettor making sure not to disturb the captured IO-Ab tagged cells that stuck on the wall of the tube where the magnetic field was

strongest. The captured pellet containing the IO-Ab tagged cells were with glutaraldyhyde and formaldehyde. After one day, the pellet is washed with PBS to remove the fixatives and then dehydrated in an alcohol series, embedded in Epon, and sliced to a thickness of 70 nm and put on the 400 mesh copper grids for TEM.

4. **SK-BR3 separation from spiked whole blood using IO-Ab:** 10 uL of whole blood was diluted at 1:100 with DPBS and mixed with the AO at a ratio of 1:1. The mixture was incubated for 10 min at room temperature; the cells were pelleted by centrifugation at 5000 rpm for 5 min. Excess AO was washed with DPBS two times. The AO stained white blood cells were counted using a hemocytometer under a fluorescence microscope.
   Cultured SK-BR3 cells were pre-stained with a live cell staining fluorescent dye CMFDA that converted the cells to green color following the manufacture's staining protocol. The cells were counted and spiked in 1 mL whole blood sample. A 0.05 mL of IO-Ab nanoparticles at a concentration expressed as 2 mg Fe/mL was added to the SK-BR3 spiked whole blood and mixed gently at room temperature for 1 h to form IO-Ab tagged cells. The mixture was placed on a SuperMag™ separator for 1 h to allow magnetic isolation of the IO-Ab tagged cells. The supernatant fluid was gently withdrawn with a pipettor making sure not to disturb the captured IO-Ab tagged cells that stuck on the wall of the tube where the magnetic field was strongest. The captured pellet containing the IO-Ab tagged cells were resuspended in 100 uL DPBS for cell inspection and counting.

Results and Discussions

Specific tagging of SK-BR3 cells

At first the affinity and specificity of the IO-Ab mediated cell tagging was studied. As shown in Fig. 1A, cells that were specifically labeled with IO-Ab showed very significant dark blue coloration, whereas cells incubated with the SHP without antibody showed less blue coloration which was due to the non-specific binding of IO to cells (Fig. 1B). Thus, the amount of non-specifically attached IO on the cell membrane was insignificant compared with the amount of attached IO-Ab.

Additionally, the evidence of the presence of IO on the cell surface was confirmed by TEM. As shown in Fig. 1C and D, IO nanoparticles were found on the cell membranes which may be attributed to the specific targeting with the anti-HER2 on IO surface. The high density of IO on the cell membrane indicated that small sized nanoparticles were

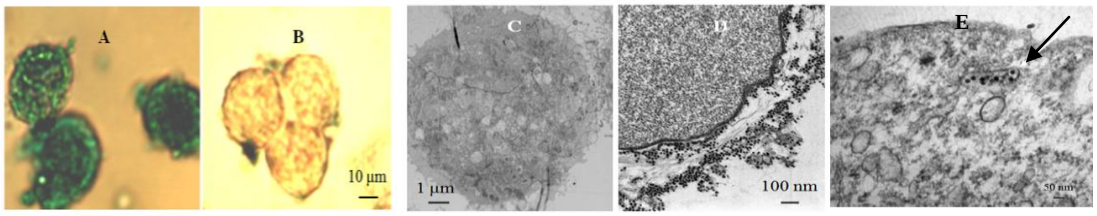

Fig. 1. Specific tagging of SK-BR3 cells with IO-Ab. Prussian blue staining of SK-BR3 cells incubated with IO-Ab (A) or SHP30 (B) showed the specific tagging of cells with IO-Ab. TEM of IO-Ab tagged cells confirmed the presence of IO on the cell surface (D) or uptaking by the cell (E, indicated by the arrow). C is the whole cell section of IO-Ab tagged SK-BR3 under low magnifications.

highly efficient in targeting the cells which allowed ease of separation without cell membrane damage, preserving the integrity of the cells. However, cellular uptake of the IO-Ab was observed under TEM, as shown in Fig. 1E, where cellular vesicles were found to contain IO. Compared with the number of particles specifically attached on the cell surface, the intracellular cell uptake of the IO was insignificant.

Separation of SK-BR3 cells from whole blood

To demonstrate the effectiveness of IO-Ab to capture circulating cancer cells in whole blood, we used SK-BR3 cells spiked in fresh human whole blood. The results are shown in Fig. 2. To generate this data, the number of white blood cells in commercial female whole blood was counted at 4.2×10^7 cells/mL and the red blood cells at 4.4×10^9 cells/mL. During the cancer cell separation process, 300 of the CMFDA pre-stained SK-BR3 cells (Fig. 2A) were added to 1 mL whole blood with a ratio of cancer cells to blood cells (WBCs and RBCs) at about 1:10,000,000. The spiked whole blood was exposed to the IO-Ab to allow formation of the IO-Ab tagged SK-BR3 cells. After separation on the magnet, the IO-Ab tagged SK-BR3 cells were resuspended and smeared on a clean microscope glass slide for inspection and counting under a microscope. The CMFDA stained SK-BR3 cells appeared greenish yellow in color which were bigger in diameter than the WBCs and RBCs. As shown in Fig. 3B, the SK-BR3 cells were successfully captured directly from spiked fresh human whole blood. The recovery was as high as 86% with 73.6% in average (N = 5) in an unoptimized system. An enrichment factor of 1:10,000,000 were attained based on the number of WBCs and RBCs in the spiked whole blood using our 30 nm diameter iron oxide nanoparticles in the presence of a magnetic field gradient of 100 T/m. The increased cell enrichment was attributed to the 30 nm diameter magnetic nanoparticles that allowed for greater number of particles to attach on the cell surface. The unique features of these hydrophilic biocompatible polymer coated iron oxide nanoparticles with high mobility, stronger binding ability, and high colloidal stability in whole blood contributed to the high enrichment factor. The system developed for isolation of specific cells using IO nanoparticles that are conjugated to specific antibodies holds promise for the isolation and enrichment of circulating cancer cells in fresh human whole blood.

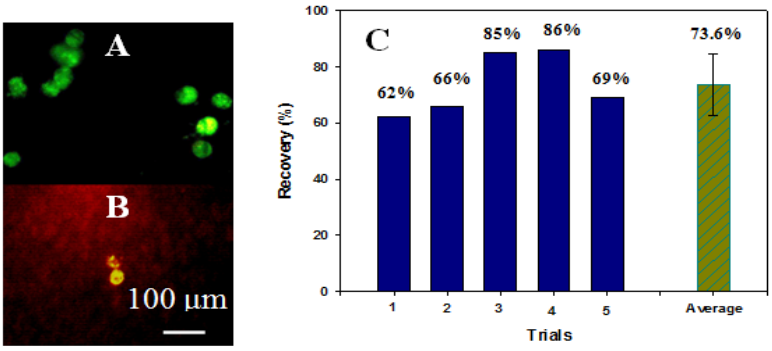

Fig. 2. SK-BR3 cell separation in spiked female whole blood. A) SK-BR3 cell pre-stained with CMFDA, B) isolated CMFDA pre-stained SK-BR3 cells from whole blood, C) IO-Ab captured cell from spiked whole blood.