Effective management of microbial contamination in cultured skin substitutes after grafting to athymic mice

STEVEN T. BOYCE, PhD; M. DANA HARRIGER, PhD; ANDREW P. SUPP, BS; GLENN D. WARDEN, MD; IAN ALAN HOLDER, PhD

Cultured skin substitutes have become therapeutic alternatives for treatment of acute and chronic skin wounds, but all models of these substitutes are avascular and susceptible to microbial destruction during vascularization. To develop a practical management protocol for increased survival of skin substitutes, experimental wounds were contaminated with *Pseudomonas aeruginosa* and treated with a formulation of noncytotoxic antimicrobial agents (polymyxin B, neomycin, ciprofloxacin, mupirocin, amphotericin B) in a nutrient medium (vehicle). Cultured skin substitutes consisting of human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan sponges were grafted to 2 x 2 cm full-thickness wounds on athymic mice that were contaminated with the strain SBI-N of *P. aeruginosa* at 1 x 10⁴, 1 x 10⁵, and 1 x 10⁶ organisms/wound. Experimental wounds were irrigated with 1 ml/day topical antimicrobial solution for 10 days, and controls received vehicle only. Two, three, and four weeks after grafting, wounds were traced and swabbed for microbial culture and areas were measured with planimetry. At 4 weeks, biopsy samples were scored histochemically for immunoreactivity to HLA antigens. Data analysis by chi-square, analysis of variance, and Tukey’s test shows that treatment of contaminated wounds with noncytotoxic topical antimicrobials is associated with an increased area of healed wounds, positive detection of HLA antigens, and negative cultures for *P. aeruginosa*. These results show that microbial contamination of cultured skin substitutes on full-thickness wounds may be managed effectively during graft vascularization. However, this formulation of antimicrobial agents is not currently approved for human use and is investigational only. Effective management of microbial contamination suggests that clinical efficacy of avascular tissue analogs may be increased by local application of noncytotoxic antimicrobial agents. (WOUND REP REG 1997;5:191-7)

Cultured skin substitutes (CSS) have become alternative treatments for burns and chronic wounds. Because none of these analogs of skin contains a vascular plexus and epidermal analogs do not form a functional barrier, cultured keratinocytes are more susceptible to microbial destruction than grafts of native skin.

As with other causes of graft loss, microbial destruction of CSS results in additional requirements for regrafting or in secondary healing of wounds which increases scar. Both burns and chronic wounds become colonized with various microorganisms that include species of gram-negative and gram-positive bacteria and fungi. Effective coverage of these groups of organisms must be provided without cytotoxicity to cultured cells. In addition, effective treatment of microbial contamination in wounds requires topical rather than parenteral administration of agents. These general requirements...
for management of microbial contamination of CSS has qualified certain agents (i.e., polymyxin B, neomycin, mupirocin, nystatin) and disqualified others (i.e., sulfamylon, silver sulfadiazine) predominantly on the basis of in vitro assays. A simple algorithm was developed for testing individual agents by concentration-dependent inhibition of human cell (keratinocyte and fibroblast) growth and subsequent retention of antimicrobial activity. After identification of individual nontoxic agents that retained antimicrobial activity, combined formulations of individual agents were retested on human cells and panels of microorganisms isolated from wounds of burn patients.

Currently, there are few preclinical studies of topical antimicrobial agents (AM) on cultured skin in vivo. The current study was performed to determine whether topical irrigations with a formulation of nontoxic agents can control bacterial contamination in full-thickness skin wounds after treatment with CSS.

MATERIALS AND METHODS

Experimental design

Full-thickness wounds were prepared on the flanks of athymic mice. Wounds were contaminated with Pseudomonas aeruginosa (strain SBI-N). Strain SBI-N was isolated from the wounds of a patient at this institution and has been shown to be an O serotype G strain. A strain with known serotype was chosen so that P. aeruginosa organisms recovered from wounds contaminated experimentally could be tested to ensure that they were the same serotype as the contaminating organism inoculated into the wounds. This strain was also selected on the basis of its determination by in vitro testing to be susceptible to the experimental formulation of agents. A preliminary experiment was performed to determine a concentration of SBI-N ($1 \times 10^6$ colony-forming units per 4 cm² wound; $n = 3$ or 4 per concentration) that resulted in uniform graft destruction without drug treatment (negative control). After determination that $1 \times 10^6$ organisms destroyed CSS, wounds ($n = 7$) were inoculated with that density of SBI-N, CSS were applied, and grafted wounds were irrigated for 10 days with a formulation of nutrients and AM as described in Table 1. Grafted wounds were evaluated at 10, 14, 21, and 28 days after surgery for detection of SBI-N organisms and wound area. At 4 weeks after surgery, animals were euthanized and scored by immunohistochemistry for graft survival (HLA-ABC). Controls included CSS grafts without microbial contamination (positive controls) +AM ($n = 7$) or −AM ($n = 5$), and inoculation of wounds with $1 \times 10^6$ colony-forming units of SBI-N (negative control) −AM ($n = 7$).

Preparation of CSS

CSS were prepared as described previously from cultured human epidermal keratinocytes and dermal fibroblasts attached to collagen-glycosaminoglycan substrates. In brief, substrates that were sterilized by gamma irradiation were inoculated with fibroblasts to the porous side and keratinocytes to the opposite side the following day. CSS were incubated (5% CO₂, 37°C, saturated humidity) for 14 days in a lipid-supplemented medium at the air-liquid interface of the culture medium. CSS were cut into $2 \times 2$ cm grafts the day preceding surgery. Histologic anatomy of CSS is divided into epidermal and dermal compartments, and partial formation of epidermal barrier occurs in vitro.

Animal surgery

All studies with animals were performed with full approval of the University of Cincinnati Institutional Animal Care and Use Committee. Grafting of CSS to mice was performed as in previous studies under strictly sterile conditions. Female athymic mice age 8 to 12 weeks were anesthetized by intraperitoneal injection with avertin (tri-bromo-ethanol in tertiary amyl alcohol). Full-thickness skin wounds ($2 \times 2$ cm) were prepared to a depth of the panniculus carnosus. Experimental wounds were inoculated with $1 \times 10^4$, $1 \times 10^3$, or $1 \times 10^6$ P. aeruginosa SBI-N contained in 0.1 ml normal saline solution. CSS were applied, covered with N-Terface (Winfield Laboratories, Richardson, Texas), and stent-sutured to the cor-

Table 1. Formulation of nutrient and antimicrobial irrigation solution

<table>
<thead>
<tr>
<th>Solution component</th>
<th>Individual agents</th>
<th>Amount per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient medium</td>
<td>Modified MCDB* 153</td>
<td>As reported^1,^20</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Polymyxin B</td>
<td>700,000 U</td>
</tr>
<tr>
<td></td>
<td>Neomycin</td>
<td>40 mg</td>
</tr>
<tr>
<td></td>
<td>Mupirocin</td>
<td>20 mg</td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
<td>20 mg</td>
</tr>
<tr>
<td>Antimycotic</td>
<td>Amphotericin B</td>
<td>1 mg</td>
</tr>
<tr>
<td>Human cell mitogens</td>
<td>Insulin</td>
<td>5 mg</td>
</tr>
<tr>
<td></td>
<td>Hydrocortisone</td>
<td>0.5 mg</td>
</tr>
</tbody>
</table>

*MCDB, Molecular, cellular and developmental biology.
Table 1 Concentration-dependent scoring of *P. aeruginosa* (strain SBI-N) in murine wounds treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM).

<table>
<thead>
<tr>
<th>SBI-N Inoculum</th>
<th>AM</th>
<th>(10^6)</th>
<th>(10^5)</th>
<th>(10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n/group</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

**Figure 1** Concentration-dependent scoring of *P. aeruginosa* (strain SBI-N) in murine wounds treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM).

Figure 2 Concentration-dependent scoring of HLA-ABC antigens in murine wounds treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM).

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<tr>
<td>AM</td>
<td>+</td>
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<tr>
<td>n/group</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**

On day 28 after grafting, animals were euthanized and wounds were excised, cryostat-sectioned, and reacted by a direct method with a fluorescein-conjugated, murine monoclonal antibody to HLA-ABC (Accurate Chemical and Scientific Corp, Westbury, N.Y.). Cryostat sections included the entire width of the healed skin as determined by absence of hair follicles between hair-bearing margins of the wounds. Biopsy samples with any staining of HLA-ABC in plasma membranes of keratinocytes of healed epidermis was scored as immunopositive by examination on a Nikon FXA epifluorescence microscope. Data were analyzed by chi-square testing and are expressed as percent HLA-ABC-positive animals per group.

**Data collection and analysis**

At the time of dressing changes (days 10, 14, 21, and 28), treated wounds were photographed and data were collected for microbial assay, as described previously, and wound area by tracing onto an acetate sheet followed by computerized image analysis. Data from microbial cultures were analyzed statistically by one-way analysis of variance and are presented as *P. aeruginosa*-positive wounds per group. Wound areas were analyzed by repeated-measures analysis of variance and Tukey’s test and are expressed as percentage of original wound area.

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**WOUND REPAIR AND REGENERATION**

VOL. 5, NO. 2

BOYCE ET AL. 193
area (mean ± standard error of the mean). Data were considered statistically significant at a value of $p < 0.05$ unless indicated otherwise.

RESULTS

Concentration-dependent study

Figure 1 presents results of tests for detection of *P. aeruginosa* from swab cultures of animal wounds after inoculation with $1 \times 10^6$, $1 \times 10^5$, $1 \times 10^4$ SBI-N per wound (4 cm²). All wounds that did not receive the topical AM irrigation were positive for *P. aeruginosa*. One wound treated with AM and inoculated with $1 \times 10^4$ SBI-N was positive for *P. aeruginosa*, but the CSS graft was not adversely affected. All other grafts treated with AM were negative for *P. aeruginosa*. As shown in Figure 2, all wounds treated with AM were positive for HLA-ABC independent of inoculation density of SBI-N. In the absence of AM, wounds were negative for HLA-ABC except one inoculated at $1 \times 10^4$ organisms. Wound area in Figure 3 was statistically greater in wounds treated with AM after inoculation with $1 \times 10^6$ or $1 \times 10^5$ organisms. In the absence of AM, an inverse relationship between wound area and inoculation density is suggested, although no statistical differences were detected.

Time-dependent study

Physical examination of treated animals at 10 days after grafting showed obvious differences among treatment conditions as shown in Figure 4. CSS inoculated with $1 \times 10^5$ SBI-N and irrigated with nutrients, antimicrobials, and mitogens were fully epithelialized at day 10 (+Psa +AM). Irrigation of CSS without the antimicrobials (+Psa -AM) resulted in partial or complete destruction of all grafts associated with the characteristic green color of *P. aeruginosa*. CSS that were not inoculated with *P. aeruginosa* were fully epithelialized by day 10 in the presence (--Psa +AM) or absence (--Psa --AM) of AM. Wound healing with CSS in the presence of these concentrations of AM verifies in vitro studies that determined this formulation is not cytotoxic to transplanted cells. By day 28 after grafting, contaminated CSS treated without AM had desiccated and sloughed (not shown). Figure 5 shows that all CSS inoculated with SBI-N --AM were positive for *P. aeruginosa* during the first 21 days after grafting. Decrease of *P. aeruginosa* detection at day 28 may reflect elimination of the organism from the wound by healing. Figure 6 shows that all CSS inoculated with SBI-N --AM were positive for *P. aeruginosa*. In the absence of SBI-N inoculation, no microbial contamination of any kind was detected. In no instances were *P. aeruginosa* other than O serotype G (SBI-N) detected by serotyping of *P. aeruginosa* --positive wounds.

Engraftment of CSS was scored by expression of HLA-ABC as shown in Figure 6. CSS that were contaminated and did not receive antimicrobials showed statistically fewer wounds that expressed human class I histocompatibility antigens at day 28. All other grafted wounds were HLA-ABC positive.

As shown in Figure 7, a plot of wound area versus time shows a statistically significant reduction in the wound area in contaminated grafts without antimicrobial treatment. No differences were detected in any other condition.

DISCUSSION

Data presented in this report support the hypothesis that gram-negative contamination of CSS can be controlled with topical application of noncytotoxic AM. As suggested in preclinical studies, this formulation does not inhibit wound closure and healing of cultured grafts. Preclinical testing of the specific formulation used in this study showed no significant reduction in proliferation of human keratinocytes or fibroblasts and effective antimicrobial activity against clinical isolates of gram-negative (i.e., *P. aeruginosa*, *Klebsiella* sp., *Escherichia* sp., *Enterobacter* sp., *Serratia* sp.), gram-positive (*Staphylococcus aureus*), and fungal (i.e., *Candida* sp.) microorganisms. This formulation was designed to provide simultaneous activity against major categories of organisms and redundant activity for bacterial strains. Gram-negative organisms are sensitive to neomycin, polymyxin B, and ciprofloxacin in this formulation; gram-positive *Staphylococcus aureus* to muopirocin and ciprofloxacin; and fungi to amphotericin B. For redundant management of fungi, nystatin may be added to the formulation, but the less
Figure 4 Photographs of skin wounds on athymic mice inoculated with P. aeruginosa (Psa) and treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM).

Figure 5 Time-dependent scoring of P. aeruginosa (strain SBI-N) in wounds treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM). Treatments was as follows: ▼, -AM +SBI-N; ▲, +AM +SBI-N; ○, +AM -SBI-N; ■, -AM -SBI-N.

Figure 6 Scoring of HLA-ABC at postoperative day 28 in wounds of athymic mice inoculated with P. aeruginosa and treated with cultured skin substitutes and noncytotoxic antimicrobial agents (AM).
The virulent character of fungi in skin wounds has not required clinical use of multiple antifungal agents in topical applications. By this approach, the probability to manage a mixed contamination of organisms is increased and the probability of generating resistant organisms is decreased. Omission of this topical therapy in this study resulted in significant reduction of wound area and HLA-ABC expression. It is concluded from these results that this formulation and route of administration of agents provides direct and indirect advantages to survival of CSS grafts on wounds.

Direct advantages for graft survival include control of microbial growth that results in graft destruction. Although this study used a single strain of gram-negative bacteria, the formulation includes agents for control of gram-positive and fungal organisms. Data from in vitro studies\textsuperscript{20,21} shows that a variety of bacteria from these groups are susceptible to this AM formulation. Results of those studies suggest that microbial contaminations of mixed species can also be controlled with the formulation described here. Those studies have also shown that combination of noncytotoxic agents at certain concentrations does not reduce their individual activities. Clinical studies\textsuperscript{2,3,5} have shown qualitatively that microbial destruction of CSS is greatly reduced by topical application of this formulation.

Indirect advantages of this formulation include, but are not limited to, the following: low cytotoxicity that is permissive to survival of transplanted cells and graft vascularization, redundancy of antimicrobial activity for bacteria that reduces algebraically the probability that drug-resistant or super-infecting organisms will emerge, and administration as a solution which increases rate of drug delivery and reduces the concentration of drug required. Penetration of the agents is particularly important because the origin of most microbial contamination is the wound surface that is under the graft. Therefore, detection of contamination by swab cultures of the wound surface may occur only after the graft has been destroyed, which is too late for treatment. However, after CSS have engrafted and keratinized, a mixture of ointments consisting of equal parts Neosporin, Bactroban, and nystatin also provides simultaneous coverage of mixed wound contaminations.\textsuperscript{8}

Although this soluble formulation has broad-spectrum activity, it contains drugs (ciprofloxacin, and mupirocin) that are not approved for administration as topical solutions. Therefore, study of this formulation is currently investigational only and requires approval of a local institutional review board. However, the value and importance of simultaneous administration of multiple agents is verified by practices of burn care in which multiple parenteral agents are administered to manage sepsis. Similarly, certain topical ointments (i.e., Neosporin) are combinations of individual drugs. With appropriate considerations of regulatory and medical practices, introduction of this kind of broad-spectrum formulation is expected to improve the clinical efficacy of CSS for treatment of burns and chronic wounds. Greater efficacy of skin substitutes may contribute to reduced morbidity and mortality from full-thickness skin loss injuries.

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