Temporin A as a prophylactic agent against methicillin sodium–susceptible and methicillin sodium–resistant *Staphylococcus epidermidis* vascular graft infection

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Objective: **The purpose of this study was to investigate the efficacy of temporin A as a prophylactic agent in a rat model of vascular graft infection from methicillin sodium–susceptible and methicillin sodium–resistant *Staphylococcus epidermidis*.**
Methods: **The prospective, randomized, controlled animal study set in a research laboratory in a university hospital used 280 adult male Wistar rats (weight range, 280 to 350 g). Graft infections were established in the back subcutaneous tissue of rats with implantation of 1-cm^2 sterile Dacron grafts followed by topical inoculation with 2×10^7 colony-forming units of *S epidermidis*. The study for each staphylococcal strain included: one control group (no graft contamination), one contaminated group that did not receive any antibiotic prophylaxis, one contaminated group that received temporin A–soaked graft, two contaminated groups that received perioperative intraperitoneal cefazolin (30 mg/kg) or vancomycin hydrochloride prophylaxis (10 mg/kg), and two contaminated groups that received temporin A–soaked graft and perioperative intraperitoneal cefazolin (30 mg/kg) or vancomycin hydrochloride (10 mg/kg) prophylaxis. All grafts were explanted at 7 days after implantation. The main outcome measure was quantification of bacterial contamination.**
Results: **Overall, the perioperative prophylaxis based on soaked grafts was not significantly different to that of parenteral vancomycin hydrochloride. Only the combination between temporin A and vancomycin hydrochloride produced a complete bacterial inhibition for both strains.**
Conclusion: **Temporin A showed a similar antibacterial in vitro activity against the two different strains. The in vivo results suggest its potential use in providing prophylaxis to direct graft contamination when used in combination with parenteral vancomycin hydrochloride. (J Vasc Surg 2002;36:1027-30.)**

Staphylococci are among the most common pathogens that cause biomaterial infections. The patient can have late-appearing signs of infection develop as commonly as early postoperative infection, frequently resulting in prolonged hospitalization, organ failure, amputation, and death.[1,2] Vascular prosthetic graft infection is one of the most feared complications that the vascular surgeon treats, and *Staphylococcus epidermidis*, a skin commensal, is well known to be the most frequent cause of vascular graft infection in humans.[3,4] The mainstay of prophylaxis is the perioperative administration of systemic antibiotics. Since the emergence of methicillin sodium–resistant (MR) staphylococci, several protocols for the prevention of prosthetic infection have been introduced in different devices. For vascular grafts, antimicrobials bound in high concentrations to prosthetic grafts have been proposed as adjunctive prophylaxis.[5-9]

In recent years, several polycationic antimicrobial peptides, compounds that play an important role in innate immunity, have been isolated from bacteria, plants, insects, fish, amphibians, birds, mammals, and humans.[10] Particularly, amphibian skin has proven to be an especially rich source of peptides. Several molecules from amphibian species have been analyzed in detail and found to have antimicrobial activity against a broad spectrum of bacteria.[11-13] Among these molecules, temporins, a group of peptides isolated from the skin of the European red frog *Rana temporaria*, showed activity against clinically important gram-positive cocci, including MR staphylococci and vancomycin hydrochloride–resistant *Enterococcus faecium*. 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 specifically against gram-positive bacteria and *Candida albicans*. They are among the smallest antimicrobial peptides.[14-16] Temporin A is a basic, highly hydrophobic, antimicrobial peptide amide (FLPLIGRVLSGIL-NH^2) that has variable antibiotic activity against a broad spectrum of microorganisms. Like the other temporins, it is active against clinically important antibiotic–resistant gram-positive cocci. Currently, the following different hypotheses concern the mechanism of action by which temporins kill organisms: inserting into the hydrophobic core of the cell membrane, interaction with

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Competition of interest: nil.

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Published online Sep 9, 2002.

0741-5214/2002/$35.00 + 0 **24/1/127530**
doi:10.1067/mva.2002.127530

anionic heads and hydrocarbon tails of bacterial phospholipids, binding to DNA, or altering enzyme activities.[17,18] In this study, we investigated the efficacy of temporin A as a prophylactic agent in a rat model of vascular graft methicillin sodium–susceptible (MS) and MR *S epidermidis* infection.

MATERIALS AND METHODS

Organisms. The commercially available quality control strain of MS *S epidermidis* ATCC 12228 (Oxoid SPA, Milan, Italy) and one clinical isolate of MR *S epidermidis* (Se15-00) were used.

Synthetic peptides. Temporin A was synthesized manually with the solid phase method with the Fmoc/Bu^t procedure (Faculty of Pharmacy, Medical University of Gdańsk, Poland) and was purified with reversed-phase (Vydac C-18; 10 × 250 mm) high-pressure liquid chromatography on a Knauer K501 two-pump system (Berlin, Germany). The product was analyzed with high-pressure liquid chromatography, chemical analysis, and matrix-assisted laser-desorption ionization mass spectrometry. Temporin A was dissolved in distilled H_2O at 20 times the required maximal concentration. Successively, serial dilutions of the peptide were prepared in 0.01% acetic acid containing 0.2% bovine serum albumin in polypropylene tubes.

Drugs. Cefazolin, vancomycin hydrochloride and oxacillin sodium were obtained from Sigma-Aldrich S.r.l. (Milan, Italy). Powders were dissolved in accordance with manufacturers' 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.

Susceptibility testing. The antimicrobial susceptibilities of the strains were determined with the microbroth dilution method, according to the procedures outlined by the National Committee for Clinical Laboratory Standards.[19] The minimum inhibitory concentration (MIC) was taken as the lowest antibiotic concentration at which observable growth was inhibited. However, the MIC of temporin A was determined according to the procedures recently proposed for testing antimicrobial peptides.[20] Particularly, because cationic peptides bind polystyrene, polypropylene 96-well plates (Sigma-Aldrich) were substituted for polystyrene plates and incubated for 18 hours at 37° C in air. Plates were shaken throughout the study. Experiments were performed in triplicate.

Rat model. Adult male Wistar rats (weight range, 280 to 350 g) were studied. Graft infections were established in the back subcutaneous tissue of rats with implantation of 1-cm^2 sterile Dacron grafts (Saluggi VC, Italy) followed by topical inoculation with 2×10^7 colony-forming units (CFUs) of *S epidermidis*. The study for each staphylococcal strain included: one control group with no graft contamination and no antibiotic prophylaxis (uncontaminated control; C_0), one contaminated group that did not receive any antibiotic prophylaxis (treated control; C_1), one contaminated group that received temporin A–soaked graft (T_T); two contaminated groups that received perioperative intraperitoneal 30 mg/kg cefazolin (T_C) or 10 mg/kg vancomycin hydrochloride (T_V) prophylaxis, and two contaminated groups that received temporin A–soaked graft and perioperative intraperitoneal 30 mg/kg cefazolin ($T_{T\text{-}C}$) or 10 mg/kg vancomycin hydrochloride ($T_{T\text{-}V}$) prophylaxis. Each group was formed by 20 animals. Rats underwent anesthesia with ether, the hair of the back was shaved, and the skin was cleaned with 10% povidone-iodine solution. The groups T_C, T_V, $T_{T\text{-}C}$, and $T_{T\text{-}V}$ received intraperitoneal antibiotic prophylaxis 10 minutes before the surgical procedure. One subcutaneous pocket was made on each side of the median line with a 1.5-cm incision. Aseptically, 1-cm^2 sterile collagen-sealed Dacron grafts (Albograft, Sorin Biomedica Cardio, S.p.A., Saluggi VC, Italy) were implanted into each pocket. Before implantation, the Dacron graft segments were impregnated with 10-μg/mL temporin A (groups T_T, $T_{T\text{-}C}$, and $T_{T\text{-}V}$). Bonding of the peptide was obtained immediately before implantation with soaking grafts for 20 minutes in a sterile solution of the compound. The pockets were closed with skin clips, and sterile saline solution (1 mL) containing *S epidermidis* ATCC 12228 or the Se15-00 strain at a concentration of 2×10^7 CFU/mL was inoculated onto the graft surface with a tuberculin syringe to create a subcutaneous fluid-filled pocket.[21] The animals were returned to individual cages and thoroughly examined daily. All grafts were explanted at 7 days after implantation.

Assessment of the infection. The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 mL of phosphate-buffered saline solution, and sonicated for 5 minutes to remove the adherent bacteria from the grafts. Quantification of viable bacteria was performed with culturing serial 10-fold dilutions (0.1 mL) of the bacterial suspensions 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 with counting the number of CFUs per plate. The limit of detection for this method was approximately 10 CFU/mL.

Statistical analysis. MIC values were presented as the geometric mean of three separate experiments. Quantitative culture results regarding the in vivo studies were presented as the mean ± the standard deviation of the mean; comparisons of the results were performed with analysis of variance on the log-transformed data with Tukey-Kramer significant difference test. As specified previously, to strengthen the statistical analysis, each animal received two graft specimens, so that 40 graft specimens were analyzed from each group. Significance was accepted when the *P* value was .05 or less.

RESULTS

In vitro studies

According to the broth-microdilution method, vancomycin hydrochloride showed the greatest potency against the strains tested, with MICs of 0.25 mg/L and 0.50 mg/L for *S epidermidis* ATCC 12228 and *S epidermidis* Se15-00, respectively. Interestingly, the two organisms were similarly susceptible to temporin A that showed MICs of 8 mg/L for both strains. Finally, the two strains showed different susceptibility patterns for the betalactam antibiot-

ics: *S epidermidis* ATCC 12228 was susceptible to oxacillin sodium and cefazolin (MIC values, 0.5 and 2 mg/L, respectively), and *S epidermidis* Se15-00 was resistant (MIC values, 8 and 32 mg/L, respectively).

In vivo studies

***S epidermidis* ATCC 12228.** None of the animals included in the control group C_0 had microbiologic evidence of graft infection. On the contrary, all rats included in the untreated control C_1 showed evidence of graft infection, with quantitative culture results showing $1.9 \times 10^7 \pm 5.1 \times 10^6$ CFU/cm^2 graft.

Culture from the group that received temporin A–soaked graft (T_T) showed $3.4 \times 10^3 \pm 7.1 \times 10^2$ CFU/cm^2 graft, and similar results were obtained from the groups that received intraperitoneal cefazolin (T_C) and vancomycin hydrochloride (T_V) prophylaxis ($5.9 \times 10^3 \pm 4.7 \times 10^2$ CFU/cm^2 graft, and $4.4 \times 10^2 \pm 2.0 \times 10^2$ CFU/cm^2 graft, respectively). For the group that received temporin A–soaked graft associate to intraperitoneal cefazolin (T_{T-C}) the quantitative graft cultures showed weak bacterial growth ($2.5 \times 10^1 \pm 0.7 \times 10^1$ CFU/cm^2 graft, respectively). Finally, the group that received temporin A–soaked graft associate to perioperative intraperitoneal vancomycin hydrochloride (T_{T-V}) showed no evidence of staphylococcal infection, with negative quantitative cultures (Fig 1).

***S epidermidis* Se15-00.** Similar to the series concerning MS strain, none of the animals included in the control group C_0 had microbiologic evidence of graft infection. The rats included in the untreated control C_1 showed evidence of graft infection with quantitative culture of $3.9 \times 10^7 \pm 1.2 \times 10^7$ CFU/cm^2 graft. Culture from the group that received temporin A–soaked graft (T_T) and intraperitoneal vancomycin hydrochloride (T_V) prophylaxis showed significant results if compared with C_1 ($6.1 \times 10^3 \pm 2.2 \times 10^2$ CFU/cm^2 graft, and $5.9 \times 10^2 \pm 2.3 \times 10^2$ CFU/cm^2 graft, respectively), and the group that received intraperitoneal cefazolin prophylaxis (T_C) showed no statistically significant difference versus C_1 with quantitative culture of $6.7 \times 10^7 \pm 7.9 \times 10^6$ CFU/cm^2 graft. For the group that received combined treatment, only the group T_{T-V} showed no evidence of staphylococcal growth, and quantitative cultures from group T_{T-C} showed bacterial growth ($3.0 \times 10^3 \pm 0.7 \times 10^3$ CFU/cm^2 graft) that was significantly lower when compared with C_1 (Fig 2).

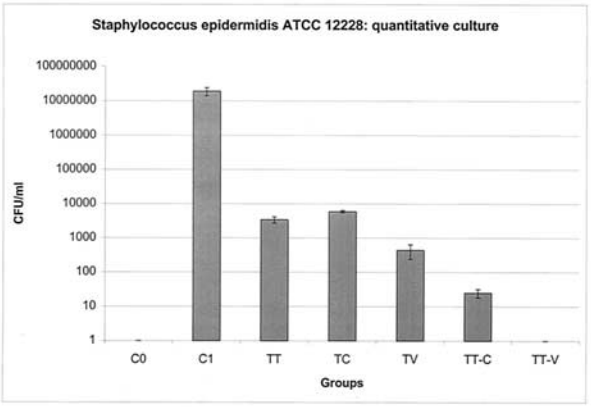

Fig 1. MS *S epidermidis* ATCC 12228: Quantitative microbiologic results of in vivo experiments.

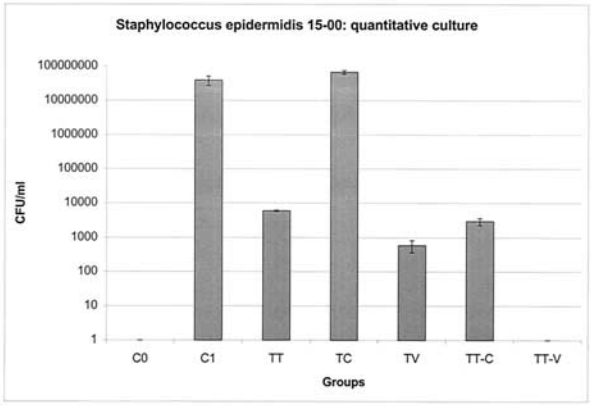

Fig 2. MR *S epidermidis* 15-00: Quantitative microbiologic results of in vivo experiments.

DISCUSSION

Prosthetic vascular grafts are foreign bodies that can harbor bacteria within their interstices. Most graft infections are thought to arise with contamination of the prostheses at the time of insertion. Contamination may occur because of inadequate sterilization or breakdown in the sterilization technique.[1] In the clinical practice, strategies for the prevention of infections are considered with respect to the periods before, during, and after operation. Parenteral antibiotic prophylaxis has been shown to reduce the incidence of wound and graft infection. Nevertheless, during the last decades, resistance among clinical isolates against currently available antimicrobial agents has emerged at an alarming rate.[22-24] The emergence of the resistant organisms has stimulated the search for new bactericidal agents, naturally occurring antimicrobial molecules that may have clinial utility. The temporins are a group of small, basic, and hydrophobic peptides that have been isolated from the skin of the European red frog. They show activity against both gram-positive and gram–negative bacteria and are found to be nontoxic to human red blood cells.[14-16,25] Particularly, our results show that the activity of temporin A appears similar against both the MS and the MR strain, which suggests that methicillin sodium susceptibility is not related to the mechanism of action of this peptide. Actually, although methicillin sodium, similar to all betalactams, inhibits the bacterial peptidoglycan synthesis, temporin A is thought to act with the formation of ion channel or pores in the bacterial cytoplasmic membrane.[17] The essential property of temporins is their net positive charge at neutral pH (usually +1 or +2) by virtue of their possession of the basic aminoacids arginine or lysine. The antibiotic activity of the temporins is determined by their mode of interaction with the bacterial

surface.[14,25] Nevertheless, although the activity against gram-negative rods is probably from their positively charged residues that interact with the negatively charged lipids of the outer bacterial membrane before damaging the function of the cytoplasmic membrane, there is still little information about the initial events that could explain the activity of cationic compounds against gram-positive organisms. However, the site for the lethal action of these molecules is the cytoplasmic membrane, where the association of several molecules would form water-filled pores that would serve as ion-conducting, anion-selective channels.[14-16,25] In addition, recent reports have shown that the peptides may act by triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of the peptidoglycan and lysis of the cell.[26-28] Finally, it has been suggested that the lytic activity of temporins could be not affected by the membrane composition of the target organism, differing from other antimicrobial peptides. This lack of selectivity of temporins may be related to the low number of positive charges; binding to the outer membrane could be the result of hydrophobic interaction.

Taken together, the results of this study showed that the use of temporin A–soaked Dacron graft in vascular surgery can result in significant bacterial growth inhibition even if high concentrations of organisms are topically inoculated on the Dacron prostheses. Statistical analysis showed that most of the antibiotic prophylactic treatments were useful; nevertheless, only the combination between temporin A–soaked graft and intraperitoneal vancomycin hydrochloride was able to completely inhibit the bacterial growth for both staphylococcal strains.

It is notable that the surface of several synthetic materials used by surgeons, such as polyesters, are negatively charged at pH 7 and that this property permits binding of cationic molecules.[29-31] In virtue of this binding, the retention of the biologically active molecules is not from passive entrapment in the plastic tissue but reflects an ionic interaction between the anionic ligands and the cationic compounds that allow a lasting antimicrobial effect.

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Submitted Mar 21, 2002; accepted May 28, 2002.