Delivery of siRNA by Electroporation Into Primary Human Neutrophils Using the Gene Pulser MXcell™ System

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Introduction

Neutrophils are the most abundant circulating leukocytes, responsible for engulfing and destroying bacteria that enter the body through wounds. These leukocytes possess an array of adhesion molecules and chemical sensors at their surface that allow them to respond to acute inflammation caused by bacteria, adhere to the blood vessel wall, and migrate into the infected tissue. Neutrophils are widely used as a model for acute inflammation studies because they are easily isolated from human blood. However, they are very difficult to maintain in culture because of their short life span (approximately 6 hr) outside of the body. The neutrophils' life span can be prolonged to approximately 48 hr in culture by addition of granulocyte macrophage colony stimulating factor (GM-CSF). This lengthened life span allows possible genetic manipulations and other longer-term experimental assays. Although GM-CSF treated neutrophils cannot be made to survive long enough to obtain a stable transfectant, transient transfection is a feasible approach if sufficiently efficient. The leukemic cell line HL-60 has also been used as a model of long-term neutrophil behavior, but differentiated HL-60 cells lack several features of mature neutrophils, including bactericidal granules. Therefore, transfection of primary human neutrophils would be a significant advance, allowing the study of pathways that have proven elusive due to the difficulty of culturing neutrophils.

Methods

Neutrophils were isolated from donated human blood by centrifugation over a Ficoll-Paque density medium, as previously described (Simon et al. 1995). Isolated neutrophils were suspended in Gene Pulser® electroporation buffer (Bio-Rad Laboratories, Inc.) at a density of 5×10^6 cells/ml, aliquoted in 200 µl fractions into the wells of a 96-well electroporation plate (Bio-Rad), and electroporated with the Gene Pulser MXcell system (Bio-Rad) using an exponential waveform at 1,000 Ω resistance, which was previously determined to be optimal for HL-60 cells (Schaff and Simon 2008). Using these conditions, a range of voltage and capacitance conditions were tested. Following electroporation, neutrophils were diluted 1:6 in GIBCO Advanced RPMI Medium 1640 (Invitrogen Corporation) containing 10% fetal bovine serum, and incubated overnight at 37°C.

Following transfection with siRNA (AllStars Negative Control siRNA, QIAGEN; 50 nM) conjugated to Alexa Fluor 546 (Invitrogen), neutrophils were aliquoted onto slides and imaged with a Nikon 1200 microscope equipped with a 100 W mercury arc lamp, 546 nm excitation filter, and 20x air objective. Transfection efficiency was quantified by obtaining the median fluorescent intensity (MFI) of neutrophils through an automated image analysis program (Image-Pro 5.1, Media Cybernetics, Inc.) that averaged pixel intensity over the surface of each cell image, producing population statistics from the individual brightness measurements. Formyl peptide (fMLP), an agonist of inflammatory neutrophil activation, was obtained from Tocris Bioscience. The fluorescent calcium indicator Fura-2 AM (Invitrogen) was used to assay cell survival and to measure calcium fluxes. Molecules that contain an acetoxymethyl ester group, such as Fura-2 AM, pass through cell membranes. In living cells, esterases cleave the acetoxymethyl ester group, activating Fura-2 and retaining it inside the cell. Survival was assayed by the percentage of total input cells that were alive 1 day after electroporation. Each data point shown in figures is the average of three wells.
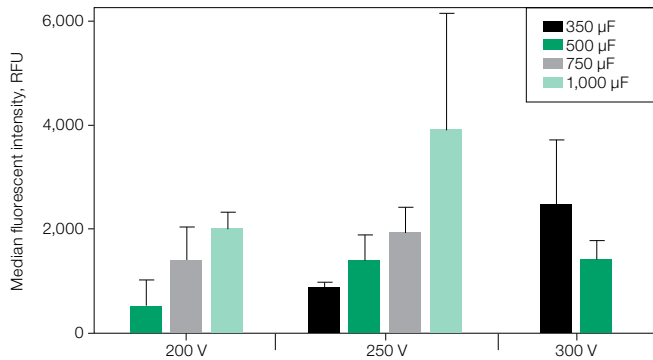
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Results

Fig. 1. siRNA delivery into neutrophils using a range of electroporation conditions. Neutrophils were electroporated using the indicated conditions in the presence of siRNA conjugated to Alexa Fluor 546, then imaged by fluorescence microscopy. MFI was used as an index of siRNA delivery into the cells. Under conditions below 300 V, transfection efficiency increased when capacitance was increased.
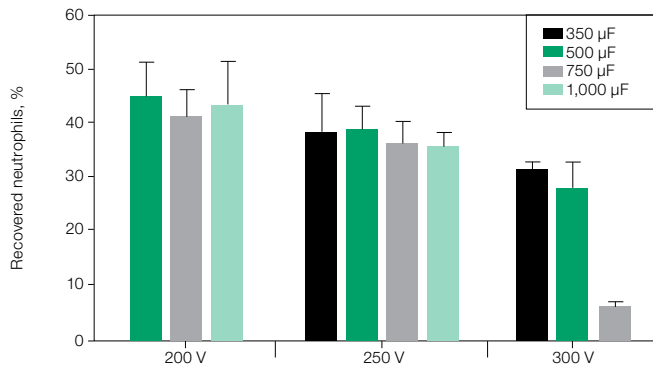

Fig. 2. Cell survival under a range of electroporation conditions. Post-electroporation survival of neutrophils was assayed after 24 hr in culture by counting the number of intact cells that excluded trypan blue, and dividing by the number of neutrophils initially transfected. Cell survival was substantially reduced at 300 V and 1,000 µF.
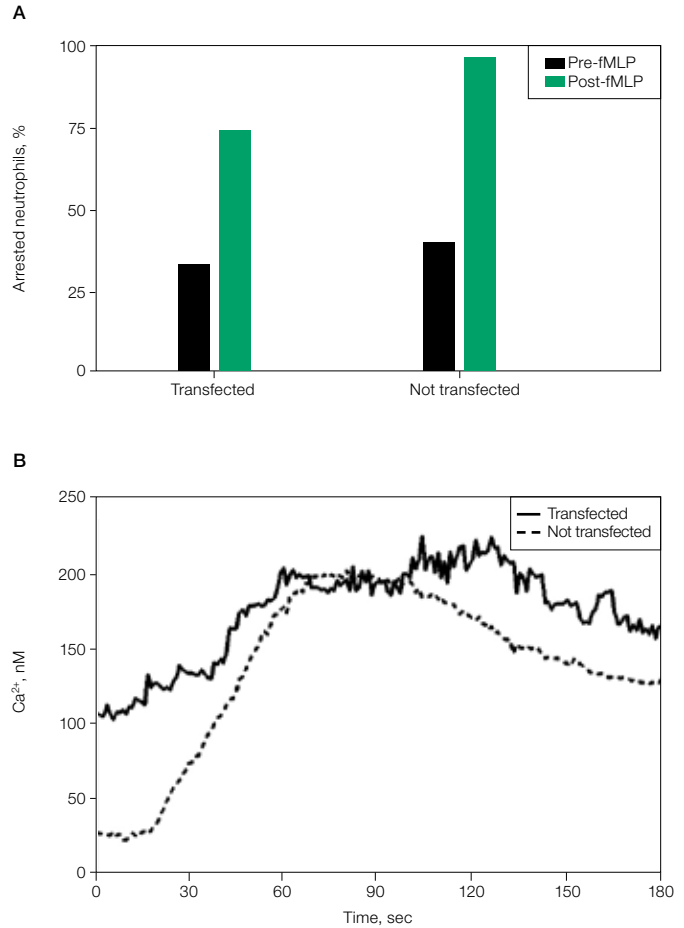
A

B

Fig. 3. Inflammatory response of electroporated neutrophils. Two days (48 hr) after electroporation (at 300 V, 350 µF, and 1,000 Ω) neutrophils were analyzed for calcium flux in response to 1 µM fMLP by fluorescence microscopy after loading with the calcium-sensitive dye, Fura-2 AM. **A**, neutrophils interacting with E-selectin underwent a transition from rolling to arrest in response to addition of fMLP, regardless of electroporation; **B**, neutrophils loaded with Fura-2 AM underwent calcium flux in response to addition of fMLP at time 0. The fact that transfected cells were detectable by fluorescence imaging at 340 nM and 380 nM indicates that these cells were alive because they possessed active esterase to process the dye.

Results and Discussion

Using the Gene Pulser MXcell electroporation system, neutrophils were successfully transfected with siRNA. Among all the conditions tested, 250 V and 1,000 µF using an exponential waveform resulted in the best transfection efficiency (Figure 1). Neutrophils required greater voltages for optimal electroporation and siRNA delivery than the analogous HL-60 cells, but did not incur as much cell death in the process (Schaff and Simon 2008). Many neutrophils survived for 24 hr posttransfection (a portion were still functional 48 hr after transfection) and their survival was not substantially affected by electroporation up to 300 V, with the exception of 300 V in combination with 750 µF and 1,000 µF settings (Figure 2), suggesting that cell death might be partly due to apoptosis under culture conditions.

Intracellular calcium in neutrophils rises in response to chemotactic stimuli, triggering the downstream signaling that leads to neutrophil arrest (Schaff et al. 2008). Thus, the change in intracellular calcium concentration in response to a chemokine is an excellent assay for checking the health and sensitivity of neutrophils (DiVietro et al. 2001). To assess any changes in the inflammatory response due to electroporation, the cultured neutrophils were incubated 1 hr with Fura-2 AM, a calcium-sensitive dye, and rolled on E-selectin infused with 1 µM fMLP (Figure 3). Calcium flux across the plasma membrane was assessed by measuring intracellular fluorescence of Fura-2 AM, an indicator of calcium concentration in the cell. Elevation in resting calcium indicates a level of preactivation that is expected in neutrophils that have been exposed to physical stressors or bacterial products (Anderson and Goolam Mahomed 1997). Preactivated neutrophils tend to be desensitized to nanomolar concentrations of chemokines, such as fMLP, but can be stimulated with higher concentrations of chemokines (Eierman et al. 1994). The fact that electroporated neutrophils were able to raise intracellular calcium above this elevated baseline in response to 1 µM fMLP (a high concentration) indicates it is likely that the most important signaling pathways for response to inflammation were intact.

Neutrophils had elevated resting intracellular calcium and slightly reduced arrest efficiency following electroporation and culture, indicating modest desensitization to chemokine. Nonetheless, neutrophils retained significant responsiveness to chemokine above the initial calcium levels, indicating that this transfection technique may allow genetic study of processes that occur in activated neutrophils. For instance, following chemokine-mediated arrest on inflamed vascular endothelium, neutrophils migrate through the wall of the blood vessel in order to reach bacteria in the tissue. If transfection of siRNA against a particular calcium channel prevented this migration in an in vitro model of inflammation, it would conclusively demonstrate that this channel was an important component directing migration. Many leukocyte signaling pathways are known only from studying longer-lived cell types, such as T cells, because of the availability of genetically modified variants. The ability to transfect neutrophils may allow similar studies in shorter-lived cells.

References

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