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Direct Observation of the p188 Mediated Membrane Sealing with Atomic Force Microscopy

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1 Introduction

The realization that Poloxamers can be used to restore the structural integrity of permeabilized cell membranes has established new methods for repairing damaged cell membranes after various physico-chemical insults and neurotoxic events (for a review, see Agarwal, J, Walsh, A.M. and Lee, RC. "*Multimodal Strategies for Resusitation of Injured Cells*" *Annuals NYAS Vol.**1066** 295-309 2005*). The three blocks of this surfactant class contain a central hydrophobic chain capped at both ends with hydrophilic chains of equal length. Due to the associated amphiphilic character, multiple interaction modes with a lipid bilayer can be imagined. Our hypothesis is that these multi-block copolymers absorb onto exposed hydrophobic domains at lipid bilayer defects and/or membrane-bound glycoproteins at bilayer defects to induce sealing. To test this hypothesis we monitored the restoration of the structural integrity of the compromised membranes by direct visualization using atomic force microscopy (AFM) images.

2 Materials and Methods

We characterize structural changes in fibroblast cell membranes associated with the observed permeability increases due to a chemical injury by saponin and successive Poloxamer 188 (P188) mediated membrane sealing by using AFM. The AFM is a non-destructive, high resolution method for investigating adsorbed film structure at length scales from 0.1 nm to 100 μm, and can help us determine the distribution of pores within the membrane by scanning a sharp tip across the surface. Cells were cultured onto glass cover slips in low density to ensure that individual cell can be singly imaged. After treatment with drugs, cells are fixed with 2.5% glutaraldehyde and 2% osmium tetroxide, and the slips are transferred onto the AFM fluid cell. Scanning is carried out in water using contact mode with a low force applied to surface (0.1-0.5nN). In combination with the AFM imaging, we also performed a cell viability measurement. Ethidium homodimer-1 (EH) saponin/water and calcien-AM (Molecular Probes, Oregon) were used to stain the cells. EH fluoresces with a red color after binding to DNA within the cell. Entrance of this molecule into the cell indicates significant cell membrane destabilization and cell death. Calcein-AM flourescence requires ATP-dependent cleavage which occurs in the cell's cytosol. Its green fluorescence indicates that the cell maintains both metabolic capabilities as well as a stable membrane. Cells demonstrating any accumulation of EH were deemed nonviable even if green fluorescence was still appreciated. Fifteen minutes after the dye was added to these dishes, their fluorescence was assessed using a Nikon Diophot inverted microscope with fluorescent optics.

3 Results

We report direct observations of the interactions of P188 with damaged cell membranes under physiological conditions with AFM. Representative images of (a) intact, (b) damaged and (c) sealed membranes are displayed in **Fig. 1**. We observed a surface morphology change of the cells after injury (b) and a ~30-50% increase in the number of pores in saponin injured cell

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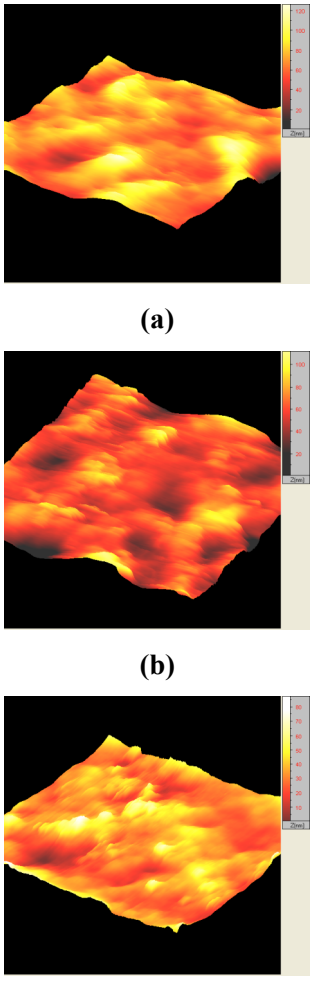
membranes. After P188 treatment (c), the cell membrane integrity was restored, as revealed by the evolution of the pore density in treated cells. **Fig. 2** shows the survival of control (non-injured) cells (a), injured, nontreated cells (b) and P188 treated cells (c). Treated cells maintain their viability to a level which is nearly commensurate with that of non-injured cells.



(a)

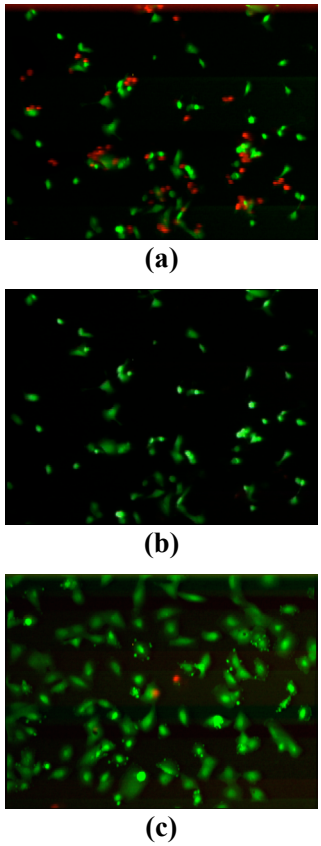
(b)

(c)

Figure 1 : Cell membrane morphology of normal (non-injured) cells (**a**), saponin-injured cells (**b**), and injured P188 treated cells (**c**). AFM images of fixed fibroblast cells in water. Scan size is 2x2 µm.

4 Conclusion

We presented direct evidence that P188 restore the integrity of compromised cell membranes and increase the survival of the treated cells. The time line of membrane sealing is short. Apparently, the structural recovery of the damaged membranes induced by P188 occurs on a molecular time scale, and the beneficial effect at the cellular level can be seen in less than 15 minutes. These may indicate that the remedy of ruptured membranes will not require a continuous intravenous administration of P188. The delivery of P188 boluses for short periods of time can be highly effective and can also reduce the adverse effects caused by its low renal excretion.



(a)

(b)

(c)

Figure 2 : Control (non-injured) cells (**a**), injured, nontreated cells (**b**) and P188 treated cells (**c**). Green fluorescence indicates healthy cells. Red color stained irreversible injured cells.

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