Prophylactic Efficacy of Topical Temporin A and RNAIII-Inhibiting Peptide in a Subcutaneous Rat Pouch Model of Graft Infection Attributable to Staphylococci With Intermediate Resistance to Glycopeptides

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Background—Bacteria that adhere to implanted medical devices play an important role in industry and in modern medicine. Staphylococci are among the most common pathogens that cause biomaterial infections. Vascular prosthetic graft infection is one of the most feared complications that the vascular surgeon treats, frequently resulting in prolonged hospitalization, organ failure, amputation, and death. A rat model was used to investigate the topical efficacies of temporin A and the quorum-sensing inhibitor RNAIII-inhibiting protein (RIP) as prophylactic agents of vascular prosthetic graft infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* with intermediate resistance to glycopeptides.

Methods and Results—Graft infections were established in the back subcutaneous tissue of adult male Wistar rats by implantation of Dacron prostheses 1 cm² followed by topical inoculation with 2×10⁷ colony-forming units of bacterial strains. The study included, for each staphylococcal strain, a control group (no graft contamination), a contaminated group that did not receive antibiotic prophylaxis, and 6 contaminated groups that received grafts soaked with temporin A, RIP, rifampin, temporin A plus RIP, RIP plus rifampin, or temporin A plus RIP. The infection was evaluated by quantitative agar culture. When tested alone, temporin A and RIP showed comparable efficacies, and their efficacies were significantly higher than that of rifampin against both strains. All combinations showed efficacies significantly higher than that of each single compound. The combinations of temporin A and RIP exerted the strongest antistaphylococcal efficacies, eliminating infection by 100%.

Conclusions—The results of the present study make these molecules potentially useful for antimicrobial chemoprophylaxis in vascular surgery. (Circulation. 2003;108:767-771.)

Key Words: vasculature • grafting • infection • peptides

Medical devices used in the cardiovascular system as prosthetic heart valves or central venous catheters are subject to risk of microbial infection.¹,² Surgical implantation of the device leads to tissue damage and inflammation, with increased susceptibility of microbial colonization in sites of injury. Infection can lead to prosthetic valve endocarditis, with rates ranging between 0.5% and 4%,³,⁴ whereas in the case of central venous catheter implantation, the risk of infection is greater than in any other indwelling medical devices, rates of infection reach 3% to 5%.⁵ Initial colonization is followed by development of a biofilm structure, where the microorganisms are encased in a polysaccharide matrix. This matrix protects microorganisms from the attack of antimicrobial therapy and from the immune system; therefore, biofilm-based infections are rarely resolved.¹,⁶,⁷ Staphylococci on prosthetic valves and central venous catheters are most often found in biofilms, especially *Staphylococcus epidermidis* and *Staphylococcus aureus*. During the first year of postvalve replacement, staphylococci in biofilms are responsible for 65% of postwound infections.⁸–¹⁰ Antibiotic therapy and prophylaxis to fight bacterial biofilm often fail to eradicate the infection because of the capacity of the bacteria encased into the biofilm matrix to be more resistant to treatment compared with planktonic bacteria.

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heads and hydrocarbon tails of bacterial phospholipids, binding to DNA, or altering enzyme activities. However, the precise mechanisms remain incompletely understood. In this study, we investigated the in vivo efficacy of temporin A and RIP in preventing S. epidermidis and S. aureus graft infection in a rat model previously described. Peptides were spontaneously bound to the graft, and efficacy was compared with that of rifampin, a commonly used antibiotic in graft precoating.

Methods

Organisms

The strains of S. epidermidis and S. aureus with intermediate resistance to vancomycin were isolated from clinical specimens submitted for routine bacteriological investigation to the Institute of Infectious Diseases and Public Health, University of Ancona, Italy. These isolates were described using the acronyms VISE (vancomycin-intermediate S. epidermidis) and VISA (vancomycin-intermediate S. aureus).

Drugs

Vancomycin and rifampin were obtained from Sigma-Aldrich S.r.l. Powders were dissolved in accordance with the manufacturer’s recommendations. Solutions were made fresh on the day of assay or stored at –80°C in the dark for short periods. The concentration range assayed for each antibiotic was 0.25 to 256 μg/mL.

Synthetic Peptides

Temporin A was synthesized manually by the solid-phase method using Fmoc/Br procedure (Faculty of Pharmacy, Medical University of Gdańsk) and was purified by reverse-phase (Vydac C-18, 10×250 mm) high-pressure liquid chromatography (HPLC) on a Knauer K501 2-pump system. The product was analyzed by HPLC, chemical analysis, matrix-assisted laser-desorption ionization mass spectrometry. Temporin A was dissolved in distilled H2O at 20 times the required maximal concentration. Successively, serial dilutions of the peptide were prepared in 0.01% acetic acid containing 0.2% BSA in polypropylene tubes.

The amide form of RIP (YSPWTNF-NH2) and FITC-labeled RIP [Cys(S, Fluorescein)-YSPWTNF-NH2] were synthesized by Neo-system as previously described and purified by HPLC to 99%. RIP was dissolved in distilled H2O at 20 times the required maximal concentration. Solutions of RIP and temporin A were made fresh on the day of assay or stored at –80°C in the dark for short periods.

Binding of RIP and Temporin A to Dacron

To determine how much RIP impregnates Dacron, FITC-RIP (10 mg/L) was applied to 1 cm² sterile collagen-sealed Dacron graft (Allograft, Sorin Biomedica Cardio, S.p.A) for 20 minutes at room temperature. Fluorescence in unbound solution was determined at OD 485/530 nm in a Microplate Fluorescence Reader (F, FL 600, Bio-Tek) using KC4 software. The binding level of temporin A to Dacron was estimated using UV spectroscopy. Collagen-sealed Dacron graft 1 cm² (Allograft, Sorin Biomedica Cardio, S.p.A.) was first washed with distilled water for 10 minutes. Next, Dacron was allowed to be impregnated by Fmoc-temporin A 10 mg/L) for 20 minutes at room temperature. Absorption spectra of Fmoc-temporin A was measured at A 266 nm with UV-vis spectrometer (Lambda 40P, Perkin-Elmer) either before or after impregnation.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities of the strains to vancomycin, rifampin, RIP, and temporin A were determined by the broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS). The MIC was taken as the lowest antibiotic concentration at which observable growth was inhibited. In addition, the strains were tested for susceptibility to

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vancomycin by the NCCLS reference disk diffusion method by using 30-μg vancomycin disks.44 Experiments were performed in triplicate.

Rat Model
Adult male Wistar rats (weight range, 250 to 300 g; Instituto Nazionale Riposo e Cura Anziani Instituto di Ricovero e Cura a Carattere Scientifico [INRCA IRRCS] animal facility, Ancona, Italy) were studied. The study included, for each staphylococcal strain, a control group (no graft contamination), a contaminated group that did not receive any antibiotic prophylaxis, and 6 treated groups (VISE1-6 and VISA1-6) that received grafts soaked with RIP, temporin A, rifampin, temporin A plus RIP, RIP plus rifampin, and temporin A plus RIP, respectively. Each group contained 15 animals. Rats were anesthetized with ether, the hair on the back was shaved, and the skin was cleansed with 10% povidone-iodine solution. One subcutaneous pocket was made on each side of the median line by a 1.5-cm incision. Aseptically, 1-cm² sterile collagen-sealed double velour knitted polyethylene terephthalate (Dacron) grafts (Albograft, Sorin Biomedica Cardio) were implanted into the pores. Before implantation, the Dacron graft segments were impregnated with 10 mg/L temporin A, RIP, and rifampin, alone or combined. Binding of the compounds was obtained immediately before implantation by soaking grafts for 30 minutes in a sterile solution of the above-mentioned agent. The pockets were closed by means of skin clips, and physiological solution (1 mL) containing the VISE and VISA strains at a concentration of 2 × 10⁸ colony-forming units (CFU) per mL was inoculated onto the graft surface using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals were returned to individual cages and thoroughly examined daily. Based on experiments demonstrating peak bacterial growth and biofilm formation within 72 hours (data not shown), all grafts were explanted at 7 days after implantation. This study was approved by the Animal Research Ethics Committee of the INRCA IRRCS, A. I. University of Ancona.

Assessment of the Infection
The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 mL PBS solution, and sonicated for 5 minutes to remove the adherent bacteria. Quantitation of viable bacteria was obtained by performing serial dilutions (0.1 mL) of the bacterial suspension in 10 mmol/L of sodium HEPES buffer (pH 7.2) (Sigma-Aldrich) to minimize the carryover effect and by culturing each dilution on blood agar plates. All plates were incubated at 37°C for 48 hours and evaluated for the presence of the staphylococcal strains. The organisms were quantified by counting the number of CFUs per plate. The limit of detection for this method was approximately 10 CFU/cm².

Statistical Analysis
MIC values are presented as the geometric mean of 3 separate experiments. Quantitative culture results from all groups are presented as the geometric mean of 3 separate experiments. The results and the statistical comparisons between groups are summarized in the Table. Statistical analysis was performed using ANOVA on the log-transformed data with Tukey-Kramer honestly significant difference test. Significance was accepted when P ≤ 0.05.

Results

Binding of RIP and Temporin A to Dacron
To determine how much RIP and temporin A bound to the Dacron graft, the graft was soaked in FITC-RIP or Fmoc-temporin A and absorbance was determined. These experiments show that when 1 cm² Dacron was soaked in 10 mg/L RIP or 10 mg/L temporin A solutions, 26 μg of RIP and 37 μg of temporin A bound to it.

In Vitro Data
According to the broth microdilution method recommended by the NCCLS, the isolate confirmed its intermediate resistance to vancomycin. Vancomycin exhibited MICs of 8 μg/mL and zone sizes of 11 mm by the disk diffusion test. Both Temporin A and rifampin showed MICs of 8 mg/L for both strains, whereas RIP did not demonstrate any in vitro activities against the 2 strains (MICs ≥ 128 mg/L), as expected considering its mechanism of action.

In Vivo Data
Our results indicate that none of the animals included in the negative control group (no graft contamination) had anatomic and microbiological evidence of graft infection. On the other hand, all 30 rats included in the contaminated controls (VISE and VISA) demonstrated evidence of graft infection, with quantitative culture results showing 4.3 × 10⁷ ± 16.8 × 10⁶ CFU/cm² graft and 6.0 × 10⁷ ± 2.4 × 10⁷ CFU/cm² graft, respectively, although there were no local signs of perigraft inflammation.

Among groups treated with a single agent, the RIP-treated groups (VISE1 and VISA1) showed the lowest bacterial growth 4.9 × 10⁸ ± 1.1 × 10⁸ CFU/cm² graft and 7.5 × 10⁶ ± 2.9 × 10⁶ CFU/cm² graft, respectively. Otherwise, the groups treated with local temporin A (VISE2 and VISA2) and local rifampin (VISE3 and VISA3) demonstrated bacterial growth of 5.3 × 10⁷ ± 7.7 × 10⁶ CFU/cm² graft, 4.5 × 10⁷ ± 8.1 × 10⁶ CFU/cm² graft, 5.0 × 10⁷ ± 8.8 × 10⁷ CFU/cm² graft, and 7.3 × 10⁷ ± 2.4 × 10⁷ CFU/cm² graft, respectively.

Finally, the RIP and temporin A combination showed the lowest bacterial growth with no evidence of staphylococcal infection and negative quantitative cultures for group VISE4 and 6.9 × 10⁷ ± 1.3 × 10⁸ CFU/cm² graft for group VISA4, whereas the other combinations showed bacterial numbers within 10⁷ and 10⁸ CFU/cm² graft (Table).

In the results from quantitative bacterial graft cultures, there were always significant differences when the data obtained from treated groups were compared with those obtained from the untreated groups with the exception of rifampin-treated groups. Interestingly, for the S. epidermidis series, combination between RIP and temporin A was shown to be statistically significant compared with any other treatment (P ≤ 0.05). Similar statistical results were obtained in the S. aureus series. The results and the statistical comparisons between groups are summarized in the Table.

All agents did not show any toxicity, and none of the animals included in any group died or had clinical evidence of drug-related adverse effects.

Discussion
The difficulty in eradicating a vascular chronic infection related to microcolony and biofilm formation relies on the fact that bacteria in a biofilm are able to resist higher antibiotic concentrations than bacteria in suspension.35 Moreover, it was recently shown that biofilm formation can be considered a multicellular developmental process regulated by the exchange of chemical signals between cells.20,36 The recognition of the presence of a cell to cell communication
Efficacy of RIP, Temporin A, and Rifampin Against Staphylococcus epidermidis and Staphylococcus aureus With Intermediate Resistance to Glycopeptide Strains Causing Graft Infection in a Rat Model

<table>
<thead>
<tr>
<th>Group</th>
<th>Graft-Bonded Drug†</th>
<th>Quantitative Graft Culture, CFU/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncontaminated control</td>
<td>...</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Untreated control VISE</td>
<td>...</td>
<td>4.3×10³±6.8×10⁴</td>
</tr>
<tr>
<td>VISE1‡</td>
<td>RIP</td>
<td>4.9×10⁵±1.1×10⁶</td>
</tr>
<tr>
<td>VISE2‡</td>
<td>Temporin A</td>
<td>5.3×10⁵±7.7×10⁵</td>
</tr>
<tr>
<td>VISE3</td>
<td>Rifampin</td>
<td>5.0×10⁶±8.8×10⁶</td>
</tr>
<tr>
<td>VISE4‡§</td>
<td>RIP plus temporin A</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VISE5‡</td>
<td>RIP plus rifampin</td>
<td>2.2×10⁵±0.8×10⁵</td>
</tr>
<tr>
<td>VISE6‡</td>
<td>Temporin A plus rifampin</td>
<td>8.9×10⁶±2.5×10⁵</td>
</tr>
<tr>
<td>Uncontaminated control</td>
<td>...</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Untreated control VISA</td>
<td>...</td>
<td>6.0×10³±2.4×10⁵</td>
</tr>
<tr>
<td>VISA1‡</td>
<td>RIP</td>
<td>7.5×10⁵±2.9×10⁵</td>
</tr>
<tr>
<td>VISA2‡</td>
<td>Temporin A</td>
<td>4.5×10⁶±8.1×10⁵</td>
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<tr>
<td>VISA3</td>
<td>Rifampin</td>
<td>7.3×10⁶±2.4×10⁶</td>
</tr>
<tr>
<td>VISA4</td>
<td></td>
<td>‡</td>
</tr>
<tr>
<td>VISA5‡</td>
<td>RIP plus rifampin</td>
<td>5.6×10⁵±1.7×10⁵</td>
</tr>
<tr>
<td>VISA6‡</td>
<td>Temporin A plus rifampin</td>
<td>3.2×10⁵±1.1×10⁵</td>
</tr>
</tbody>
</table>

VISE indicates Staphylococcus epidermidis with intermediate resistance to glycopeptides; VISA, Staphylococcus aureus with intermediate resistance to glycopeptides.

†Dacron graft segments were impregnated with 10 mg/L of RIP, temporin A, or rifampin.
‡Statistically significant when compared with control groups VISE and VISA.
§Statistically significant when compared with all VISE groups.
||Statistically significant when compared with all VISA groups, with the exception of VISA5.

will allow new approaches for prevention and treatment of the persistent infections stemming from biofilm.

With the aim of determining the antibiotic efficacies on biofilm bacteria, studies were carried out involving in vivo formation of microcolonies and biofilms, but most or all the antibiotics used were only partially effective.6,8,10 Furthermore, the recent emergence of multidrug resistance in coagulase-negative staphylococci heightens concerns and need of new prophylactic and therapeutic strategies. Since the emergence of methicillin-resistant staphylococci, glycopeptides were often the only effective drugs. For this reason, the emergence of staphylococcal strains exhibiting reduced sensitivity to vancomycin is of particular concern.15,37,38

In these experiments we used clinical isolates of vancomycin-resistant strains because they represent the most recent pattern of resistance that can be observed in the clinical practice and are more challenging to cure with classical antibiotics.12

One way to overcome the problems of emergence of resistance is the use of new antimicrobial compounds or combination therapy. Although several agents have been subjected to chemical manipulations to yield increased resistance to enzymatic inactivation, antibiotic-resistant mutants have usually emerged quickly. Recently, polycationic peptides, crucial components of the innate immunity of both invertebrates and vertebrates and conserved theme in host antimicrobial defenses, have been proposed as a new class of antimicrobial agents. Among these peptides and their potential role in stimulating the defense of the host, defensins59,40 and temporins have been suggested as a new alternative to classical antibiotics, and some of them are quickly moving toward clinical applications.41,42 Temporins are among the smallest antimicrobial peptides so far described. They are all amidated at the C-terminus; those containing one basic residue, either lysine or arginine, in the sequence (net charge +2) were found to be active against Gram-positive pathogens, specifically staphylococci.59,39 Previous studies showed the efficacy of temporin A to provide prophylaxis against methicillin-susceptible and methicillin-resistant S. epidermidis graft contamination. In fact, it was demonstrated that prophylaxis based on temporin A–soaked grafts was as effective as parenteral vancomycin.42

Other prophylactic and therapeutic strategies could be based on compounds such as RIP, able to interfere with cell to cell communication, required for biofilm formation, and able to reduce bacterial adherence and the contact area each bacterium makes with the plastic surface.20–25 RIP is a quorum-sensing inhibitor of staphylococci that can adsorb to surfaces and act against bacterial biofilm on several synthetic polymers. For these reasons, in this study both the antistaphylococcal activity of temporin A and the disruption of cell to cell communication by RIP absorbed onto Dacron graft were tested.

For testing, we used a topical treatment model for prophylaxis instead of a more common systemic treatment. We believe that treatment in situ if successful will limit the systemic side effect of drugs given parenterally and allow a lower dosage. Indeed, we show that although a limited amount of the drugs bind to the graft, efficacy is maintained. Although we believe that the drugs will eventually be washed off the graft, their binding and their efficacy should be long enough to prevent the initial bacterial infection that could arise mostly during the surgical procedure. Additional experiments will be needed to establish in vivo duration of drug binding and efficacy and protection from secondary infections.

Our data show that the use of temporin A–soaked and RIP-soaked Dacron graft can result in significant bacterial growth inhibition even if high concentrations of organisms are topically inoculated on the Dacron prostheses. In particular, the results of the present study, if compared with those obtained in our recent report, point out the high antistaphylococcal activity of temporin A independently of the level of resistance shown by the isolates.42 On the other hand, RIP was shown to be highly effective also when tested alone and was more effective against both staphylococcal strains than either temporin A or rifampin alone.

In conclusion, we have shown in this paper that the use of novel drugs that do not belong to the classic antibiotics but that are derived either from the host or the bacteria itself prove to be very effective and even superior to antibiotics in inhibiting biofilm formation induced by drug-resistant staphylococci. The administration of a parenteral peptide such as
temporin A and topical RIP may become an important future consideration for chemoprophylaxis in vascular surgery.

References

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