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RNAIII-inhibiting Peptide and/or Nisin Inhibit Experimental Vascular Graft Infection with Methicillin-susceptible and Methicillin-resistant *Staphylococcus epidermidis*

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Objective. *To investigate the efficacy of RNAIII-inhibiting peptide (RIP) and nisin as prophylactic agents in a rat model of vascular graft infection.*
Design. *Prospective, randomized, controlled animal study.*
Materials. *Two hundred and twenty adult male Wistar rats.* Staphylococcus epidermidis *ATCC 12228 and one clinical isolate of methicillin-resistant* S. epidermidis. *Drugs: RIP, nisin and rifampin.*
Methods. *Graft infections were established in the dorsal subcutaneous tissue by implantation of 1 cm^2 sterile Dacron grafts, followed by topical bacterial inoculation: grafts were retrieved at 7 days. The study included a control group (without inoculation) and two series composed of five groups for each staphylococcal strain: one contaminated group that did not receive any antibiotic prophylaxis, three contaminated groups that received grafts soaked with 10 mg/l RIP, 10 mg/l nisin, 10 mg/l rifampin, or RIP + nisin. The main outcome measure was the extent of bacterial at graft harvest.*
Results. *The bacterial counts for methicillin-resistant* S. epidermidis *on explanted grafts were $6.1 \pm 2.8 \times 10^2$, $7.8 \pm 3.0 \times 10^3$ and $5.5 \pm 2.9 \times 10^4$ for RIP, nisin and rifampin, respectively. RIP and nisin used in combination reduced the bacterial count to <10. The results for* S. epidermidis *were similar.*
Conclusions. *RIP and nisin could be used in combination to coat medical devices to prevent drug resistant* S. epidermidis *infections.*

Key Words: Surgical prophylaxis; Antibiotic prophylaxis; Vascular graft; Cationic peptides; RIP.

Introduction

Bacteria that adhere to implanted medical devices play an important role in industry and in modern medicine. Since adherent microrganisms can become the cause of persistent infections, it is an important problem that has been the subject of numerous investigations.[1–3] The bacteria encase themselves in a hydrated matrix of polysaccharide and proteins, forming a slimy layer known as a biofilm. Prevention of biofilm formation is a necessary step in the successful prophylaxis of medical device infections.[1,3,4] Vascular prosthetic graft infection is one of the most feared complications that frequently results in prolonged hospitalization, organ failure, amputation, and death.[5,6]

Coagulase-negative staphylococci are among the most common pathogens that cause biomaterial-associated infections. *Staphylococcus epidermidis*, a skin commensal, is the most frequent cause of late-appearing vascular graft infection in humans.[4,6,7]

Biofilm-based infections are rarely resolved. Antibiotics may suppress symptoms of infection by killing free-floating bacteria, but fail to eradicate bacterial cells still embedded in the biofilm.[3] In addition, tissue adjacent to the biofilm can undergo collateral damage by immune complexes and neutrophils. For this reason, the centerpiece of the antimicrobial strategy remains prevention by using systemic and topical antibiotics.[1–4,8,9] Nevertheless, the development of microorganism resistance to several antimicrobial agents emphasizes the need to develop new drugs to prevent graft-associated infections.[10–13] Consequently, several extended-spectrum or selective antimicrobial agents are under investigation as new approaches in the prevention of vascular prosthesis infections.

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In this study, we tested the efficacy of two peptides, RIP and nisin that have proven activity against staphylococci, and compared them to the standard drug, rifampin. RIP is a heptapeptide YSPWTNF-NH2 having inhibitory activity against *Staphylococcus aureus* and *S. epidermidis*.[14] RIP inhibits staphylococcal quorum sensing and in addition, it reduces bacterial adherence to mammalian cells and to synthetic materials. This indicates that it could be used as a prophylactic and therapeutic agent against staphylococcal infection.[14–16] Nisin is a 34-residue-long peptide belonging to the group A antibiotics and produced by *Streptococcus lactis* subsp. *Lactis*. Nisin has antibacterial activity against a wide range of Gram-positive bacteria including several important contemporary multidrug-resistant Gram-positive pathogens.[17–19] Nisin has been used in foods as a direct additive to inhibit the growth of Gram-positive cells and spores. Nisin can adsorb to surfaces, maintain its anti-bacterial activity and kill adherent cells. Purified nisin has been evaluated for its safety *in vivo* and has been found harmless, or of very low toxicity in rat and guinea pig models. Finally, in 1988, the Food and Drug Administration granted approval for the use of nisin in pasteurized processed cheese spreads and established the legal precedent for application of bacteriocins as food additives in the US.[17–19]

RIP and nisin were tested for their ability to prevent *S. epidermidis* graft infection in a well described vascular graft rat model.[20,21] Peptides were spontaneously bound to the graft, and their efficacy in preventing graft infection was compared to rifampin, an antibiotic commonly used for pre-coating grafts.[20]

Materials and Methods

Organisms

Methicillin-susceptible (MS) *S. epidermidis* ATCC 12228 (Oxoid S.p.A., Milan, Italy) and a clinical isolate of methicillin-resistant (MR) *S. epidermidis* (Se10-01) cultured from a patient with surgical wound infection coming from Central Italy and admitted to the Hospital Umberto I, Ancona, Italy, in February 2001.

Drugs

Bacterially derived nisin (Sigma-Aldrich S.r.l. Milan, Italy) and RIP (Neosystem, Strasbourg, France) were dissolved in distilled H_2O at 20 times the required maximal concentration. Rifampin (Sigma-Aldrich S.r.l.) was dissolved in 50% methanol–50% acetone at a concentration of 1 mg/ml. Fresh solutions were made daily.

Binding of RIP and nisin to Dacron

To determine how much RIP impregnates Dacron, fluorescein isothiocyanate (FITC)-RIP (10 mg/l) was applied to 1 cm^2 sterile collagen-sealed Dacron graft (AlbograftTM, Sorin Biomedica Cardio, S.p.A., Saluggi VC, Italy) for 20 min at room temperature. Fluorescence in unbound solution was determined at OD 485/530 nm in a Microplate Fluorescence Reader (FL 600, Bio-Tek, Vermont, USA) using KC4 software. The binding level of nisin to Dacron was estimated using UV spectroscopy. 1 cm^2 collagen-sealed Dacron graft (AlbograftTM, Sorin Biomedica Cardio, S.p.A., Saluggi VC, Italy) was first washed with distilled water for 10 min. Then the Dacron was impregnated with 9-fluorenylmethyl chloroformate (Fmoc)-nisin (10 mg/l) for 20 min at room temperature. The absorbance of Fmoc-nisin was measured at λ 266 nm with UV–vis spectrometer (Lambda 40P, Perkin–Elmer, Norwalk, USA), either before or after impregnation.[22]

Antimicrobial susceptibility testing

The antimicrobial susceptibilities of the strains to rifampin, RIP, and nisin were determined by the broth microdilution method described by the National Committee for Clinical Laboratory Standards.[23] Minimal inhibitory concentration (MIC) was taken as the lowest antibiotic concentration at which observable growth was inhibited. Experiments were performed in triplicate.

Rat model

The study was approved by the animal research ethics committee of the I.N.R.C.A. I.R.R.C.S., University of Ancona, Ancona, Italy. Adult male Wistar rats (weight range, 280–350 g) were studied. The study included a control group (without graft inoculation) and two series composed of five groups (MS1 to MS5 and MR1 to MR5) for each staphylococcal strain. Each of the series included one contaminated group, three contaminated groups that received grafts soaked in RIP, nisin or rifampin, and one contaminated group that received grafts soaked with nisin + RIP. Each group contained 20 animals. Rats were anesthetized with ether, the hair on the back was shaved, and the skin 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-cm^2 sterile collagen-sealed Dacron grafts (Albograft™, Sorin Biomedica Cardio, S.p.A., Saluggi VC, Italy) were implanted into the pockets. Prior to implantation, the Dacron graft segments were impregnated with 10 mg/l RIP (MS2 and MR2), 10 mg/l nisin (MS3 and MR3), 10 mg/l rifampin (MS4 and MR4), 10 mg/l RIP plus 10 mg/l nisin (MS5 and MR5). Drug absorption on the graft was performed immediately before implantation by soaking the graft for 30 min in a sterile solution of the agents mentioned above. To remove any non-adherent antimicrobial agent, each graft was rinsed 1 min in 50 ml sterile saline before implantation. The pockets were closed by means of skin clips and sterile saline solution (1 ml) containing *S. epidermidis* ATCC 12228 or the methicillin-resistant strain *S. epidermidis* Se10-01 at a concentration of 2×10^7 colony forming units/ml (cfu/ml) was inoculated onto the graft surface by using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals were returned to individual cages and thoroughly examined daily. Toxicity was evaluated on the basis of the presence of any drug related adverse effects, i.e. local signs of perigraft inflammation, anorexia, weight loss, vomiting, diarrhea, fever and behavioral alterations. All grafts were explanted at 7 days following implantation.

Assessment of the infection

The explanted grafts were placed in sterile tubes, washed in a sterile saline solution, moved into tubes containing 10 ml of sterile phosphate-buffered saline solution and sonicated (Fisher Scientific 300, PBI International, Milan Italy) for 5 min to remove adherent bacteria from the grafts. Quantitation of viable bacteria was obtained by performing serial dilutions (0.1 ml) of the bacterial suspension in 10 mM of sodium HEPES buffer (pH 7.2) (Sigma-Aldrich, Milan, Italy) to minimize the carryover effect and by culturing each dilution on blood agar plates. All plates were incubated at 37 °C for 48 h and evaluated for the presence of the staphylococcal strains. The organisms were quantitated by counting the number of cfu/plate. The limit of detection for this method was approximately 10 cfu/cm^2.

Statistical analysis

MIC values are presented as the geometric mean of three separate experiments. Quantitative culture results from all groups are presented as mean ± standard deviation and the statistical comparisons between groups were made using analysis of variance (ANOVA) on the log-transformed data. Significance was accepted when the P value was ≤ 0.05.

Results

Binding of RIP and nisin to Dacron

To determine how much RIP and nisin bound to the Dacron graft, the graft was soaked in FITC-RIP or Fmoc-nisin and absorbance determined. These experiments showed that when 1 cm^2 Dacron was soaked in a 10 mg/l RIP or 10 mg/l nisin solutions there was binding of 25.9 μg ± 3.6 of RIP or 35.2 ± 4.1 μg of nisin, respectively.

In vitro *studies*

Using the broth-microdilution method, rifampin exhibited MICs of 0.25 and 4.00 mg/l for *S. epidermidis* ATCC 12228 and *S. epidermidis* Se10-01, respectively. In contrast, the two strains demonstrated similar susceptibility patterns for nisin, which showed MICs of 4.00 mg/l for both strains. Finally, RIP did not demonstrate any *in vitro* activity against either strain (MICs &greater;128 mg/l).

In vivo *studies*

None of the animals included in the negative control group (no inoculation of graft) had anatomic and microbiological evidence of graft infection. On the other hand, all 40 rats included in the groups MS1 and MR1 demonstrated evidence of graft infection, with $4.8 \times 10^7 \pm 2.0 \times 10^7$ cfu/cm^2 graft and $5.1 \times 10^7 \pm 1.3 \times 10^7$ cfu/cm^2 graft, respectively, but without local signs of perigraft inflammation. Among the groups treated with a single antimicrobial agent, the groups MS2 and MR2 showed the lowest bacterial growth, $5.2 \times 10^2 \pm 1.3 \times 10^2$ cfu/cm^2 graft and $6.1 \times 10^2 \pm 2.8 \times 10^2$ cfu/cm^2 graft, respectively. The groups MS3 and MS4 demonstrated similar quantitative culture results with $6.2 \times 10^3 \pm 1.3 \times 10^3$ cfu/cm^2 graft and $5.8 \times 10^3 \pm 1.6 \times 10^3$ cfu/cm^2 graft, respectively. In contrast, MR3 showed a lower bacterial growth ($7.8 \times 10^3 \pm 3.0 \times 10^3$) than MR4 ($5.5 \times 10^4 \pm 2.9 \times 10^4$). Finally, only the groups MS5 and MR5, treated with the RIP + nisin combination, showed no evidence of staphylococcal infection, with quantitative cultures below the limit of detection (Table 1). For all treated grafts the bacterial counts were significantly lower than for the untreated grafts. Interestingly, RIP-treated group MR2 had a significantly higher

bacterial count than the rifampin-treated group MR4. The bacterial counts from the single agent treatment groups (MS2 to MS4 and MR2 to MR4) were significantly higher than the counts for grafts treated with RIP + nicin.

Finally, no agent appeared to have toxic side-effects. No animal had clinical evidence of drug related adverse effects, such as local signs of perigraft inflammation, anorexia, weight loss, vomiting, diarrhea, fever and behavioral alterations.

The results and the statistical comparisons between groups are summarized in Table 1.

Discussion

Recent studies on bacterial biofilm suggest that its formation is the cause of a multicellular developmental process regulated by the exchange of chemical signals between bacteria. It is well known that specific gene products are required for the initial association of bacteria with a surface and that during this process several genes are turned on and off as bacteria move onto a surface, suggesting a pathway of differentiation.[1,3,24] The recognition of biofilm formation as a multicellular process is important because this insight will allow new approaches for prevention and treatment of the persistent infections stemming from biofilm. Most or all the antibiotics currently used were identified on the basis of their activity against growing cultures of individual organisms. Screening of existing or novel drugs selected for activity against non-growing or biofilm organisms might yield antimicrobial agents with clinical efficacy. New prophylactic and therapeutic strategies could use analogues of microbial signalling molecules that interfere with cell-to-cell communication required for normal biofilm formation, and proteins or peptides able to reduce bacterial adherence. RIP falls into this category. It interferes with cell-to-cell communication reducing bacterial adhesion in the early exponential phase and inhibiting toxins and virulence factors production in the late exponential phase.[14] As evidenced by MIC results, RIP does not kill the bacteria, but eliminates their ability to adhere and form a biofilm. RIP can, therefore, be used for prevention of biofilm formation.

We explored the potential synergy of RIP with nisin, a peptide from *Streptococcus lactis*. It is well known that nisin exert very low toxicity, but even though the Food and Drug Administration granted approval for its use as food additives, *in vivo* report are lacking. Due to the low toxicity of nisin and its high antimicrobial activity against staphylococci, we decided to investigate, for the first time *in vivo*, its activity in an animal model of staphylococcal Dacron graft infection. Nisin has the advantage that it adheres strongly to several synthetic materials due to its particular chemical structure. It is a cationic amphipilic peptide (electric charge approximately + 4) with several unusual dehydro residues and thioether-bridged lanthionines.[18,25] The surface of several synthetic materials used by microbiologists and surgeons, such as polystyrene, polytetrafluoroethylene, and polyethylene terephthalate (Dacron), bind this type of cationic molecules strongly.[26,27] As a result of this binding, the retention of the biologically active molecules is not due to passive entrapment in the plastic tissue but results from ionic interaction between the anionic ligands and the cationic compounds, thus allowing a longer adhesion on the graft and improved anti-bacterial activity. In contrast, it has been shown that if antimicrobial agents have an insufficient number of positively charged residues, they usually bind to the prosthetic graft via a protein such as collagen, albumin and fibrin.[28–32]

When nisin and RIP were tested alone, RIP showed the higher efficacy against both staphylococcal strains, but only the combination of nisin and RIP was able to completely inhibit bacterial growth, even when high concentrations of bacteria had been inoculated on the Dacron prostheses. Interestingly, rifampin was not effective as RIP and nisin against the resistant strain, indicating that the two peptides are good candidates for treatment of drug-resistant staphylococci.

Previously, we have investigated the effects of RIP, both alone and in combination with other cationic

Table 1. Efficacy of RIP, nisin, and rifampin against methicillin-susceptible and methicillin-resistant *Staphylococcus epidermidis* strains causing graft infection in a rat model

Group*	Graft-bonded drug†	Quantitative graft culture (CFU/cm^2)
Control	–	<10
MS1	–	$4.8 \times 10^7 \pm 2.0 \times 10^6$
MS2	RIP‡	$5.2 \times 10^2 \pm 1.3 \times 10^2$
MS3	Nisin‡	$6.2 \times 10^3 \pm 1.3 \times 10^3$
MS4	Rifampin‡	$5.8 \times 10^3 \pm 1.6 \times 10^3$
MS5	RIP-nisin[c,d]	<10
MR1	–	$5.1 \times 10^7 \pm 1.3 \times 10^7$
MR2	RIP[c,e]	$6.1 \times 10^2 \pm 2.8 \times 10^2$
MR3	Nisin‡	$7.8 \times 10^3 \pm 3.0 \times 10^3$
MR4	Rifampin‡	$5.5 \times 10^4 \pm 2.9 \times 10^4$
MR5	RIP-Nisin[c,d]	<10

*Each group was formed by 20 animals; MS methicillin-susceptible; MR methicillin-resistant.
†The Dacron graft segments were impregnated with 10 mg/l RIP (group 3), 10 mg/l nisin, 10 mg/l rifampin.
‡Statistically significant when compared with group MS1 or MR1 ($P < 0.001$)..
§Statistically significant when compared with groups MS2-MS4 or MR2-MR4 ($P < 0.001$)..
‖Statistically significant when compared with group MR4 ($P = 0.007$)..

peptides, against different multiresistant staphylococcal strains.[33–35] Data from these studies demonstrated that the co-administration of agents able to reduce bacterial adherence to prosthetic materials together with agents able to kill the bacteria can be an useful way to prevent vascular graft infection.

The present study confirms the usefulness of this alternative approach for antimicrobial perioperative chemoprophylaxis in vascular surgery.

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