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Single-cell Electrical Lysis of Erythrocytes Detects Deficiencies in the Cytoskeletal Protein Network

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Abstract

The network of erythrocyte cytoskeletal proteins significantly influences erythrocyte physical and biological properties. Here we show that the kinetics of erythrocyte lysis during exposure to an electric field is sensitively correlated with defects in the cytoskeletal network. Histograms compiled from single-cell electrical lysis data show characteristics of erythrocyte populations that are deficient in a specific cytoskeletal protein, revealing presence of cell subpopulations.

Introduction

The erythrocyte membrane is embedded with membrane-spanning proteins that are in turn connected to a spectrin/actin cytoskeleton via protein bridges such as ankyrin^{1, 2} and adducin^{3, 4} (as shown in Fig. 1A). Because the phospholipid bilayer is intrinsically unstable in the absence of structural support from the cytoskeleton, defects in cytoskeletal proteins commonly lead to membrane instability, resulting in loss of membrane surface during circulation in the vasculature. This loss of surface area can result in gradual conversion of the erythrocyte's morphology to a smaller, more rounded cell termed a spherocyte, which is more efficiently removed by macrophages in the spleen and liver^{5, 6}.

Classical methods for studying changes in erythrocyte morphology have been largely based on measurement of the ensemble properties of large cell populations.⁷ These include both analyses of the rate of erythrocyte filtration through microscale pores and evaluation of the rate and extent of their deformation under shear stress in an ektacytometer. In order to extract physical properties of red blood cells (RBCs) at the single cell level, techniques including micropipette aspiration, mechanical deformation with optical tweezers, and atomic force microscopy (AFM) have also been introduced. However, these latter approaches are limited by their low throughput capacity and do not have the capability of examining large cell populations. With significantly higher throughput and single cell resolution, the rheoscope has been explored as a method to examine single cell deformation in a shear flow using a high-speed camera⁸. A number of microfluidic devices have also been developed to

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Electronic supplementary information (ESI) available. Additional histograms of the lysis time and Wilcoxon signed ranks tests, as well as the optical images of red blood cells.

examine RBC biomechanics with single cell resolution. In addition to miniaturizing macroscopic methods, some of these microfluidic devices have employed microfabricated structures with subcellular dimensions⁹ or taken advantage of microscale fluid dynamics¹⁰ for screening cell deformability. However, these tools have not been shown to reveal the links between physical properties and molecular anatomy of the RBC cytoskeleton.

In this report, we have employed microfluidics to rapidly examine the duration needed for individual erythrocytes in a heterogeneous cell suspension to burst in a high electric field as a consequence of excessive transmembrane potential. Because this threshold duration is determined by cell size and membrane stability, the method would be anticipated to allow for a rapid and sensitive diagnosis of defects in membrane structure. In here we explored the utility of microfluidic electrical lysis for detection of erythrocyte membrane abnormalities in mice with known membrane protein mutations.

Experimental section

Microchip fabrication

The microfluidic chip for electric lysis of red blood cells was fabricated in polydimethylsiloxane (PDMS) using standard soft lithography¹¹. Briefly, the microchannel patterns were designed using FreeHand MX (Macromedia, San Francisco, CA) and printed on a transparency with a resolution of 5080 dpi. The printed transparency was used as the photomask for patterning a negative photoresist SU-8 2025 (MicroChem, Newton, MA) on a 3-inch silicon wafer to form the master. A PDMS microfluidic chip was produced by pouring and curing a PDMS prepolymer mixture (containing monomer A and curing agent B) (GE Silicones RTV 615, MG Chemicals, Toronto, ON, Canada) on the master. The depth of the microchannels was measured to be ~23 μm . A glass slide was cleaned with solution ($\text{H}_2\text{O}:\text{NH}_4\text{OH}$ (27%): H_2O_2 (30%) = 5:1:1, volumetric ratio) at ~75 °C. The PDMS chip (punched to form access holes) and the clean glass slide were oxidized with a plasma cleaner (Harrick, Ossining, NY) and pressed against each other to form irreversible bonding and the microfluidic device.

Sample Preparation

Blood samples obtained from normal mice and mice with deficiencies in ankyrin (nb/nb) and β -adducin were obtained from the Jackson Laboratory^{12–14}. Blood was collected into heparin tubes (Sigma H-3393) as previously described^{12–14}. The tropomodulin1 (Tmod1)-null erythrocytes and associated travel control samples, from whole blood collected into K₃EDTA Vacutainer tubes, were obtained at the Scripps Research Institute¹⁵. Whole blood was washed three times with PBS (phosphate buffered saline, pH 7.4, 330 mOsm) by centrifuging at 1000g and removing the supernatant containing the plasma and buffy coat (white cells). The pelleted red blood cells were used for further analysis.

Microfluidic electroporation

Red blood cells were suspended in the electroporation buffer (10 mM Na_2HPO_4 , 10 mM KH_2PO_4 , and 250 mM sucrose). The microchannel was conditioned by the same buffer for several minutes prior to experiments. RBC suspension was flowed into the device from the central inlet at the flow rate of 0.06 $\mu\text{l}/\text{min}$ under hydrodynamic focusing by the flows from two side inlets, each having a flow rate of 0.03 $\mu\text{l}/\text{min}$. After the flows became stable, a constant voltage was applied across the channel to establish the electroporation field in the narrow section (the field in the wide sections was too weak to produce lysis). RBC lysis was recorded using a CCD camera with a frame rate of 93 frames per second as cells flowed through the electroporation section (the narrow section). Hydrodynamic focusing ensured that the cells entered the narrow section in a single profile along the centerline, thus exposed

to the identical field intensity for the same duration when observed at a given channel length.

Image analysis

A MATLAB program was written and used to analyze the image series that recorded RBC lysis. The program tracked each flowing RBC and determined the lysis time for the cell (i.e. the period between the entry of the narrow section and the lysis). The lysis was determined based on the rapid change in the light intensity of a cell upon lytic release of intracellular materials. The lysis times of all the cells from each sample (~2000–3000 cells) were then plotted in histograms and the mean lysis time was determined from the center of a Gaussian fit of the distribution using Origin 8.0 (OriginLab, Northampton, MA).

Results and Discussion

We have applied a flow-through electroporation technique¹¹ to determine, under a specific field intensity, the time needed for individual cells in an ensemble of erythrocytes to lyse (with the setup shown in Fig. 1B). Upon exceeding a threshold field duration under which the red cell membrane is stable, the membrane is ruptured by electroporation via generation of nanoscale pores in the phospholipid bilayer. In general, larger cells are more susceptible to electroporation (or electrical lysis) due to the higher transmembrane potential built up across the membrane (e.g. for a spherical cell, transmembrane potential $\Delta\psi_E = 0.75 g(\lambda)aE \cos\theta$, where $g(\lambda)$ is a complex function of the membrane and buffer conductivities, a is the diameter of the cell, E is the field strength and θ is the angle between the normal to the membrane surface and the field direction). Lysis of individual cells is monitored with a CCD camera (Fig. 1C) and image analysis is used to determine the time of lysis at which a RBC releases its intracellular contents. With this methodology, throughput capacity is only limited by the frame rate of the camera, which was 93 frames per second in these experiments, allowing interrogation at 50 cells/s. Several thousand cells from each sample were analyzed and the lysis times (i.e. the duration needed for cell lysis to occur at a specific field intensity) of these individual cells were compiled into histograms that reveal the erythrocyte population characteristics.

RBCs from normal mice, β -adducin-null mice, and Tmod1 (tropomodulin1)-null mice were examined using a field intensity of 1100 V/cm in the narrow section of the flow channel, and the distribution of electrical lysis times was plotted for each (Fig. 2A). Normal mouse RBCs, which are rather homogeneous in size and shape, displayed only a single distribution of lysis times, with a centroid value of 92 ms. Analysis of erythrocytes from Tmod1-null and β -adducin-null also yielded single Gaussian distributions. However, while the Tmod1-null RBCs showed no significant difference in the distribution compared to normal cells (with detailed statistical analysis in ESI Table S1), β -adducin-null RBCs exhibited a significant delay in the lysis (~153 ms) that was ~67% longer than normal. This lengthening of lysis time could theoretically derive from either an increase in membrane stability or decrease in cell size. Since RBCs from β -adducin-null mice have been shown to be less stable than normal RBCs,¹⁴ we hypothesize that this shift to longer lysis times is likely due to a gradual loss of membrane surface as a result of their heightened instability. The lack of significant difference between Tmod1-null and normal samples is consistent with the fact that the Tmod1-null erythrocytes showed no obvious difference in membrane stability or size, despite their somewhat spherocytic shapes as compared to normal cells¹⁵ (ESI Fig. S1).

Interestingly, to induce quantitative lysis of the ankyrin-deficient (nb/nb) cells, the field intensity in the narrow section had to be increased to 1200 V/cm (Fig. 2B). Under these conditions, RBCs from ankyrin-deficient mice showed a dramatically different distribution

in the lysis time compared to normal cells treated similarly (~74 ms as the centroid value). Thus, the histogram of ankyrin-deficient RBC lysis times was best fit by three superimposed Gaussian distributions with mean lysis times of 77, 155, and 357 ms. This distribution was consistent among two independent samples of nb/nb mouse blood analyzed on separate dates (ESI Fig. S4). Because the nb/nb blood contained erythrocytes of highly variable sizes, including many spherocytes and microspherocytes¹³ (see ESI Fig. S1), we propose that the diversity of lysis times likely arises from the heterogeneity in cell sizes, especially the presence of microspherocytes, which prominently contribute to the population with the longest lysis times.

It is important to note that while only representative distributions are shown for each mutant cell type in Fig. 2, we tested additional samples of each blood type (as shown in ESI Fig. S1–S7) and used the Wilcoxon signed-rank tests to assess any statistically significant difference between the various histograms¹⁶. These analyses showed no statistical difference among samples of the same blood type (ESI Table S1). Moreover, there was no significant difference in the histograms of Tmod1-null and normal RBCs. In contrast, Wilcoxon analysis revealed significant differences among normal, β -adducin-null and ankyrin-deficient (nb/nb) RBCs.

In Fig. 3, we established the distributions of the cell size (in terms of the disk diameter of the RBC biconcave shape) for different cell types from image data such as the ones in ESI Fig. S1. The distributions suggest that the cell size affects the lysis time. For example, for nb/nb cells, their small sizes are likely to contribute to the very long lysis time and there appear to be multiple Gaussians in both the cell size and the lysis time distributions. However, it is also apparent that the cell size does not entirely determine the lysis time distribution. β -adducin-null cells are similar to normal cells in terms of their sizes but exhibit a large shift toward longer lysis time than normal cells. Thus we believe that both the cell size and the cytoskeletal mechanics significantly influence the lysis time.

Our technique based on single-cell electrical lysis provided a rapid, high throughput method for analysis of the size and stability of individual RBCs in a large cell population. Our analyses revealed that distributions of the electrical lysis times are different between normal and most of the mutated types. Moreover, statistically different profiles were observed for each membrane mutation, and subpopulations of RBCs could be detected within a single blood sample. These data suggest that microfluidic electroporation might constitute a rapid and sensitive tool for detection of erythrocyte membrane defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The cytoskeletal structure and single-cell electric lysis of red blood cells (RBCs). (A) A schematic of the RBC membrane and the underlying cytoskeletal structure. (B) The setup of the microfluidic device for electrical lysis of RBCs at the single cell level. A constant voltage was applied across the channel. The widths of the narrow section and the wide sections were 120 μm and 1200 μm , respectively, producing a field intensity ratio of 10:1 in the narrow section versus in the wide sections. Electrical lysis of flowing RBCs occurred only in the narrow section due to the high field intensity there. The cell lysis was recorded by a CCD camera. (C) The schematic (left) and time-lapse images (right) of RBC lysis in the narrow section (labeled by the times for the cell to be in the lysis field). The light intensity of the cell decreased due to the release of intracellular materials. The lysis time was defined as the period between the entry into the narrow section and the time that the cell became ghost and exhibited an undetectable intensity.

Figure 2. Representative lysis time histograms compiled based on data taken using (A) RBCs from normal, Tmod1-null, β-adducin-null mice at the field intensity of 1100 V/cm and (B) RBCs from normal, nb/nb (ankyrin-deficient) mice at the field intensity of 1200 V/cm. Each histogram consists of data from ~2000–3000 cells. Fitting by one or multiple Gaussian(s) (outlined in red or blue) reveals the centroid lysis time(s).

Figure 3.

Cell size histograms for normal, Tmod1-null, β -adducin-null, and nb/nb RBCs. The RBC cell size is described by the disk diameter of the biconcave shape. The disk diameter of each cell was calculated based on the area occupied in the 2D plane and the assumption of circular disks for all the cells. Each histogram includes data from roughly ~800 cells. The average disk diameters for normal, Tmod1-null, β -adducin-null, and nb/nb cells were 5.1, 5.6, 5.1, and 3.9 μm , respectively.