Treatment of Infected Wounds with the Antimicrobial Peptide D2A21

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Background: **Infected wounds impose a significantly negative effect on patient care and recovery, as infection hinders normal wound healing, resulting in increased patient morbidity and mortality. More attention is being focused on addressing the problem of multidrug-resistant bacteria and the staggering costs and consequences resulting from this. Recently, newly evaluated antimicrobial peptides have been shown to be active against a wide variety of bacteria in in vitro studies. This study evaluates the use of a particular antimicrobial peptide, D2A21 (Pittsburgh, PA), to combat infection in an acutely infected wound model.**

Methods: **Forty-eight Wistar rats were used to compare the effects of D2A21 to control vehicle, silver sulfadiazine (SSD), and Sulfamylon in this model. Two 1.5 × 1.5-cm full-thickness defects were created on the rat dorsum and were subsequently inoculated with 10^8 *Pseudomonas aeruginosa*. Animals underwent daily treatment with either D2A21 gel, control vehicle, SSD, or Sulfamylon. Animals were evaluated for survival differences.**

Results: **Survival analysis at 21 days for the different treatment groups were as follows: 100% for the D2A21-treated animals, 50% for control-treated animals, 83% for Sulfamylon-treated animals, and 33% for SSD-treated animals.**

Conclusion: **D2A21 antimicrobial peptide demonstrates significant activity compared with controls and standards of therapy. The promising effect of this topical peptide is clearly evident as shown by this study, and its further investigation as a potential agent in the fight against infected or chronic wounds is warranted.**

Key Words: **Infected wounds, Antimicrobial peptide, D2A21, Sulfamylon.**

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Antimicrobial peptides represent a relatively new discovery in the immune system pathway. These small peptides are inducible elements of the immune system that serve as nonspecific effector molecules to eradicate infection caused by bacteria, yeast, and viruses, protecting host epithelial surfaces such as the tracheal mucous membrane and genitourinary tract.[1–4] In mammals, several of these compounds are known to be present in high concentrations in neutrophilic granules and phagocyte vacuoles. They are believed to exert their antibacterial effects through the insertion and formation of voltage-sensitive channels in bacterial cell membranes, creating cell lysis.[5–7]

Recent research has demonstrated the significant in vitro effect of many of these peptides against a large variety of pathogens.[3,8–15] When applied exogenously, however, the peptide's activity can be attenuated as a result of wound proteases or minimal electrolyte or pH alterations in the wound milieu.[1,16–20] Some peptides in higher concentrations exhibit cytotoxicity that might adversely affect wound healing.[3,21–23] In this light, synthetically engineered antimicrobial peptides have been designed to increase potency and activity against bacteria and fungi and yet remain noncytotoxic.[24,25]

Current therapeutic regimens for infected wounds often encompass debridement, irrigation, and the use of topical antimicrobials to help combat bacterial load. Previous literature, however, has pitted the potential significant benefits against the possible deleterious effects of many agents on the wound-healing process.[26–35] We have previously demonstrated use of one synthetic antimicrobial peptide, D2A21, in an infected burn model in improving survival and recovery.[24] This study attempts to compare the effect of this peptide on survival in an infected wound model with that of traditionally used wound therapies, silver sulfadiazine (SSD) and Sulfamylon.

MATERIALS AND METHODS

Procedures for animal care were reviewed and approved by the Laboratory Animal Care and Use Committee at Southern Illinois University. Animals were maintained in a central research animal facility where an environment of controlled temperature and humidity was provided. Animals were maintained in individual cages in a 12-hour light-dark cycle with food and water provided ad libitum.

Forty-eight male Wistar rats weighing approximately 420 g (Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with Nembutal (42 mg/kg intraperitoneally), and their dorsal hair was clipped. Two 1.5 × 1.5-cm full-thick-

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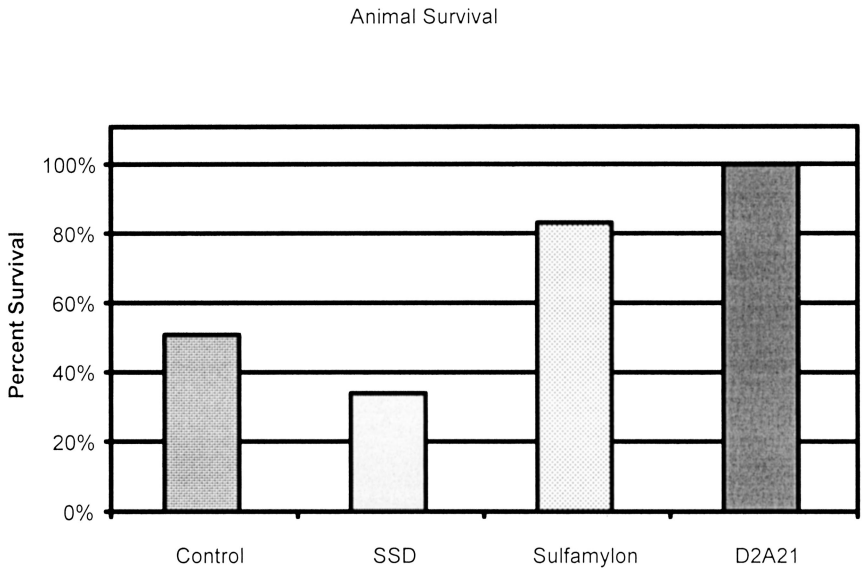

Fig. 1. *Survival of acutely infected animals at 3 weeks treated with control vehicle, SSD, Sulfamylon, or D2A21.*

ness wounds were created on the rats' dorsum by sharp excision of skin and panniculus carnosus. Butorphanol (23 mg/kg subcutaneously) was administered for analgesia. *Pseudomonas aeruginosa* bacteria (strain 1244) were grown in tryptic soy broth overnight at 37°C. An aliquot of the overnight culture was transferred to fresh tryptic soy broth and incubated at 37°C, shaking for 4 hours to an optical density of 0.3 (measured at 620 nm). The cell suspension was centrifuged at 2,000 × *g* for 15 minutes, washed twice with 0.9% NaCl, and resuspended at 10×10^8 colony-forming units/mL (for a 10^8 inoculum). Animals were infected 30 minutes later by applying *Pseudomonas aeruginosa* (1×10^8 colony-forming units in 0.1 mL saline) into each dorsal wound. Bacterial concentration in the inoculum was confirmed by plating serial dilutions on trypticase soy agar and counting colonies after 24 hours' incubation at 37°C. The rats were equally divided into four treatment groups (12 in each group): topical application of 2% D2A21 peptide at 3 hours after wound creation and daily; water-based control gel vehicle 3 hours after wound creation and daily; SSD (Thermazene, Kendall Healthcare Products, Mansfield, MA) at 3 hours after wound creation and daily; or Sulfamylon creme (Bertek Pharmaceuticals, Morgantown, WV) at 3 hours after wound creation and daily. Animals were then monitored for survival differences between treatment groups.

Survival analysis was performed using the Kaplan-Meier procedure with a log-rank χ^2 test. Statistical analyses were performed with SAS (Cary, NC), and statistical significance was set at the 5% level.

RESULTS

Gross observations of the control- and SSD-treated animals showed more lethargy than the Sulfamylon- or D2A21-treated animals. None of the D2A21-treated animals demonstrated any evidence of illness, toxicity, or side effect related to the peptide. Animals surviving to 21 days were uniformly found to be long-term survivors.

Animal survival was as follows: 100% for the D2A21-treated group, 50% for control vehicle, 83% for the Sulfamylon-treated group, and 33% for the SSD-treated group (Figs. 1 and 2). Improvement was significant between D2A21-treated animals and SSD or control groups ($p < 0.001$). Comparison between D2A21 and Sulfamylon showed a trend without significance of improvement for the D2A21 group ($p = 0.08$). Sulfamylon-treated animals also demonstrated significant improvement over SSD and control animals ($p < 0.01$ and $p < 0.05$, respectively). There were no significant survival differences between SSD and control animals.

Previous research with the gel vehicle demonstrated no improvement or worsening compared with animals that did not receive any topical treatment after bacterial inoculation. Pilot studies also demonstrated no mortality in this model when bacterial inoculation was not used.

DISCUSSION

Infected and colonized wounds impose an extraordinary burden to patients and the hospital care system. Whereas chronic wounds invariably involve a multitude of bacterial species, acutely infected wounds are more frequently the result of isolated or few species. Oral or intravenous antibiotics are often used in conjunction with topical antimicrobials to decrease the bacterial burden on tissue. As more attention is focused on the problem of multidrug-resistant bacteria, choices for effective selection of antimicrobial agents can become limited. For example, increased use of vancomycin for methicillin-resistant *Staphylococcus aureus* (MRSA) therapy has been demonstrated to help select for vancomycin-resistant *Enterococcus*, which can secondarily elevate cross-

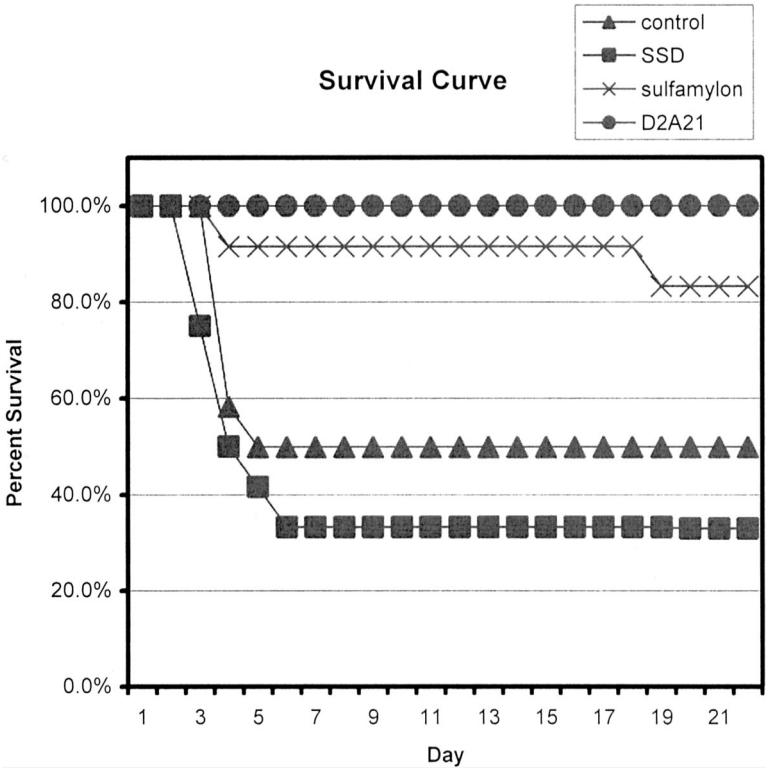

Fig. 2. *Survival curve of animals treated with control vehicle, SSD, Sulfamylon, or D2A21.*

resistance in MRSA through plasmid transfer.[36] Recent analyses of hospital discharges showed that 1% of all discharges were for *S. aureus* infections alone.[37] Those patients had hospital costs that were twice those of other patients. Treating MRSA infections, which are frequently related to open wounds, costs at least 6% to 10% more than a methicillin-sensitive *S. aureus* infection and has a death rate that is greater than 2.5 times higher than that attributable to methicillin-sensitive *S. aureus* alone.[37] Clearly, effective antimicrobial choices are needed as drug resistance continues to emerge.

Traditional topical agents such as silver sulfadiazine and Sulfamylon have been well documented in the literature regarding their use and benefit in wound care.[29,33,34] However, these agents can present particular difficulties related to resistance or inhibition of the wound-healing process. The use of silver sulfadiazine, for example, has been demonstrated to increase wound epithelialization but can impair wound contraction.[30–32,35] Sulfamylon has been demonstrated to enhance angiogenesis, epithelialization, and dermal thickening in some studies, whereas in others it has been linked to decreases in keratinocyte growth rates and is a known source of acidosis through its inhibition of carbonic anhydrase.[26,27,30–32] These agents have limited spectra of antibacterial activity.[38–41] Other topical agents used to decrease wound bacterial load have included Dakin's solution, Betadine, acetic acid, and hydrogen peroxide. Dakin's solution exhibits deleterious effects on fibroblasts and endothelial cells and can impair neutrophil migration and wound neovascularization.[26,42] Studies of Betadine have shown slower rates of reepithelialization compared with other topical antimicrobial agents and impairment of microcirculation at higher levels of concentration.[43,44] Acetic acid does not demonstrate effective control of bacterial levels and is cytotoxic at its traditionally used concentration of 0.25%.[26,44] Hydrogen peroxide can also be toxic to fibroblasts.[45]

The ideal topical agent should be extremely active against pathogens and have a neutral or even beneficial effect on the wound-healing process. Newly evaluated antimicrobial peptides have shown great potential for activity against a wide variety of pathogens in in vitro testing, including MRSA.[1,3,4,8–16,24,25] These antimicrobial peptides work through a process different from traditional antibiotics, creating pores in the bacterial cell wall culminating in cell lysis.[5–7] The naturally occurring antimicrobial peptides, however, are extremely susceptible to minute variations in their local environment. Slight alterations in the sodium, magnesium, phosphate, or pH may render them inactive. A portion of the sensitivity of these peptides may reside in fragile disulfide bonds that maintain their distinct configuration. Under nonphysiologic conditions, such as infection, these bonds are cleaved and an inactive metabolite remains. Synthetic antimicrobial peptides, such as D2A21, are fortified in that they are not only more resistant to degradation but also have greater antimicrobial activity. The active peptide is

maintained in hostile, nonphysiologic environments. Alterations in the wound milieu do not affect the potency or characteristics of the peptides, making their use of value in managing infected or adversely colonized wounds.

This study demonstrates the significant effect of one antimicrobial peptide. Topical application of *Pseudomonas* resulted in sepsis and death in 50% of control-treated animals, whereas those animals treated with D2A21 showed no evidence of systemic or local infection. Statistical analysis showed the D2A21 animals to have significant benefit compared with SSD- or control-treated groups and a trend toward improvement compared with Sulfamylon. The silver sulfadiazine animals had surprisingly low survival that was equivalent statistically to control animals. We suspect that this may be secondary to bacterial resistance to the drug.

Although naturally occurring peptide antimicrobials are susceptible to factors in the wound milieu that cleave the peptide or render it inactive, this does not appear to occur with D2A21, as it continues to demonstrate significant activity as demonstrated by the excellent survival ratio in this study and in previous in vivo studies.[24,25] D2A21 does not appear to have any deleterious effects on keratinocyte growth (unpublished company data). Future studies should further delineate effects on wound healing, vascularization, contraction, epithelialization, and so forth, as has been accomplished with both Sulfamylon and silver sulfadiazine over the years. We believe this peptide shows significant promise and warrants further investigational study for future use in infected wounds.

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