Effect of a Synthetic Peptide Designed on the Basis of the Active Site of Insect Defensins on the Proliferation of Methicillin-resistant Staphylococcus aureus under the Conditions for External Application

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A short antibacterial peptide was designed and synthesized on the basis of the active sites of insect defensins. The 9-mer peptide, ALYLAIRRR-NH₂, was tested to determine whether it can suppress the proliferation of methicillin-resistant Staphylococcus aureus (MRSA) in vivo and in vitro. In vivo evaluation of the peptide against MRSA was carried out histopathologically. Silk sutures pretreated or non-treated with the peptide were embedded in the back skin of mice for 24 h. Sutures treated with the peptide showed strong inhibition of the proliferation of MRSA judging from examination by light microscopy of biopsy samples. On the contrary, non-treated sutures showed numerous Gram-positive loci. For in vitro experiments, silk fibroin films containing different concentrations of the 9-mer peptides were prepared. MRSA seeded on the culture plates was covered with a transparent fibroin film and incubated at 37°C for 24 h. No MRSA colonies were detected under the films containing the 9-mer peptide, whereas many colonies appeared under the control film without the peptide. These results suggest this synthetic antibacterial peptide is a useful lead peptide for development of novel therapeutic agents against infection with antibiotic-resistant bacterial pathogens.

Key words: synthetic antibacterial peptide, insect defensin, MRSA, mouse, fibroin film, insect immunity

INTRODUCTION

Antibacterial peptides are an important constituent of the innate immune mechanisms used by multicellular organisms to control the natural flora and fight a broad range of bacterial targets (Hancock, 2001; Sitaram and Nagaraj, 2002). Characteristics that make antibacterial peptides attractive as alternatives to conventional antibiotics and pharmaceuticals include rapidity of action, a wide antibacterial spectrum, low likelihood of resistance development and the ability to act in conjugation with existing regimens (Zasloff, 2002).

Insect defensins are ca. 4 kDa peptides containing 6 cysteine residues with strong antibacterial activity against mainly Gram-positive bacteria. Only a few Gram-negative bacteria, fungi and yeast are affected by defensins (Hetru et al., 1998). Insect defensins contain 3 disulfide bonds, and have a loop, an α-helix and two β-sheets from the N-terminus as structural characteristics (Bulet et al., 1998). Insect defensins contain 3 disulfide bonds, and have a loop, an α-helix and two β-sheets from the N-terminus as structural characteristics (Bulet et al., 1998). Insect defensins contain 3 disulfide bonds, and have a loop, an α-helix and two β-sheets from the N-terminus as structural characteristics (Bulet et al., 1998). Two insect defensins were isolated in our laboratory from larvae of beetles, Allomirina dichotoma (Miyanoshita et al., 1996) and Oryctes rhinoceros (Ishibashi et al., 1999). These defensins consist of 43 amino acid residues and their active sites were determined (Saido-Sakanaka et al., 1999; Ishibashi et al., 1999). Some modified synthetic peptides based on the active sites were effective against both Gram-positive and Gram-negative bacteria including antibiotic-resistant pathogens (Saido-Sakanaka et al., 2004; Yamada et al., 2004). These peptides exhibited no growth inhibition activity against murine fibroblasts or macrophages and no hemolytic activity against rabbit erythrocytes in vitro (Saido-Sakanaka et al., 2004). In addition, the administration of these peptides protected mice from a lethal challenge with methicillin-resistant Staphylococcus aureus (MRSA) (Saido-Sakanaka et al., 2004). Moreover, these peptides suppressed tumor necrosis factor α (TNF-α) gene expression by lipopolysaccharide or lipoteichoic acid in murine macrophages, and also blocked nuclear factor κB (NF-κB) activation (Saido-Sakanaka et al., 2004; Motobu et al., 2004).

The rapid spread of antibiotic-resistant pathogenic bacteria, such as MRSA, is a severe problem in therapy of bacterial infection (Hiramatsu et al., 1992, 1997). It has been suggested that S. aureus cells interact with foreign bodies to form a biofilm, thereby evading the effect of antibacterial agents. For example, S. aureus cells epicutaneously inoculated with silk stitches on the back skin of mice were shown to form microcolonies on the surface of the silk thread enclosing them in membrane-like structures. The membrane-like structures were stained with ruthenium red, suggesting that these structures contain polysaccharide components (Akiyama et al., 1993). Silk sutures used in surgical operations have thus a possibility
to be sites for formation of biofilms once they are infect-
ed by MRSA. From this viewpoint, it is very important
to test whether synthetic antibacterial peptides designated
on the basis of insect defensins can prevent MRSA prolif-
eration around surgical sutures in vivo.

Another possible utilization of these synthetic peptides
is the development of novel wound dressings containing
the peptides to prevent infection of antibiotic-resistant
pathogenic bacteria. Recently, a transparent fibroin film
(silk film) was newly developed and its effect on full-
thickness skin wounds tested (Sugihara et al., 2000). Re-
results showed that the healing time of wounds dressed with
the fibroin film was shorter than those dressed with a con-
ventional hydrocolloid dressing. The fibroin film showed
a similar or slightly better promotive effect as lyophilized
porcine dermis, which is used as a dressing for burns, ul-
cers and decubitus.

In this study, we chemically synthesized a 9-mer pep-
tide designated on the basis of the active site of insect de-
fensins and observed its effect on MRSA proliferation
around surgical sutures in vivo. We also prepared fibroin
films containing different concentrations of the synthetic
peptide and examined effects on the growth of MRSA in vitro. Here we report the results and discuss the possible
application of peptide antibiotics for therapy of infection
with antibiotics-resistant bacteria.

MATERIALS AND METHODS

Peptide synthesis
A 9-mer peptide ALYLAIRRR-NH₂ was synthesized by
a solid method using f-moc chemistry in a 9050 Plus Pep-
tide Synthesizer (Millipore) as described previously (Sai-
do-Sakanaka et al., 1999; Ishibashi et al., 1999). The
peptide was purified by ÄKTA Explorer using a reverse
column of source RPC (3 ml) (Pharmacia). The peptide
was eluted with a linear gradient (0-30%, v/v) of acetoni-
trile containing 0.05% trifluoroacetic acid (v/v). The ami-
no acid sequence and molecular mass were confirmed by
a protein sequencer (Procise cLC, PE Biosystems) and
Matrix-assisted laser desorption/ionization time-of-flight
mass spectrometry (Voyager, Perseptive Biosystems), re-
spectively.

Bacterium
The MRSA C-3 strain used in this work was isolated
from the urinary tract of patients. The MRSA was cul-
tured in Müller-Hinton broth (MHB) (Difco) at 37°C for
18 h (ca. 1 × 10⁶ colony forming units/ml).

Antibacterial assay
The plate growth inhibition was according to the meth-
ods described previously (Hultmark et al., 1982). Briefly,
melted MHB agar (Difco) (10 ml) containing 1 × 10⁶ cells
of MRSA was poured into a plate. Wells (2 mm) were cut
into the freshly poured plate after solidification. Each well
received 2 μl of different concentration of peptide in phos-
phate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl,
8.1 mM Na₂HPO₄·12H₂O and 1.5 mM KH₂PO₄). The plate
was incubated for 18 h at 37°C and diameter of the clear
zone were recorded after subtraction of the well diameter.

In vivo experiment
Silk sutures (Natume, No. 3 black soft type) were cut,
immersed for 10 min in phosphate-buffered saline (PBS,
130 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM
KH₂PO₄, pH7.4) containing the 9-mer peptide (2.5 mg/ml)
and air-dried. The sutures were then immersed for 10 min
in MRSA culture solution described above or in PBS and
air-dried. The sutures were epicutaneously stitched on the
back skin of hairless mice (Hos: HR-1) under anesthetic
conditions. The mice were purchased from Japan SLC Inc. The skin containing the sutures were excised after 24
and 48 h. The biopsy specimens were fixed in 20% neu-
tral buffered-formalin for 7 days. The tissues were dehy-
drated with ethanol and xylene, and embedded in paraffin.
Serial paraffin sections (4 µm thickness) were prepared
and stained with hematoxylin and eosiń for tissue stain-
ing, and with MacCallum Goodpasture stain for Gram
staining of MRSA. The sectioned samples were examined
with a light microscope (Nikon, ECLIPSE E 800). Num-
bers of the bacterial cells on the surface of sutures and 1 cm² area of skin along the suture were counted to analyze
the progression of bacterial infection. The suture in-
mersed in MRSA culture solution was suspended in PBS
for 10 min. A 1 cm² area skin along the suture was ex-
cised from each mouse. The tissue was homogenized in
PBS using glass grinder. A part of bacterial solution was
plated on MHB agar plates. The plates were incubated for
18 h at 37°C and the viable bacterial cells counted.

In vitro experiment
Fibroin solution was prepared as described previously
(Yamada et al., 2001). Briefly, silk fibers containing no
sericin were prepared from raw silk fibers by using 8 M
urea and stirred in 30-100 times (v/v) of saturated (about
9 M) aqueous LiSCN at room temperature until dissolved.
The solution was dialyzed against 100 times (v/v) water
for 2 h, while refreshing the outer solution every 30 min.
The 5% fibroin solution containing the synthetic 9-mer
peptide (100, 200 or 300 μg/cm²) was poured into plastic
culture dishes and air-dried at room temperature. The
films were cut into 1 cm². As a control, fibroin films con-
taining no peptide were also prepared. For antibacterial
activity assay of the fibroin films, the mid-exponential
phase culture of MRSA was diluted with fresh MHB to a
Inhibition of Bacterial Growth by a Synthetic Peptide

final concentration of $2 \times 10^4$ cells/ml, where 0.01 OD at 600 nm indicated ca. $2 \times 10^6$ cells/ml. One hundred µl of the diluted MRSA culture was spread on the MHB agar plate, the fibroin film placed on the plate, and the plate incubated at $37^\circ$C for 24 h.

RESULTS

Antibacterial activity of the 9-mer peptide against MRSA

Antibacterial activity of the peptide against different bacteria was assayed in the liquid medium and the minimal inhibitory concentration (MIC) determined previously (Saido-Sakanaka et al., 1999). The MIC against MRSA was 24 µg/ml. Antibacterial activity against MRSA in the solid medium, however, has not yet been examined. Thus, the antibacterial activity was measured in the solid medium by growth inhibition assay in this study. The peptide inhibited the growth of MRSA under the conditions used, when the peptide concentration was more than 20 µg but not less than 10 µg, confirming the activity against MRSA in the solid medium (Table 1).

<table>
<thead>
<tr>
<th>Peptide dose (µg)</th>
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<th>10</th>
<th>20</th>
<th>40</th>
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<tr>
<td>Inhibition zone (mm)</td>
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<td>0</td>
<td>1.5</td>
<td>5.5</td>
<td>9.5</td>
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Growth inhibition zones were recorded after subtraction of the well diameter. The solid culture medium contained $10^3$ cells/ml of MRSA.

Fig. 1. Light micrographs of biopsy specimens. Surgical silk sutures treated with or without the 9-mer peptide, ALYLAIRRR-NH₂, were used to examine effects of the peptide on the proliferation of MRSA. Panels a, c, e and g: Specimens stained with hematoxylin and eosin. Panels b, d, f and h: Specimens stained with Gram stain. Panels a and b: Sutures alone. Panels c and d: Sutures treated with the peptides. Panels e and f: Sutures treated with MRSA alone. Panels g and h: Sutures treated with peptides and then with MRSA. A scale bar inserted in each figure indicates 50 µm. Inset: Hipower magnification of filtrating cells. Most cells show continuous segmentation of the nucleus into lobes. They are typical mature neutrophils (Panels a, c, e and g). A scale bar inserted in insets indicates 10 µm.
Effect of the 9-mer peptide on the proliferation of MRSA in vivo

Minimal inhibition concentrations of the peptide for bacteria in the liquid medium were determined (Saido-Sakanaka et al., 1999). In this study, antibacterial activity of the peptide against MRSA was assayed in solid medium.

Effect of the synthetic 9-mer peptide ALYLAIRRR-NH₂ modified from the active sites of insect defensins on the proliferation of MRSA in vivo was tested using silk sutures for surgical operation. The sutures were considered to absorb the peptide by capillary action of the silk fibers. Biopsy specimens were taken from the back skin containing the sutures and examined with light microscopy. No Gram-stained MRSA was detected after 24 h around the silk fibers of the suture (Fig. 1h) as seen in the non-treated suture alone (Fig. 1b) or the suture treated with peptides alone (Fig. 1d). On the contrary, the suture treated with MRSA alone showed numerous Gram-positive loci around the silk fibers of the suture (Fig. 1f). These results indicated that the 9-mer peptide absorbed by the suture suppressed the multiplication of MRSA in vivo. The paraffin sections from the same sample were also stained by hematoxylin and eosin. Hematoxylin stains nuclei bluish purple and eosin stains cytoplasm red. Most infiltrating cells in the lesions were mature neutrophils characterized by continuous segmentation of the nucleus into lobes (Insets of Fig. 1a, c, e and g). Fig. 1e also showed the neutrophil infiltration, but their morphological characteristics were not clear because of various degree of the degeneration. Similar results were obtained after 48 h inoculation except that the suture treated with MRSA alone showed fewer bacterial loci compared with the results inoculated for 24 h. The bacterial numbers before surgical operation were ca. $2.3 \times 10^4$ cells per cm² of suture containing the peptide, whereas the suture just treated with MRSA gave ca. $1.2 \times 10^6$ cells per cm². The bacterial numbers 24 h after inoculation under the skin of the mice were ca. $7.0 \times 10^2$ cells per cm² skin along the suture containing the peptide and ca. $2.3 \times 10^4$ cells per cm² skin along the suture treated with MRSA alone.

Effect of the fibroin film containing 9-mer peptide on the proliferation of MRSA in vitro

Fibroin films containing different concentrations of the 9-mer peptides were prepared to determine whether such fibroin films as wound dressings can suppress the multiplication of MRSA in vitro. For this in vitro experiment, MRSA was seeded onto MHB agar plates, covered with fibroin films containing the peptides and incubated at 37°C for 24 h. No MRSA colonies were seen under the fibroin films containing 100, 200 or 300 μg/cm² of the peptides (Fig. 2b, c and d), whereas many colonies were detected under the film containing no peptides (Fig. 2a). Many MRSA colonies equally appeared in the area not covered by fibroin film with or without the peptides (Fig. 2a, b, c and d). These results suggest that the 9-mer peptides in the fibroin film have the potential to inhibit the prolifera-
tion of antibiotic-resistant pathogenic bacteria.

**DISCUSSION**

Our results showed that fibroin sutures treated with ALYLAIRRR-NH$_2$ can suppress the proliferation of MRSA *in vivo*. This peptide also protected mice from lethal MRSA challenges and suppressed the induction of TNF-α gene expression triggered by lipopolysaccharide or lipoteichoic acid in murine macrophages (Saido-Sakanaka *et al.*, 2005). These results suggest that the synthetic peptide has antibacterial potential not only *in vitro* but also *in vivo*. In general, the primary target of antibiotic peptides is the bacterial membrane (Ojicius and Young, 1991; Hara and Yamakawa, 1995; Saido-Sakanaka *et al.*, 1999, 2004). No conventional antibiotics have been shown to directly target the bacterial membrane as an antibacterial mechanism. Synthetic antibacterial peptides designed on the basis of antibiotic peptides have the same unique properties to inhibit bacterial proliferation (Yamada and Natori, 1994; Alvarez-Bravo *et al.*, 1994; Helmerhost *et al.*, 1997; Saido-Sakanaka *et al.*, 1999, 2004). However, Nakajima *et al.* (1997) reported that an 11-mer synthetic peptide and its D-enantiomer modified from the active site of sapecin B also showed chemotherapeutic activity in MRSA-infected mice, namely a neutrophil-stimulating activity that resulted in the production of superoxide. This was also tested with the 9-mer peptide used in this study and we found the peptide is not involved in the stimulation of superoxide production, suggesting our 9-mer peptide inhibits the proliferation of MRSA by disrupting the bacterial membrane (Saido-Sakanaka *et al.*, 2005). Light microscopic observations indicated that neutrophil leukocytes and monocytes accumulated around the fibroin fibers of the sutures regardless of the presence or absence of the 9 mer-peptide. It is conceivable that these leukocytes may recognize the sutures embedded in the back skin of mice as foreign materials.

Appearance of antibiotic-resistant bacteria, such as MRSA, is a serious problem in bacterial infection therapy. Therefore, we tested the effect of the fibroin film containing the 9-mer peptide as a novel wound dressing on the multiplication of MRSA *in vitro* based on the idea that combination of the advantages of our 9-mer peptide with the fibroin film may strengthen the function of the film to prevent bacterial infection especially from antibiotic-resistant pathogens. As the 9-mer peptide could show a clear inhibition zone against MRSA on the solid culture medium at appropriate peptide concentrations, we tested the effect of the fibroin films containing different concentration of the peptide on the proliferation of MRSA using the solid culture medium. The results revealed that no MRSA colonies appeared under the wound dressing containing more than 200 μg/cm$^2$ of the peptide. Our results indicated that the peptide can form a clear inhibition zone on the plate when the fibroin film contains 300 μg/cm$^2$ of the peptide, suggesting that the 9-mer peptides in the film may have gradually permeated into the bacterial culture and inhibited the proliferation of MRSA. Wound dressings must fulfill the following functions: (1) drainage of exudates, (2) prevention of evaporative water loss, (3) control of insensitive perspiration from the wound surface and (4) prevention of bacterial infection (Sugihara *et al.*, 2000). The fibroin film demonstrated many advantages such that it can be obtained easily, be sterilized, allows observations of wound healing, supports collagen synthesis, reduces edema and scarring due to inflammatory responses, and promotes epithelization (Sugihara *et al.*, 2000). As our 9-mer peptide suppresses TNF-α gene expression (Saido-Sakanaka *et al.*, 2005), the fibroin film containing this peptide may contribute to reducing the inflammatory responses *in vivo*. From this view point, it is important to determine whether the fibroin film containing our 9-mer peptides as a wound dressing can suppress the multiplication of MRSA or other antibiotic-resistant bacteria *in vivo* and to examine effects on the inflammatory responses *in vivo*. Furthermore, detailed analyses on the safety of this peptide as a component of the novel wound dressing should be conducted.

In conclusion, we synthesized a 9-mer peptide, ALYLAIRRR-NH$_2$, and examined its effect on MRSA proliferation *in vivo* and *in vitro*. Surgical silk sutures treated with the 9-mer peptide suppressed multiplication of MRSA in the back skin of mice. Fibroin film containing the peptide as a wound dressing also inhibited the proliferation of MRSA *in vitro*. These results suggest this 9-mer peptide is a good candidate as a lead peptide for development of novel peptide antibiotics for the external therapy of infectious diseases from antibiotic-resistant bacteria.

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