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Aggregation of erythrocytes: a novel activity of Human Beta-Defensin-2

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Aim. To investigate an ability of HBD-2 to induce aggregation of erythrocytes. **Methods.** Aggregation assay, turbidimetry, light microscopy. **Results.** We have identified aggregation of erythrocytes in the presence of at least 12.5 μM HBD-2 that was confirmed by light microscopy. Effect was strongly depended on HBD-2 concentration but was masked by hemolysis of erythrocytes. Also, aggregation was diminished in presence even 10 mM of NaCl that may indicate electrostatic nature of interactions between HBD-2 and erythrocytes. **Conclusions.** HBD-2 can induce the salt-sensitive aggregation of erythrocytes in a dose-dependent manner.

Keywords: β-defensins, dose-dependent, salt-sensitive

Introduction

HBD-2, a member of the β-defensin family of antimicrobial peptides, is an important component of innate immunity [1]. Its antimicrobial properties rely mainly on the ability to bind and permeabilize cell membranes of bacteria, viruses and fungi [2]. It also possesses some immunomodulatory functions [3]. Some of the defensins, such as HNP-2 [4] or cryptdin [5], can also induce the formation of peptide-membrane aggregates after the incubation with negatively charged vesicles. For HBD-2 or any other β-defensin, this activity has never been reported before. So, our goal was to show that HBD-2 can aggregate membranes as well. To reach the aim erythrocytes were used instead of negatively charged vesicles. We also observed some features of this interaction such as dose-dependency and salt-sensitivity.

Materials & Methods

Production and purification of recHBD-2. HBD-2 was produced by the recombinant

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E.coli Rosetta™ strain transformed by pGEX-2T-HBD-2 construct as previously described [6]. The production of recombinant protein was performed in the same way as in our previous report [7]. In short, the expression of recombinant protein was inducted by addition of 1 mM IPTG when bacterial growth was in the log-phase. After 5 h incubation the bacteria were pelleted and lysed. Lysate was applied on the glutathione-agarose column for purification. After washing the column the GST-HBD-2 fusion protein was cleaved by thrombin and HBD-2 was separated by reverse-phase chromatography. The HBD-2 containing fraction was air-dried and analyzed by PAAG electrophoresis, densitometry, western-blot and mass-spectrometry to check quantity, purity and conformity with native HBD-2.

Erythrocyte suspension preparation. Blood received from healthy volunteers after venipuncture was added to 3.6 % (w/v) sodium citrate solution in proportion 9:1. The resulting solution was carefully mixed by inverting the tube. Erythrocytes were triple washed in 270 mM sucrose solution and counted manually. No visible hemolysis was observed. Afterwards, erythrocytes were diluted to 200 million per milliliter of 270 mM sucrose pH 8 or isotonic solution of 10, 25, 50 and 100 mM NaCl.

Aggregation assay. Erythrocyte suspension was mixed with 3.125, 6.25, 12.5 and 25 μM HBD-2. Then 100 μL of each suspension were added to the wells of round-bottom 96-well plate in triplicate. Plate was incubated at 37 °C for 60 min and centrifuged at 400 g for 5 min. After that, the sediment was analyzed. Reaction was confirmed negative if sediment was dot-shaped and positive if sediment was umbrella-shaped. To confirm that the differences in sediment shape are due to the aggregation of erythrocytes, the samples containing 25 μM HBD-2 and control sample were carefully resuspended and checked by light microscopy with magnification of 100.8 and 160.

Turbidity assay. Absorbance measurement was performed at 630 nm. This wavelength was chosen to eliminate the noise caused by hemoglobin absorption that has absorbance peak at 580 nm. The change in absorbance indicates the changes in turbidity of sample due to aggregation or lysis of erythrocytes. Before the assay erythrocyte suspensions were mixed: 1) with HBD-2 in wide range of concentrations from 2.5 to 40 μM for 60 min or 2) with 20 μM HBD-2 in the presence of 10–100 mM NaCl for 60 min. Incubation was performed at 37 °C in both cases. For checking the rate of spontaneous erythrocyte aggregation and lysis, the control assay was also performed without addition of HBD2.

Statistical method. Standard statistical methods were used to process data, which were presented in terms of a mean and standard deviation. Differences between the groups were calculated using U-test, with statistical significance of $p < 0.05$.

Results

The aggregation assay shows that the addition of HBD-2 to erythrocyte suspension leads to the changes in erythrocyte sediment shape that indicate their aggregation (Table 1).

Table 1. **HBD-2 induced aggregation of erythrocytes**

HBD-2 concentration, μM	0	3.125	6.25	12.5	25
Aggregation	-	-	-	+	+

To prove this assumption, the sediments that were formed after incubation with 25 μM HBD-2 and with intact erythrocytes were analyzed by light microscopy (Figure 1). The analysis shows that HBD-2 induces the formation of erythrocyte aggregates of different size. Most erythrocytes are found in aggregates and number of free erythrocytes decreased due to the aggregation and hemolysis.

From Table 1 we can conclude that the aggregates formation may be a dose-dependent process because aggregation occurs only if the HBD-2 concentration exceeds 12.5 μM. The turbidity assay confirms this conclusion (Figure 2A). It is remarkable that HBD-2 in small amount actually decreases turbidity due to strong hemolysis. But when the HBD-2 concentration exceeds 2.5 μM the aggregation rate rapidly increases, at 5 μM reaches primary turbidity and then increases further to 20 μM. After that the aggregation stopped and additional HBD-2 did not cause more aggregation.

We also know about the inhibitory effect of salts on the HBD-2 activity [1]. To confirm that aggregation also depends on the presence of salts the turbidity was checked at different concentration of NaCl and 20 μM HBD-2

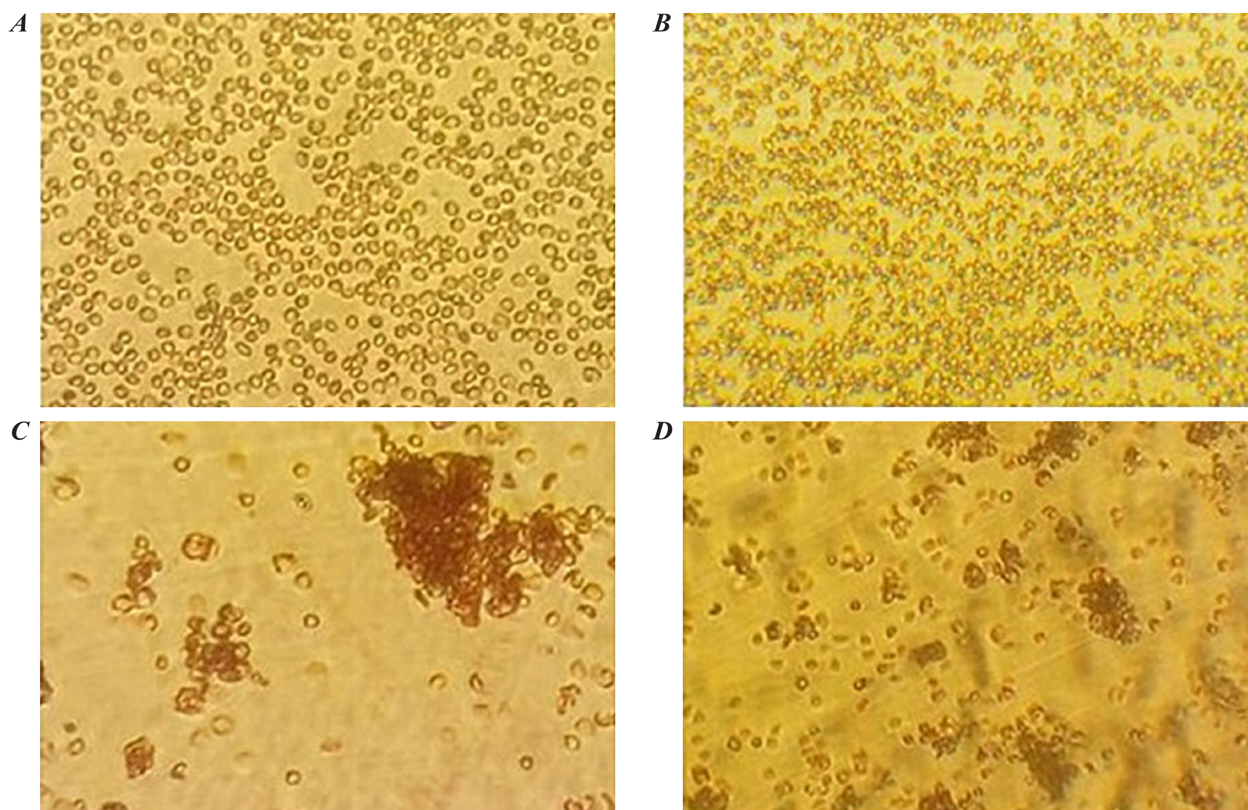

Fig. 1. Microscopic image of HBD-2 induced erythrocyte aggregates: *A* —intact erythrocytes (160 X); *B* — intact erythrocytes (100.8 X); *C* —erythrocytes after incubation with 25 μM HBD-2 (160 X); *D* — erythrocytes after incubation with 25 μM HBD-2 (100.8 X).

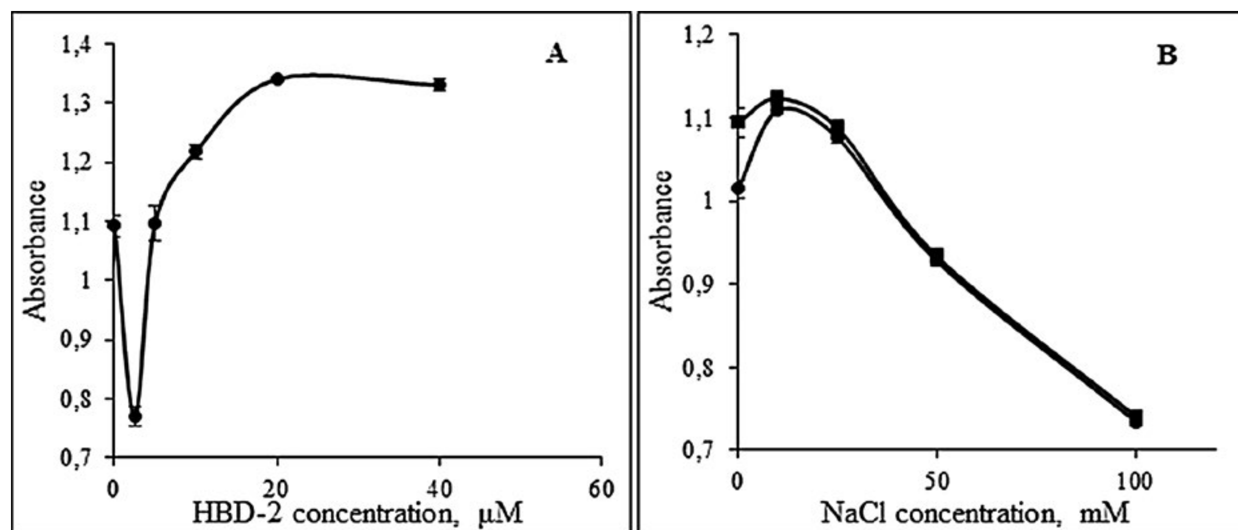

Fig. 2. Aggregation of erythrocyte A) induced by HBD-2 in different concentration; B) in the presence of NaCl in different concentration at 20 μM HBD-2; ● – sample; ■ – control; error bars indicate standard deviation of the means of four measurements.

(Figure 2B). Difference between the values of turbidity of control and tested samples was insignificant for all NaCl concentrations. Therefore, we can conclude that aggregation depends on the electrostatic interactions as well.

Despite the fact that HBD-2 and some other defensins can aggregate membranes, an explanation of physiological significance of this activity may be complicated due to the presence of salts in physiological fluids that inhibit this activity. On the other side, the activity may play a role at the low-salt conditions where defensins may aggregate some bacteria or enveloped viruses thus enhancing the pathogen clearance and preventing their penetration into the cells.

Conclusion

The above research allowed us to discover a novel mode of action of HBD-2, namely, the ability to aggregate erythrocytes, and to demonstrate that this activity is dose-dependent and salt-sensitive.

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Aggregation of erythrocytes: a novel activity of Human Beta-Defensin-2

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Мета. Дослідити здатність HBD-2 індукувати агрегацію еритроцитів. **Методи.** Агрегаційний тест, турбідиметрія, світлова мікроскопія. **Результати.** Було виявлено агрегацію еритроцитів в присутності щонайменше 12,5 μM HBD-2, що було підтверджено мікроскопічним дослідженням. Ефект сильно залежав від концентрації HBD-2, але маскувався за рахунок гемолізу еритроцитів. Також, агрегація пригнічувалась навіть у присутності 10 мМ NaCl, що може вказувати на електростатичну природу взаємодії HBD-2 та еритроцитів. **Висновки.** HBD-2 здатен індукувати солечутливу агрегацію еритроцитів дозо-залежним чином.

Ключові слова: β-дефенсини, дозо-залежний, соле-чутливий

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Цель. Исследовать способность HBD-2 индуцировать агрегацию эритроцитов. **Методы.** Агрегационный тест, турбидиметрия, световая микроскопия. **Результаты.** Было выявлено агрегацию эритроцитов в присутствии не меньше 12,5 μM HBD-2, что было подтверждено микроскопическим исследованием. Эффект сильно зависел от концентрации HBD-2, но маскировался гемолизом эритроцитов. Также, агрегация угнеталась в присутствии даже 10 мМ NaCl, что может указывать на электростатическую природу взаимодействия между HBD-2 и эритроцитами. **Выводы.** HBD-2 способен индуцировать соле-чувствительную агрегацию эритроцитов дозо-зависимым образом.

Ключевые слова: β-дефенсины, дозо-зависимый, соле-чувствительный

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