STUDY

The Longevity of a Bilayered Skin Substitute After Application to Venous Ulcers

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Background: A bilayered skin substitute composed of allogeneic keratinocytes and fibroblasts in a collagen gel has been approved by the US Food and Drug Administration for the treatment of venous and diabetic ulcers. Its mechanism of action has not been fully determined.

Objective: To determine the longevity of allogeneic fibroblasts and keratinocytes in a bilayered skin substitute in patients with venous leg ulcers.

Methods: Ten patients with venous leg ulcers were treated with a bilayered skin substitute on day 0, days 3 to 5, and weeks 1 through 3. Biopsy specimens of the grafted wound were taken. We used polymerase chain reaction analysis to determine whether allogeneic DNA was present in the biopsy specimens.

Results: We detected allogeneic DNA in 2 of 8 specimens at 1 month after initial grafting. Neither of the 2 patients showed persistence of allogeneic DNA at 2 months after initial grafting.

Conclusions: Allogeneic cells from a bilayered skin substitute do not appear to survive permanently after grafting for treatment of venous leg ulcers. Other mechanisms of action might include cytokine release, structural support, or provision of a moist wound environment.

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A BILAYERED skin substitute (BSS) (Apligraf; Novartis, Inc, Canton, Mass) is a viable skin replacement that has been approved by the US Food and Drug Administration for the treatment of venous and diabetic ulcers. The epidermal layer is formed by serial passage of human keratinocytes that are seeded onto a contracted dermal matrix in culture medium. A well-differentiated stratum corneum develops after exposure of the keratinocytes to an air-liquid interface. The dermal layer is composed of human fibroblasts in a bovine type I collagen lattice. These fibroblasts divide, multiply, and produce new collagen. Although matrix proteins and cytokines found in human skin are present in the BSS, it does not contain Langerhans cells, melanocytes, macrophages, lymphocytes, blood vessels, or hair follicles.

At present, the BSS has not been shown to be immunogenic in treated patients. In vitro studies demonstrated that the BSS does not contain cells that constitutively express class II major histocompatibility complex antigens such as macrophages, lymphocytes, and Langerhans cells. These immunogenic cells are lost during serial culture and expansion of human keratinocytes and fibroblasts.[1,2] When tested in a mixed lymphocyte reaction assay, cultured human keratinocytes and fibroblasts did not trigger a proliferative immune response, even when class II antigen expression was induced by interferon-γ.[2] Therefore, it is possible that the BSS might promote healing by surviving permanently in the wound bed. However, experience in other models suggests that the BSS may be eventually replaced by host tissues.[3-5]

The purpose of the present study was to determine whether allogeneic DNA from the BSS could be detected at different times after BSS grafting of venous ulcers.

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RESULTS

Fourteen patients were enrolled into the study. Ten patients demonstrated results of tissue typing in which HLA antigens were not common with those of the BSS. These patients underwent evaluation for persistence of BSS cells. Eight of these 10 patients underwent testing for persistence at 4 weeks. Two ulcers healed completely during the study. The DNA from the BSS could

PATIENTS AND METHODS

Patients with venous ulcers of greater than 1 month's duration (confirmed by means of duplex ultrasonographic findings) and an ankle-brachial pressure index of greater than 0.65 were enrolled into the study.

Patients enrolled in other investigational studies within the past 3 months, patients previously treated with the BSS, and patients with HLA class I antigens common to the patient and the BSS were excluded from participating.

All patients received up to 5 applications of the BSS during the 3-week treatment. At the screening visit (days −7 to −5), blood was collected as a DNA control sample. The BSS was applied to a clean, debrided wound after thorough irrigation with isotonic sodium chloride solution. Oozing or bleeding caused by debridement was stopped by means of gentle pressure. The graft was covered with a nonadherent dressing, premeasured gauze, and a self-adherent elastic wrap from the metatarsals to the tibial plateau. At 4, 8, and 12 weeks after the first BSS application, 3-mm punch biopsy specimens were taken from the area judged by the investigator (T.J.P. or V.F.) to be most likely to contain BSS. If the BSS was not visible on the ulcer, specimens were taken from the center of the wound. Biopsy sites were rotated so that the specimen was not taken from the same location at each visit. When DNA of the BSS could not be detected in the specimen, no further samples were taken.

To test for the persistence of the BSS on patients, we used the expression of specific HLA genes by BSS cells. The HLA phenotypes of the BSS have been determined previously (data not shown). We used this information to create sequence-specific primers directed toward the DNA of the BSS. In the present study, *HLA-DQB1*0201*–specific primers were used (**Figure 1**). Keratinocytes and fibroblasts from the BSS carry this gene. Primers were amplified using polymerase chain reaction (PCR) analysis.[6]

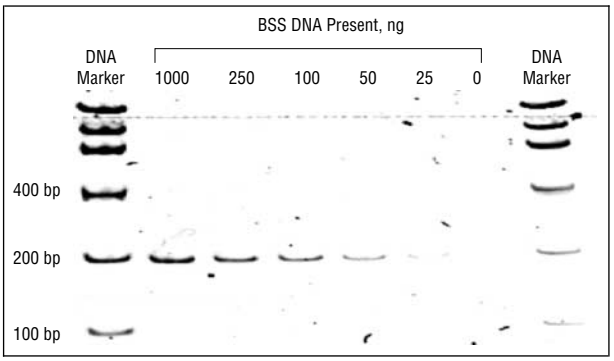

Figure 1. Titration of the bilayered skin substitute (BSS) DNA that is positive for *HLA-DQB1*0201* and mixed with specific amounts of DNA that is negative for *HLA-DQB1*0201*. The polymerase chain reaction (PCR) analysis requires 1 ng of DNA and results in a 205–base pair (bp) product. The product of the first lane (after the DNA marker) was amplified from 1000 ng of *HLA-DQB1*0201*–positive DNA. The product of the second lane was amplified from 250 ng of *HLA-DQB1*0201*–positive DNA mixed with 750 ng of *HLA-DQB1*0201*–negative DNA. The third lane has 100 ng of *HLA-DQB1*0201*–positive DNA mixed with 900 ng of *HLA-DQB1*0201*–negative DNA. The fourth lane has 50 ng of *HLA-DQB1*0201*–positive DNA mixed with 950 ng of *HLA-DQB1*0201*–negative DNA. The fifth lane has 25 ng of *HLA-DQB1*0201*–positive DNA mixed with 975 ng of *HLA-DQB1*0201*–negative DNA. The sixth lane contains 1000 ng of *HLA-DQB1*0201*–negative DNA. We used this test to establish the sensitivity of the assay, in this case 25 ng of specific target sequence. In other words, we can detect the specific sequence in a biopsy specimen that contains as little as 2.5% DNA from the BSS. The titration was used to monitor functioning of the PCR analysis within our specifications and was run every time patient samples underwent testing. It was not used as a standard curve for quantification.

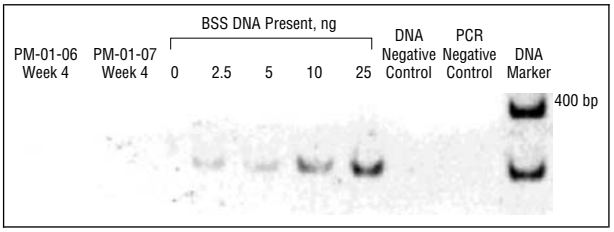

Figure 2. Lanes PM-01-06 and PM-01-07 are samples from study patients 4 weeks after application of the bilayered skin substitute (BSS). No target DNA can be detected in these lanes. A titration of DNA in the following lanes was similar to that in Figure 1, which shows that the polymerase chain reaction (PCR) analysis is working within the stated sensitivity, and that DNA from the BSS could not be detected in the biopsy specimens from these patients. bp indicates base pair.

not be detected in biopsy specimens from 6 patients (**Figure 2**). Results of PCR testing showed that 2 of these wounds demonstrated the BSS DNA at 4 weeks (**Figure 3**). No correlation between the clinical appearance of the wound and the BSS persistence was found. Neither wound showed persistence of DNA from the BSS at 8 weeks. One of these ulcers healed; the other did not.

COMMENT

The BSS has been approved by the US Food and Drug Administration for the treatment of venous and diabetic ulcers. The healing process has not been entirely elucidated.

Some authors postulated that the presence of a dermal-like substitute in the BSS may create conditions sufficiently different from cultured epidermal allografts to allow long-term engraftment.[7] In patients with acute wounds due to epidermolysis bullosa, extensive erosions were treated with the BSS, and the treated areas remained blister free, with clinical evidence of graft take and no signs or symptoms of acute graft rejection.[8,9] Molecular genetic testing using a specific marker for the tissue-engineered cells confirmed persistence of the skin substitute in 4 (33%) of 12 patients with these acute wounds at 4 weeks.[10] Delayed rejection of allograft may occur in these patients because of immune tolerance to allogeneic tissue in early life[8,11] or because of abnormalities in immune function in patients with epidermolysis bullosa.[12-15] Alternately, the mechanism of action of cultured allogeneic tissue may be different in acute wounds compared with chronic wounds. In other acute wounds (eg, split-thickness skin-graft donor sites), persistence of allogeneic DNA from the BSS occurred in 3 (27%) of 11 patients at 4 weeks.[7,10] Additional data are needed to elucidate whether the BSS persists for longer than 4 weeks in acute wounds.

In chronic wounds, clinical investigators have observed clinical graft take and temporary persistence of the

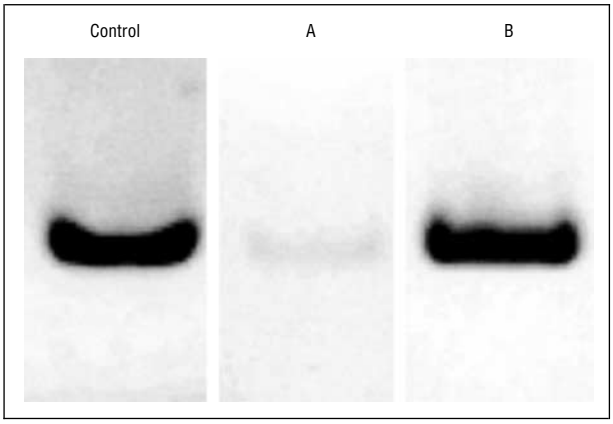

Figure 3. Samples from study patients PM-02-05 and PM-02-06 show persistence of DNA from bilayered skin substitute (BSS) at 4 weeks. We detected *HLA-DQB4*0201* DNA in biopsy specimens 4 weeks after initial treatment. Lane 1 shows the positive control, with DNA isolated from BSS keratinocytes. Lanes A and B show DNA from the BSS in specimens from both patients collected 4 weeks after the initial treatment.

BSS in approximately 41% of patients in whom the wounds healed. Clinical remodeling of the graft and probable replacement with the patient's own skin appeared to occur in at least 63% of these patients.[16] The authors commented that the BSS could benefit wounds as a temporary skin replacement or as a stimulus for wound healing.

In the small group of patients with chronic venous ulcers undergoing testing in this study, allogeneic DNA from a BSS could not be detected at 2 months after grafting. A weakness in this study is that during the period of observation, only 2 of the patients experienced complete healing. Persistence is probably related to successful initial graft take and wound closure, and it is difficult to determine persistence in an unhealed wound. If a wound heals completely and rapidly after application of the BSS, persistence of allogeneic cells would be more likely, as occurred in patient PM-02-05. However, allogeneic DNA was also detected in patient PM-02-06, who did not experience complete wound healing. Our data did not support survival of allogeneic BSS cells at 8 weeks after grafting for treatment of venous leg ulcers. However, given that the initial graft take of the BSS depends on biological and immunological factors, the results of this study do not allow us to clearly determine the reason for the lack of long-term persistence of the BSS cells. Venous ulcers are highly inflammatory, another possible mechanism for no persistence in this condition.

If the BSS does not survive permanently in chronic wounds, several possible mechanisms of action exist. The BSS acts to provide immediate wound coverage and as a barrier to protect the wound from injury, infection, and desiccation. It also provides a moist environment that would be favorable to wound healing. Fibroblasts and keratinocytes, which are found in the BSS, are known to release large numbers of growth factors and cytokines that could stimulate rapid wound healing from the wound margins and from adnexal elements from within the wound bed. These include interleukins 1, 3, 6, and 8; transforming growth factors α and β; granulocyte-macrophage colony-stimulating factor; basic fibroblast growth factor; platelet-derived growth factor; tumor necrosis factor α; and others.[17,18] The combination of human keratinocytes and human dermal fibroblasts in the skin substitute may act in a synergistic manner. For example, insulin-like growth factor-I is not found in cultures of pure human keratinocytes or of pure human dermal fibroblasts, but is present in the BSS (J.Y., unpublished data, September 1997).

The matrix components of the graft may act as a substrate to allow recruitment of cells into the wound. The dermal fibroblasts in the construct may contribute to the formation of new dermal tissue through matrix biosynthesis.[19,20] The mechanisms of action of this skin substitute are of great interest and should be investigated further. However, whatever the mechanism, the BSS offers a new treatment modality for patients with venous and diabetic ulcers, and potentially for patients with other types of wounds.

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