論文の内容の要旨

Mechanistic analysis of a novel antimicrobial compound, Kaikosin E

(新規抗生物質カイコシンの作用機序の解析)

氏名　　蘇　潔

Introduction

The emergence and spread of multi-resistant pathogenic strains declined the number of clinically available antibiotics and caused a serious public health problem. Therefore, the development of novel antimicrobial agents with effective treatment of the drug-resistant pathogens is needed to combat this situation. Our lab also worked on the discovery of novel antimicrobial compounds and isolated a novel lipopeptide antibiotic named as kaikosin E using silkworm infection model. Kaikosin E exhibits good antibiotic traits such as obvious therapeutic effect in mouse infection model with low toxicity and rapid bactericidal activity compared with other clinically used antibiotics. Cell lysis also occurred after treatment with kaikosin E. Adding kaikosin E to *Staphylococcus aureus* rapidly dissipated membrane potential. Based on these findings, disruption of membrane is one proposed mechanism of action for kaikosin E. However, the details are still unknown. In searching of target for kaikosin E, *fni* or *menA* gene mutation was found in kaikosin E resistant mutant. Both genes are involved in menaquinone

biosynthesis pathway. Furthermore mixing kaikosin E with menaquinone caused complex precipitation in water. These results guide me to focus on the importance of menaquinone for uncovering kaikosin E mechanism. In addition, a recent study showed the antimicrobial activity of kaikosin E against *S.aureus* was enhanced by addition of serum. This finding suggested that an enhancing factor for antimicrobial activity of kaikosin E exists in serum. Therefore, identification of this factor may facilitate knowing the interaction of kaikosin E with its target and provide information for improving the therapeutic activity of kaikosin E. In this study, I intend to further elucidate the antimicrobial mechanism of kaikosin E through the identification of the role of menaquinone in membrane damage and identification of the serum factor responsible for enhancing antimicrobial activity of kaikosin E.

Results

1. Identification of menaquinone as a target

In view menaquinone existed in cell membrane and kaikosin E had membrane damaging activity, identification of the role of menaquinone in membrane damage may facilitate elucidation of kaikosin E mechanism. To test the role of menaquinone on kaikosin E-mediated membrane damage effect，calcein-encapsulated liposome was prepared and used for membrane leakage assay. Kaikosin E significantly caused leakage of liposome containing menaquinone (MK) compared with liposome without menaquinone (**Figure 1**), whereas daptomycin showed no significant difference on leakage of liposome with or without menaquinone. Hence, the existence of menaquinone stimulated membrane damaging activity of kaikosin E. On the other hand, kaikosin E had no hemolytic effect on sheep red blood cells, suggesting that this membrane damaging effect is specific to bacteria. To further confirm the interaction of kaikosin E with menaquinone, isothermal titration calorimetry (ITC) assay was conducted for characterization of binding affinity between them. ITC results showed kaikosin E binds with menaquinone at a molar ratio of 1:1 with an affinity constant (K_a) of $2.2\pm0.4\times10^5 M^{-1}$. These results suggest kaikosin E binds with menaquinone in cell membrane and induces membrane damage.

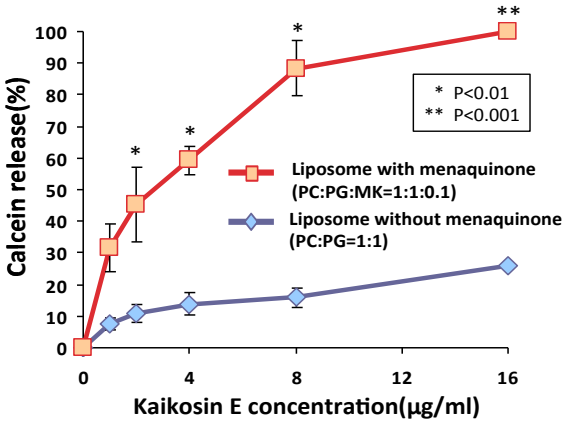

Figure 1. Membrane leakage assay. Values for 100% calcein release were obtained using Triton X-100. ∗,P<0.01; ∗∗,P<0.001.

2. Identification of a serum factor enhancing antimicrobial activity of kaikosin E

Antimicrobial activity of kaikosin E against *S. aureus* was enhanced more than 10-fold in the presence of bovine calf serum. This enhancement effect on antimicrobial activity of kaikosin E was also detected in human plasma and other mammalian serum. Considering the significant enhancement effect of

serum on kaikosin E activity, I assume some factors in serum act as enhancers. Thus I tried to purify the enhancing factor from serum. One unit of enhancing activity is defined as the minimum amount of active factor which can inhibit bacterial growth in the presence of 1 μg/ml kaikosin E. Enhancing factor in serum was purified with ethanol extraction followed by octadecyl silica (ODS) column chromatography with 190-fold increase in specific activity (**Table 1**). ODS column fraction lost 97 % of enhancing activity after trypsin treatment. This result suggested that a protein in serum is responsible for enhancing antimicrobial activity of kaikosin E. To further identify the active protein, fractions from ODS column were analyzed by SDS polyacrylamide gel electrophoresis. Twenty-four kDa protein band was observed in active fractions (**Figure 2**). These fractions were further analyzed by HPLC using size exclusion column, and the band intensity of 24 kDa protein from each fraction correlated with its enhancing activity. Peptide mass fingerprinting analysis revealed the 24 kDa protein was matched with the sequence of apolipoprotein A-I (Apo A-I). Antimicrobial activity of kaikosin E was enhanced 16-fold in the presence of 90 μg/ml of human recombinant apolipoprotein A-I, which is equivalent to 10 % serum. Apolipoprotein A-I itself had no antimicrobial activity against *S. aureus* (**Table 2**). These results suggested apolipoprotein A-I is one serum factor responsible for enhancing antimicrobial activity of kaikosin E. The mechanism of apolipoprotein A-I mediated enhancement on antimicrobial activity of kaikosin E needs to be elucidated.

Table 1. Purification of a serum factor enhancing antimicrobial activity of kaikosin E

Fraction	Weight (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Ⅰ.Serum	96	640	7	100	1
Ⅱ.60%EtOH extract	18	2600	140	406	20
Ⅲ.ODS Fr.1	16	<20	–	–	–
Fr.2	0.2	<20	–	–	–
Fr.3	0.8	<20	–	–	–
Fr.4	0.5	640	1300	100	190
Fr.5	0.1	80	800	13	110

Table 2. Effect of serum on antimicrobial activity of kaikosin E

Medium	MIC(μg/ml)
Mueller-Hinton broth	4
+ Serum(10%)	0.25
+ Human recombinant apolipoprotein A-I(90 μg/ml)	0.13

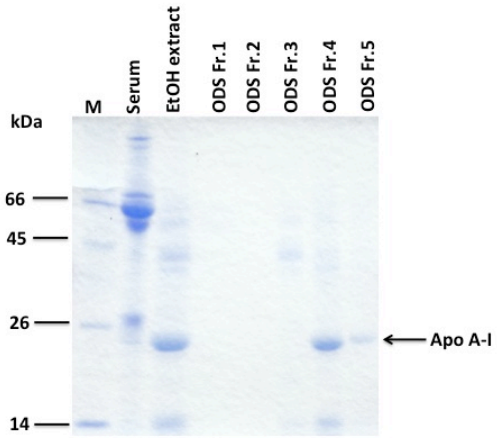

Figure 2. SDS-PAGE analysis of fractions from ODS column.

3. Interaction of apolipoprotein A-I with kaikosin E and menaquinone

Menaquinone was reported to interact with high-density lipoprotein. Apolipoprotein A-I is known as the major component of high-density lipoprotein in plasma. Hence I proposed apolipoprotein A-I enhances antimicrobial activity of kaikosin E through interaction with menaquinone and kaikosin E. To test this hypothesis, interaction assay among apolipoprotein, menaquinone and kaikosin E was performed.

After mixing kaikosin E and menaquinone at a molar ratio of 1:1 and centrifugation, yellow pellet was shown in the bottom of the tube. When mixing apolipoprotein A-I, kaikosin E and menaquinone, organge pellet was shown in the mixing solution whereas apolipoprotein A-I solution itself was transparent. Hence, I speculate that apolipoprotein A-I interacts with the binding complex of kaikosin E with menaquinone and changes the color of pellet. To check this possibility, the precipitate from mixing apolipoprotein A-I, kaikosin E and menaquinone solution was applied into SDS-PAGE analysis. Apolipoprotein A-I band was detected in the precipitate sample from mixing apolipoprotein A-I, kaikosin E and menaquinone solution **(Figure 3)**. These results suggested apolipoprotein A-I interacts with kaikosin E and menaquinone. Apolipoprotein A-I may enhance the antimicrobial activity of kaikosin E through interaction with kaikosin E and menaquinone.

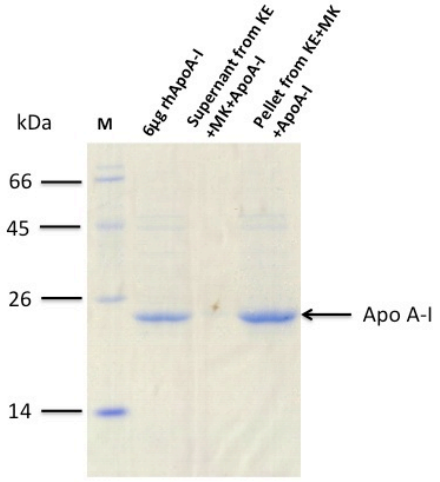

Figure 3. SDS-PAGE analysis on existence of Apo A-I.

Discussion

This study demonstrates kaikosin E binds with menaquinone in cell membrane and induces membrane damage. To my knowledge, this is the first report that menaquinone is identified as a target of antibiotic. Menaquinone is an essential component of bacterial electron transport chain. In contrast, ubiquinone is a coenzyme utilized in human electron transport chain. The finding that kaikosin E specifically binds with menaquinone not ubiquinone explains that membrane damage effect induced by kaikosin E is specific to bacteria. Therefore, menaquinone can be used as a potential target for the development of novel antibacterial drugs.

In this study, I also identified apolipoprotein A-I as a serum factor, which is responsible for enhancing antimicrobial activity of kaikosin E against *S. aureus*. This finding explains the fact that kaikosin E has good therapeutic activity compared with vancomycin in mouse infection model, whereas the antimicrobial activity of kaikosin E detected in culture medium is lower than that of vancomycin. Therefore, the enhanced antimicrobial activity of kaikosin E *in vivo* may be due to the existence of apolipoprotein A-I in serum. This finding provides evidence for kaikosin E to be successful use in future clinical situation. Study on the interaction among apolipoprotein A-I, kaikosin E and menaquinone showed that apolipoprotein A-I interacts with the binding complex between kaikosin E and menaquinone. Therefore, apolipoprotein A-I may enhance antimicrobial activity of kaikosin E through interaction with the binding complex of kaikosin E and menaquinone.