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Study of membrane defects induced by antimicrobial and hemolytic peptide Ltc1 in erythrocyte membrane

Nikita Orlov[1], Olga Geraskina[2] and Alexey Feofanov[3]

[1]Lomonosov Moscow State University, Moscow, Moskva, Russia, [2]Lomonosov Moscow State University, Russia, [3]Biological Faculty, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia

*corresponding author: n.orlov858@yandex.ru

The antimicrobial resistance is a global challenge driving a search for alternatives to classical antibiotics. Naturally occurring antimicrobial peptides are considered as promising candidates for fighting multi-resistant bacteria, but their therapeutic potential is strictly dependent on their safety to host cells. Latarcin Ltc1 is a spider venom antimicrobial peptide, which is linear, short (25 a.a.), cationic (net charge +9 at physiological pH) and undergoes coil-to-α-helix transition in membrane-mimicking environment [1]. In addition to high antimicrobial activity Ltc1 possesses cytotoxic and hemolytic action, which requires detailed investigation. Earlier it was shown that hemolytic activity of non-processed form of Ltc1 (*i.e.* Ltc1-K) is regulated by its N-terminal moiety [2]. Here we report on the features of interactions of mature Ltc1 with human red blood cells revealed with a confocal laser scanning microscopy (CLSM).

For CLSM studies, synthetic Ltc1 tagged with sulforhodamine B (Rh-Ltc1) was used. Comparative analysis of Ltc1 and Rh-Ltc1 activities in the RPMI-1640 culture medium revealed that their effective hemolytic concentrations (EC_{50}) were 1.0±0.1 and 0.4±0.1 μM, respectively. Therefore, hemolytic activity of Ltc1 was not impaired by the fluorescent label. Real time study of human erythrocyte hemolysis revealed a long phase of Rh-Ltc1 accumulation in erythrocyte membrane accompanied by erythrocyte crenation according to the pathway of discocyte – echinocyte - stomatocytes - spherocyte (Fig. 1, A, B). This phase was followed by a fast hemoglobin (Hb) release through the formed membrane openings and, finally, by a ghost formation (Fig. 1, A, B). At 3 μM Rh-Ltc1, the first phase lasts for tens of minutes, while Hb release lasts for tens of seconds. Applying the calibration-based qualitative analysis of CLSM images, membrane density of Rh-Ltc1 was estimated to be 1200±200 molecules/μm^2 at the beginning of the first phase and, it raised to 11000±900 molecules/μm^2 in ghosts. The spherocyte to ghost transformation rate allows one to suppose that Hb leakage occurs through a few membrane openings. Evidently, a diameter of membrane pores during hemolysis is higher than hydrodynamic radius (HR) of Hb (*ca*. 3.1 nm). But it is not clear, if these pores are transient or stable, and if they are preserved in ghosts.

To evaluate a size of pores, we used the size-marker influx assay described previously [2, 3]. Water-soluble 5-carboxyfluorescein (CF) or FITC-labeled dextrans of 10, 70, 250 or 500 kDa (FD10, FD70, FD250, FD500) were added to Rh-Ltc1-produced ghosts, and the degree of their influx was analyzed with CLSM. Size-markers enter into the ghosts indicating preservation of Rh-Ltc1-induced membrane pores. A size of these pores depends on the Rh-Ltc1 concentration added to erythrocytes. At high Rh-Ltc1 concentration (10 μM) FD500 (HR~16 nm) fills in 98.5% ghosts (Fig.2, A) revealing existence of large pores (>32 nm in diameter). At low Rh-Ltc1 concentration (3 μM), FD250 (HR~11.5 nm) influx is hampered, CF (HR~1 nm) fills into all ghosts, whereas FD10 (HR~2.3 nm) and FD70 (HR~6 nm) have restricted penetration into ghosts (Fig. 1, C; Fig. 2, B). Accordingly, two populations of ghosts were revealed having diameter of stable pores either 12-23 nm or less than 5 nm. A fraction of ghosts with pores, which are smaller than the Hb size, indicates that a pore size changes in the course of hemolysis because of alterations in the membrane tension and relaxes to the steady state after Hb leakage.

Real time imaging of erythrocytes pre-loaded with BCECF-AM and treated with 1 μM Rh-Ltc1 didn't reveal any BCECF efflux before Hb leakage (data not shown), thus rejecting the assumption that Hb release is preceded by small organic molecules leakage.

Ltc1 is concluded to induce hemolysis through the formation of stable lipid-peptide pores in the erythrocyte membrane. On the basis of the experimental results and the theory of transient and stable pores in lipid membranes, a model of Ltc1-induced hemolysis is proposed [4].

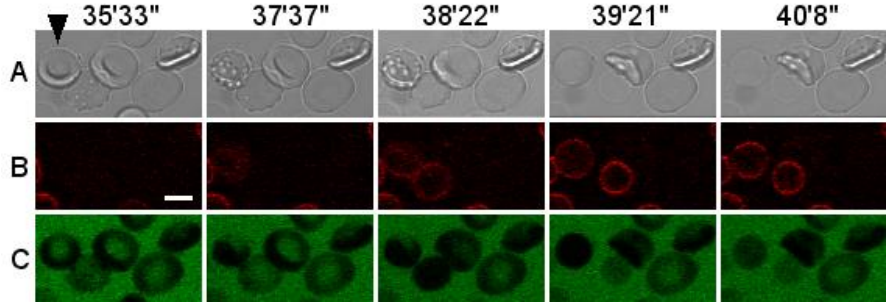

Figure 1. Time-resolved CLSM images of human erythrocytes treated with 3 μM Rh-Ltc1 and 10 μM FD70 (selected time points are top labeled). Row A - transmitted light images, rows B and C correspond to fluorescence of Rh-Ltc1 and FD70, respectively. Arrow indicates the representative erythrocyte that transforms from a discocyte to a ghost during the time of experiment. Bar - 5 □m.

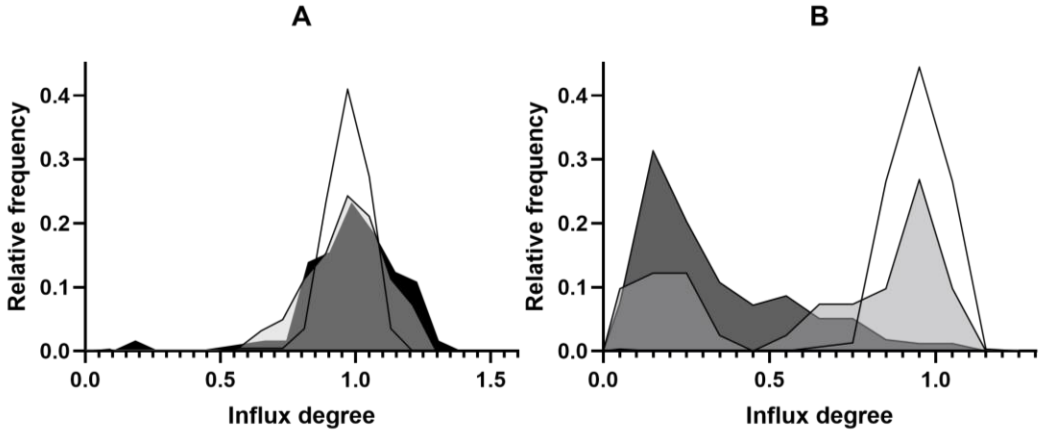

Figure 2. Frequency distributions of Rh-Ltc1-produced ghosts by the size-marker influx degree. Influx degree is the ratio of intracellular to extracellular fluorescence intensity of the size-marker. Concentration of Rh-Ltc1 was 10 □M (A) or 3 □M (B). The color scheme used: CF – transparent, FD70 - light gray, FD250 - dark gray, FD500 - black.

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