Atomic force microscope (AFM) measurements of cell stiffness

AFM uses a flexible cantilever to both measure and apply forces. As the cantilever interacts with a surface, its deflection can be measured to subnanometer precision by reflecting a laser onto the end of the cantilever and into a quadrant photodetector. The sample's position relative to the cantilever can be controlled to nanometer precision via a piezoelectric positioning platform. Stiffness can be measured by indenting a cell with a cantilever at a constant speed with respect to the sample. Deflection of the cantilever as it indents the sample is directly proportional to the applied force, since force is linearly related to loading force for small deflections. Cells were typically indented 2 μm to 3 μm. An elastic model[1] can then be fit to the force versus indentation data to determine cell stiffness. The Hertzian mechanics model was selected because of its extensive use with AFM[2-4] and previous analysis by the authors. For further discussions on the appropriateness of this model for leukocytes and leukemia cells, see Rosenbluth et al.[5]

A perfusion chamber was built to maintain fresh media during the experiments by molding a ring of polydimethylsiloxane (PDMS) and mounting it onto the microwell substrate. Media was perfused into the chamber at 0.6 mL/h and out of the chamber at 0.5 mL/h via syringe pumps (Harvard Apparatus, Holliston, MA, and Cole Parmer, Vernon Hills, IL) to account for evaporative loss. Because temperature was noted to affect cell stiffness of leukemic cells, all measurements were taken at 37°C. Closed-loop temperature control was maintained via resistive heaters (Caddock, Riverside, CA) and a thermistor mounted onto a custom-built aluminum sample platform controlled by a variable DC temperature controller (Harvard Apparatus).

Because nonactivated leukocytes and leukemia cells are nonadherent, cells were immobilized in microwells.[5] Fabrication of these wells is described in Rosenbluth et al.[5] Briefly, photocurable epoxy (SU-8 2007; Microchem, Newton, MA) was spun onto piranha-cleaned Borofloat glass wafers (Precision Glass and Optics, Santa Ana, CA), prebaked, and exposed though a mask. After postbaking and development, wells between 8 μm and 20 μm diameter were left on the glass surface. Well depth (8 μm) was controlled by spin speed during SU-8 application. Cell suspensions of approximately 100 000 cells/mL were placed onto the microwells in the perfusion chamber.

Much of the analytical methodology was previously characterized with a similar experimental setup using leukemia cell lines and normal neutrophils.[5] Cell viability and morphology were the same for cells incubated atop SU-8 as those incubated atop glass. Cells were selected to be smaller than the wells in which they were placed. No significant correlation between cell size and stiffness was observed ($P = .83$). Some cells were pushed into the microwells by the AFM cantilever before probing. These cells did not differ in stiffness from those that fell into the microwells ($P = .93$). Cells were indented at rates from 25 nm/s to more than 8000 nm/s. Viscous effects were seen at rates above 415 nm/s. At rates below 415 nm/s, there was no rate dependency on measured stiffness.

Cell size measurements of cells exposed to chemotherapy

To correlate changes in cell size with cell stiffness after exposure to chemotherapy, images of the cells of interest were acquired with each cell stiffness measurement using the brightfield and fluorescence microscope coupled to the modified AFM. Because the refraction from the microwell walls prevented an accurate size measurement of the cell of interest, the average cell area of the entire cell population in each field of view was calculated for each measurement. Images were acquired with an Axiovert 25 Microscope, 60× 0.7 numerical aperture (NA) objective (Nikon, Melville, NY), QColor3 camera (Olympus, Center Valley, PA), and ImageJ software (National Institutes of Health, Bethesda, MD).

To track changes in cell volume after exposure to chemotherapy, cells were incubated with 7 μM of the volume marker CellTracker Orange CMRA (Molecular Probes, Eugene, OR) for 30 minutes in serum-free RPMI 1640. Cells were then spun down and media was replaced with CO_2 independent medium. A concentration of 5 μM Sytox Green was added to determine cell death. Then, 1 μM daunorubicin for HL60 cells and 1 μM dexamethasone for Jurkat cells was added. Cells were then placed in a Molecular Cytomics (Boston, MA) LiveCell Array, which allows for nonadhesive cells to remain immobile without fixation for tracking and imaging over long time periods. Images were acquired at 1-minute time intervals with an

Axiovert 200 Microscope, 40× 0.6 NA objective (Carl Zeiss, Thornwood, NY), Cascade II camera (Photometrics, Tucson, AZ), and Metamorph software (Molecular Devices, Downingtown, PA).

Leukemic cells decrease in size when undergoing chemotherapeutic cell death

Cell death is often coupled with a decrease of cell size[6,7] that, if significant enough, may offset the associated increase in stiffness. To correlate in time changes in cell size with cell stiffness after exposure to chemotherapy, the average cell area of the cell population in each field of view was calculated for each cell stiffness measurement. During AFM measurements, cell stiffness was noted to markedly increase before the onset of cell shrinkage associated with cell death (Figure S1A). As the stiffness of a single HL60 cell increased with exposure to daunorubicin, the average cell area of the cell population (n = 8 to 38 cells/field of view) remained constant until cell death. Within 31 minutes, the average cell area decreased 20% and remained constant thereafter.

To confirm cell volume change with chemotherapy-induced cell death, we estimated cell volume loss by tracking the average intensity of a cell volume tracker. HL60 cells began quickly losing volume several minutes before they positively stained for Sytox Green (Figure S1B). Jurkat cells also decreased in volume before Sytox Green positivity, but closer to the point of death. HL60 cells shrunk by nearly 30% prior to cell death marker staining, and then they gradually decreased in volume after cell death. Jurkat cells shrunk by 12% prior to cell death marker staining and then remained relatively static in volume for the next 40 minutes. There was large variability in cell volume once the cells died. Whereas some cells barely changed in volume, others decreased in volume by more than 50%. From these findings we expect that the physiologic effect of stiff dead cells in the microcirculation may be partially offset by cell shrinkage although the increase in cell stiffness appears to begin before cell size decrease.