Chemotherapeutic activity of synthetic antimicrobial peptides: correlation between chemotherapeutic activity and neutrophil-activating activity

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Abstract The chemotherapeutic activity of three synthetic antibacterial peptides was investigated. KLKLLLLLKLK-NH_2 and its D-enantiomer showed significant chemotherapeutic activity in MRSA-infected mice, whereas KLKLLLKLK-NH_2, which showed the highest antibacterial activity among them in vitro, was found to have almost no ability to prevent MRSA infection. These results suggest that the antibacterial activity of peptides assessed in vitro does not necessarily correlate with their chemotherapeutic activity. We found that KLKLLLLLKLK-NH_2 and its D-enantiomer, but not KLKLLLKLK-NH_2, have the ability to activate human neutrophils to produce superoxide, suggesting that the prevention of MRSA infection by these peptides is not simply due to their direct bactericidal activity but to augmentation of the systemic defense mechanism mediated by neutrophils.

Key words: Antibacterial peptide; Chemotherapy; Infection; MRSA; Neutrophil; Superoxide

1. Introduction

It is now well known that many insects synthesize antibacterial proteins in response to a bacterial challenge or body injury [1,2]. They are thought to be defense molecules that protect the insects from bacterial infection. As the antibacterial activity of these proteins is comparable to that of various antibiotics, they are potentially useful for application to human infectious diseases. However, it is generally supposed to be difficult to use these proteins as they are for therapeutic purposes, because of their antigenicity and toxicity.

Previously, we identified the active core of sapecin B [3], a potent antibacterial protein of *Sarcophaga peregrina* (flesh fly) consisting of 34 amino acid residues [4]. This core consisted of 11 amino acid residues, residues 7–17, that form an α-helix in sapecin B [5]. We modified this peptide further, and synthesized several antimicrobial peptides exhibiting both antibacterial and antifungal activity [6]. Generally, undecapeptides having [K or R]X[K or R] motifs at both termini and internal XXXXX, where X is a hydrophobic residue, are potentially active in killing bacteria and fungi. We studied the mode of action of these peptides using one of them, KLKLLLLLKLK-NH_2, and found that bacterial membranes became permeable to low molecular mass substances when *Escherichia coli* was treated with this peptide [7]. The bacterium was also found to lose the ability to synthesize ATP and to transport amino acids, suggesting that the electrochemical membrane potential was disrupted on treatment with this peptide [7]. The D-enantiomer of this peptide, consisting entirely of D-amino acids, was resistant to tryptic digestion and persisted longer in the bacterial culture medium, showing greater antimicrobial activity than the original peptide [6].

This paper reports on the chemotherapeutic activity of three antibacterial peptides, KLKLLLLLKLK-NH_2 and its D-enantiomer, and KLKLLLKLK-NH_2, against infection by meticillin-resistant *Staphylococcus aureus* (MRSA). We found that the antibacterial activity of these peptides assessed in vitro does not necessarily correlate with their chemotherapeutic activity.

2. Materials and methods

2.1. Antimicrobial peptides

The antimicrobial peptides, KLKLLLLLKLK-NH_2 and its D-enantiomer, and KLKLLLKLK-NH_2, were synthesized by the solid-phase method with a peptide synthesizer (Shimadzu PSSM-8). Each peptide was purified to homogeneity by HPLC on a reverse-phase column of Syncropak RP-R (C_{18}), and its amino acid sequence was confirmed with a protein sequencer (Shimadzu PPSQ-10).

2.2. Assay of antibacterial activity in vitro

To assess the antibacterial activity of the peptides, we used the following convenient method in addition to the conventional method described previously [8]. An MRSA strain isolated from a patient and maintained in the hospital of Teikyo University, Tokyo (MRSA *Teikyo*), was grown in Müller-Hinton Broth (MHB) (Gibco). Then, 100 μl of a bacterial suspension containing 1×10^5 bacterial cells, 100 μl of a peptide sample dissolved in MHB, and 10 μl of Alamar Blue (Alamar Biosciences, Inc.) were mixed and incubated for 4 h at 37°C, the change in the color of the culture medium being monitored by measuring OD_{570} and OD_{610}, as described previously [9]. In this assay, the reducing activity of the growing bacteria changes the color of Alamar Blue.

2.3. Assay of protection against MRSA infection

Male BALB/c mice were each injected intraperitoneally with 250 mg/kg of cyclophosphamide. Four days later, 2×10^7 MRSA *Teikyo* cells were introduced intravenously into each mouse, and 15 min later, the peptide sample dissolved in 200 μl of saline was also injected intravenously. As a negative control, saline alone was injected. The viability of the mice was examined for 7 days after bacterial infection. We used more than five mice per group to evaluate the activity of each peptide. To prepare the MRSA *Teikyo* cell suspension, MRSA *Teikyo* was grown on a MHB plate overnight. The cells were collected, suspended in saline, and then kept at 4°C overnight. Meanwhile, a part of the bacterial suspension was plated to determine the viable cell number in the suspension. The bacterial density was adjusted to 1×10^8 cells/ml just before use.

2.4. Assay of superoxide generation

This was performed essentially as described previously [10]. The

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superoxide production by neutrophils in the presence of various peptides was monitored by measuring superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction as described before [10]. Freshly prepared human peripheral neutrophils were suspended in Hanks' balanced salt solution at a density of 3×10^5 cells/ml/well and then incubated for 5 min at 37°C in the presence of 100 μM ferricytochrome *c*. The reaction was started by the addition of 2.5 μg/ml of peptide and OD_{550} was monitored. The concentration of the peptide was increased with time, when necessary, by adding 2.5 μg/ml of the peptide each time. The control wells contained 500 units of SOD. As a positive control, 200 ng/ml of phorbol myristate acetate (PMA) was used.

3. Results

3.1. Chemotherapeutic activity of the antibacterial peptides

Previously, we demonstrated that a synthetic peptide, KLKLLLLLKLK-NH_2, and its D-enantiomer showed antibacterial activity against both Gram-positive and Gram-negative bacteria [6]. The antibacterial activity of the D-enantiomer was found to be higher than that of the original peptide. The subsequent study revealed that KLKLLLKLK-NH_2 shows much higher antibacterial activity against MRSA *Teikyo* than the D-enantiomer, as shown in Fig. 1.

As the molecular masses of these peptides were less than 1500, their antigenicity was supposed to be weaker compared with that of other antibacterial proteins having higher molecular masses. Therefore, we examined their chemotherapeutic activity using MRSA-infected mice. For this, we treated mice with 250 mg/kg of cyclophosphamide in advance to increase the efficiency of MRSA infection, and then 2×10^7 MRSA *Teikyo* cells were introduced per mouse intravenously. Fifteen minutes later, 100 μg of a peptide dissolved in 200 μl of saline was injected intravenously.

As summarized in Table 1, KLKLLLLLKLK-NH_2 showed significant chemotherapeutic activity in three independent experiments. The D-enantiomer was also effective, and its potency was assumed to be almost the same as that of KLKLLLLLKLK-NH_2. Contrary to expectation, KLKLLLKLK-NH_2, which showed the highest antibacterial activity in vitro, was found to have no ability to prevent MRSA infection. No chemotherapeutic effect was detected even when MRSA-infected mice were treated with 200 μg of this peptide. Therefore, we conclude that the antibacterial activity of these peptides does not explain their chemotherapeutic activity. Possibly, the former two peptides have the ability to augment the defense mechanism of mice to prevent infection by MRSA.

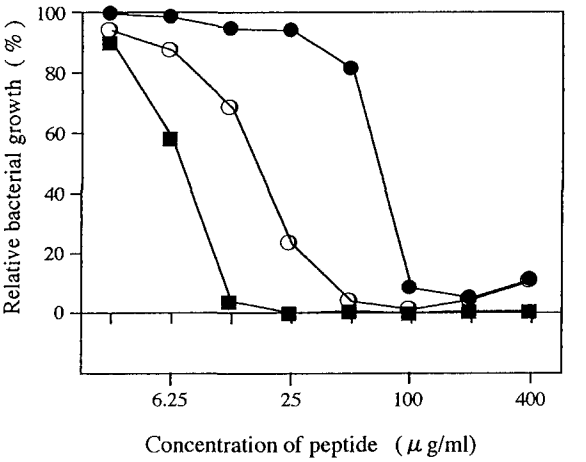

Fig. 1. Antibacterial activity of three synthetic peptides. The growth of MRSA *Teikyo* in the presence of various concentrations of peptides was monitored by means of the Alamar Blue assay, and relative bacterial growth is plotted against the amounts of the peptides. ●, KLKLLLLLKLK-NH_2; ○, D-enantiomer; ■, KLKLLLKLK-NH_2.

3.2. Activation of human neutrophils by the antibacterial peptides

Independently of the chemotherapeutic experiments involving mice, we found that KLKLLLLLKLK-NH_2 activates human neutrophils to produce superoxide in vitro. Therefore, we compared the abilities of these three peptides to activate human neutrophils, and found that KLKLLLKLK-NH_2 shows no appreciable neutrophil-activating activity. As is evident from Fig. 2, KLKLLLLLKLK-NH_2 activated neutrophils extensively. The neutrophil-activating activity of the D-enantiomer was much weaker, but it was clearly significant. On the other hand, KLKLLLKLK-NH_2, which showed no appreciable chemotherapeutic activity, showed no appreciable neutrophil-activating activity, but the same neutrophils produced superoxide when PMA was added to the wells. As it was difficult to collect sufficient mouse neutrophils to examine their activation by these peptides in vitro, we were not able to perform the same experiments with mouse neutrophils. However, we assume that the situation is the same with human and mouse neutrophils. Possibly, the chemotherapeutic activity of KLKLLLLLKLK-NH_2 and the D-enantiomer is intimately related to their neutrophil-activating activity.

Table 1
Chemotherapeutic effects of the antimicrobial peptides on MRSA-infected mice

Peptide		Survival of mice						
		day 1	day 2	day 3	day 4	day 5	day 6	day 7
L-KLKLLLLLKLK-NH_2	exp. 1	5/5	5/5	5/5	5/5	5/5	5/5	5/5
	exp. 2	8/8	7/8	7/8	7/8	7/8	7/8	6/8
	exp. 3	8/8	7/8	7/8	5/8	5/8	3/8	3/8
D-KLKLLLLLKLK-NH_2	exp. 2	8/8	7/8	6/8	6/8	6/8	6/8	6/8
L-KLKLLLKLK-NH_2	exp. 3	8/8	0/8					
Saline (control)	exp. 1	5/5	0/5					
	exp. 2	5/8	0/8					
	exp. 3	7/8	0/8					

The same experimental numbers indicate series of experiments performed at the same time.

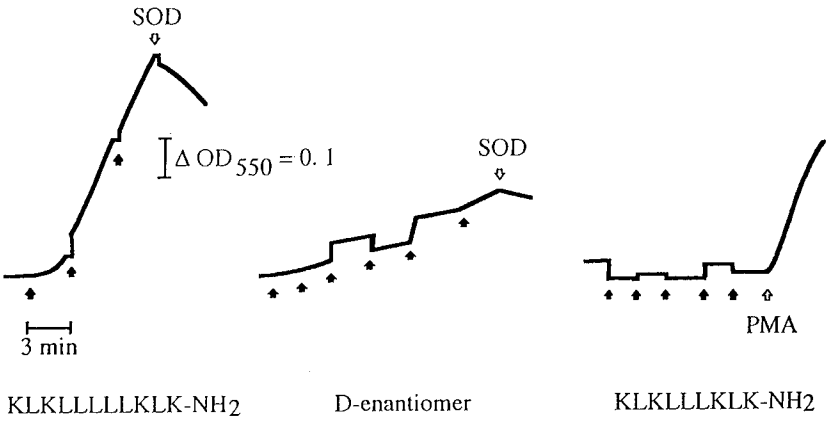

Fig. 2. Activation of human neutrophils by the antibacterial peptides. Freshly prepared human neutrophils were treated with the antibacterial peptides, and then neutrophil-activating activity was assessed as SOD-sensitive cytochrome *c* reduction (generation of superoxide). The wells contained 3×10^5 neutrophils, and 2.5 μg peptide was added at each point indicated by a solid arrow. As a positive control, 200 ng PMA was added. SOD and PMA denote superoxide dismutase and phorbol myristate acetate, respectively. Cytochrome *c* reduction was monitored over time by measuring OD_{550}.

4. Discussion

In this study, we demonstrated that KLKLLLLLKLK-NH_2 and its D-enantiomer cure MRSA-infected mice under certain experimental conditions. To the best of our knowledge, this is the first demonstration of the prevention of MRSA infection by low molecular mass antibacterial peptides. We found that the chemotherapeutic activity of these peptides is not simply due to their direct bactericidal effect, because KLKLLLKLK-NH_2, which showed the highest antibacterial activity, did not exhibit any chemotherapeutic activity. Generally, the chemotherapeutic activity of various antibiotics is due to their direct interaction with the target bacteria, but the situation with antibacterial peptides seems to be different.

We suggested that KLKLLLLLKLK-NH_2 and its D-enantiomer augment the systemic defense system of a host, resulting in prevention of MRSA infection, and that the white blood cells, such as neutrophils, play a crucial role in this process. Under our experimental conditions, the contribution of the bactericidal activity of these peptides, if any, is assumed to be small, because 100 μg KLKLLLLLKLK-NH_2 or its D-enantiomer cured a mouse infected with 2×10^7 MRSA *Teikyo*. Assuming that the blood volume circulating in a mouse is 3 ml, the number of bacteria and the concentration of the peptide in the blood can be simply calculated to be about 5×10^6 cells/ml and 33 μg/ml, respectively. This concentration of the peptide seems to be too low to explain its chemotherapeutic activity, because the minimum inhibitory concentrations of KLKLLLLLKLK-NH_2 and its D-enantiomer for 5×10^5 MRSA *Teikyo* in vitro are 100 and 50 μg/ml, respectively (see Fig. 1). Therefore, it is reasonable to predict the augmentation of the host defense mechanism by these peptides.

We found that the peptides having chemotherapeutic activity have neutrophil-activating activity, but there seems to be no quantitative relation between their chemotherapeutic potency and neutrophil activating activity. The mechanisms of contribution of neutrophils to chemotherapeutic activity of these peptides remain to be elucidated. Assuming that the augmentation of the defense mechanism by KLKLLLLLKLK-NH_2 and its D-enantiomer is due to the activation of neutrophils, the presence of a receptor for these peptides on the surface of neutrophils can be supposed. Possibly, the peptide size is important for the binding to this receptor, because only KLKLLLLLKLK-NH_2 and its D-enantiomer, not KLKLLLKLK-NH_2, can activate neutrophils. The difference between KLKLLLLLKLK-NH_2 and its D-enantiomer in the neutrophil activation potency may be explained by their different affinities to this receptor due to their configurational difference.

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