Porcine wound healing in full-thickness skin defects using Integra™ with and without fibrin glue with keratinocytes

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BACKGROUND: An artificial dermal matrix such as Integra (Integra Life Sciences Corporation, USA) provides a wound bed template for vascular and fibrocyte ingrowth as well as collagen remodelling. Dermal repair leads to epidermal and basement membrane regeneration. Burn wounds in particular have been shown to benefit from Integra by enhanced wound healing.

OBJECTIVE: To evaluate the effect of fibrin glue to modify the integration of Integra in large excised cutaneous wounds. It was hypothesized that applying fibrin glue on a wound bed would reduce the time needed for matrix vascularization and incorporation of Integra and take of the cultured keratinocytes.

METHODS: Four separate full-thickness wounds were created on the dorsum of two swine. Wound beds were randomly assigned to either application of fibrin glue or no application of fibrin glue before application of Integra. Full-thickness biopsies were performed at days 7, 14, 21, 29 and 35. On day 21, keratinocytes were applied either as sheets or aerosolized fibrin glue suspension.

RESULTS: Histological analysis revealed a wave of inflammatory cells and early granulation tissue ingrowth into the Integra from the fascia below on day 7. Only this initial phase was augmented by application of fibrin glue to the wound bed. By day 14, most and by day 21, all of the Integra thickness was incorporated. Accelerated dermal repair proceeded from the base with new collagen deposition in Integra spaces. There was no evidence of keratinocyte engraftment, although re-epithelialization occurred at wound edges extending onto the incorporated Integra.

CONCLUSIONS: It appears there is an acceleration of early phase (day 7 to day 21) dermal incorporation with fibrin glue application to the wound bed, perhaps secondary to increased cellular migration. Day 21 appears to be too early to apply cultured keratinocytes either as sheets or aerosolized suspension.

Key Words: Burns; Fibrin glue; Integra; Keratinocytes; Neodermis, Skin replacement; Tissue Engineering

Hypertrophic scars, wounds that penetrate deep into the skin (full-thickness) and burns remain major clinical problems to patients and physicians. Transplantation of the patient's own skin by way of a split-thickness skin graft remains the gold standard in the treatment of major skin loss (1). This

La cicatrisation d'imperfections cutanées pleine épaisseur de porcs au moyen d'Integra^{MD} avec et sans colle de fibrine avec kératinocytes

HISTORIQUE : Une matrice artificielle du derme, comme Integra (Integra Life Sciences Corporation, États-Unis), fournit une matrice pour le lit de la plaie, favorisant l'interposition vasculaire et fibrocytaire et le remodelage du collagène. La réparation dermique entraîne la régénération de l'épiderme et de la membrane basale. Les plaies causées par des brûlures, notamment, tirent profit d'Integra par une meilleure cicatrisation de la plaie.

OBJECTIF : Évaluer l'effet de la colle de fibrine pour modifier l'intégration d'Integra à de grosses plaies cutanées excisées. Les auteurs postulaient qu'en appliquant de la colle de fibrine sur le lit de la plaie, la vascularisation de la matrice, l'incorporation d'Integra et la prise des cultures de kératinocytes s'effectueraient plus rapidement.

MÉTHODOLOGIE : On a pratiqué quatre plaies cutanées profondes sur le dos de deux porcs. Les lits des plaies étaient répartis de manière aléatoire entre l'application et la non-application de colle de fibrine avant l'application d'Integra. On a procédé à des biopsies profondes les jours 7, 14, 21, 29 et 35. Le jour 21, les kératinocytes étaient appliqués sous forme de feuilles ou de suspension en aérosol de colle de fibrine.

RÉSULTATS : L'analyse histologique a révélé une vague de cellules inflammatoires et d'interposition précoce des tissus de granulation d'Integra à partir du fascia à compter du jour 7. Cette phase initiale était dynamisée par l'application de colle de fibrine sur le lit de la plaie. Le jour 14, presque toute l'épaisseur d'Integra était intégrée, et le jour 21, elle l'était en entier. La réparation dermique accélérée avait commencé à la base, par de nouveaux dépôts de collagène dans les espaces d'Integra. On n'a remarqué aucune trace de prise de greffe des kératinocytes, mais une reprise de l'épithélialisation aux bordures de la plaie jusqu'à l'Integra intégré.

CONCLUSION : Il semble que le début de l'incorporation dermique soit accéléré (jours 7 à 21) par l'application de colle de fibrine sur le lit de la plaie, peut-être en raison de la migration cellulaire accrue. Le jour 21 semble trop tôt pour appliquer des cultures de kératinocytes sous forme de feuilles ou de suspension en aérosol.

modality incurs significant donor site morbidity such as bleeding and scar formation (1). In addition, available harvest sites from the patient limit its supply (2). Advances in tissue engineering have allowed for decreases in the donor site morbidity and potential for a limitless supply of skin (3).

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Figure 1) Creation of four full-thickness 10 cm × 5 cm rectangular wounds and the application of Integra with and without fibrin glue

The development of an artificial dermal matrix has been a great advance in the treatment of full-thickness wounds (4). One such engineered tissue is Integra (Integra Life Sciences Corporation, USA), a bilayer membrane designed to provide immediate wound closure and permanent regeneration of the dermis (5). It is composed of an outer layer of silicone to protect the wound and an inner matrix of cross-linked fibres that act as a scaffold for regenerating dermal skin cells. Once the dermal component has regenerated, the silicone outer layer is removed and replaced with a thin split-thickness skin graft. A novel approach involves using Integra with cultured or aerosolized keratinocytes and fibrin glue. Experiments have shown that Integra, when combined with skin grafts or sprayed cells, assists in the healing of full-thickness wounds and burns (6,7). Likewise, Integra has also been approved for use in the hypertrophic scar (8).

We hypothesized that applying Integra with fibrin glue to a wound bed would reduce the time needed for matrix vascularization and incorporation of the Integra and take of the cultured keratinocytes.

METHODS

Institution review board approval was obtained and animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Housing and care for the animals was in accordance with the National Research Council guidelines (9).

Animals, anesthesia and surgery

Two female Yorkshire swine weighing approximately 20 kg each were initially induced with ketamine (20 mg/kg) and xylazine (2 mg/kg) and anesthetized with isoflurane 1% to 2%. Each animal also received a single dose of procaine/penicillin G as prophylaxis. After shaving the dorsum of the swine and prepping the skin with alcohol, four 10 cm × 5 cm rectangles were measured using a template separated by 2 cm. The four corners of each square wound were tattooed. In addition, the anterior and ventral edges of each square were tattooed in 1 cm increments. Each square was labelled A through D. A surgical preparation of betadine was applied and then washed with alcohol. Photographs were taken with a Gateway DC-T50 (5.25 megapixel) camera. The animals were draped with sterile surgical towels. Incisions were made along the wound edges with a number 15 surgical blade to the panniculus carnosus layer and the overlying skin was excised (Figure 1). A randomly selected piece of skin from each pig was defatted and sent to the The Living Skin Bank (Stony Brook University, New York) to create cultured cells (cultured epidermal grafts and keratinocytes). The sheets of Integra were soaked in two separate 2 L saline baths after having been removed from their packing. The Integra was trimmed to the wound size. The wound defects were randomized to application of fibrin glue or no application of fibrin glue on each swine such that two wounds had fibrin glue applied and two did not. The trimmed Integra was then stapled to the wound edges of each of the four defects labelled A, B, C and D. Triple antibiotic ointment (polymyxin B/sulfate-bacitracin/zinc-neomycin sulfate; Taro Pharmaceuticals USA, Inc) was applied to all of the wounds, followed by a layer of Xeroform petrolatum dressing (Kendall Healthcare Products Company, USA). The wounds were then covered with 10 cm × 10 cm sterile gauze and secured in place by a conform roll wrapped around the swine. An Elastoplast (Beiersdorf Inc, USA) bandage was then wrapped around the swine, and the distal edges were secured to the dorsum of the swine with staples. The veterinary staff evaluated the swine daily. Wound checks were performed weekly after anesthetizing the pigs with isoflurane by mask. The dressings were removed and photographs were taken. The swine received an intramuscular dose of 225,000 U of penicillin after each dressing change. In addition, they were administered subcutaneous buprenorphine 0.01 mg/kg and a 75 µg fentanyl patch. The wounds were then redressed with Xeroform. On day 21, the silicone layer was removed and either cultured autologous keratinocytes ($1 \times 10^6 \text{ cm}^{-2}$) or keratinocytes suspended in a 2 mL aliquot in swine keratinocyte culture medium mixed with 1 mL of the fibrin/apoprotein component of Tisseel fibrin glue (Baxter, USA) were applied. Dressing changes and punch biopsies were performed on days 7, 14, 21 and 35. Four-millimetre punch biopsies were taken from the central portion and top left hand corner of each wound, individually labelled and preserved in formalin. On days 7, 14, 21, 29 and 35, gross and histological comparisons were made. All wounds were photographed at right angles to the wounds and the dressings (Figure 1). On day 35, the swine were sacrificed with a lethal dose of sodium pentobarbital.

Keratinocyte culture

The harvesting of the keratinocytes was performed on day 0 at the time of application of Integra to the created wound bed. Swine keratinocytes were isolated and cultured with

Figure 2) On day 21, the silicone layer was removed and cultured keratinocytes were applied to the wound as sheets or aerosolized with fibrin glue. Dressing changes and punch biopsies were performed on days 7, 14, 21 and 35

lethally irradiated 3T3-J2 cells using media modifications previously described (10,11). Each biopsy specimen was washed in calcium-free, magnesium-free, phosphate buffered saline, pH 7.4, and incubated overnight at 4°C in a 0.25% solution of dispase type II. The tissue was then incubated for an additional 2.5 h at 37°C, and the epidermis was peeled from the underlying stroma. To isolate single keratinocytes, the epidermal sheet was incubated for an additional hour at 37°C in phosphate buffered saline containing 0.005% Trypsin 1-300 (Porcine Pancrease – MP Biomedicals Inc, USA) and 0.01% EDTA. The trypsin was neutralized by addition of fetal bovine serum (HyClone, USA) to 5% and the cells were collected by centrifugation at 200 g for 6 min. Supernatants were discarded, and the pelleted cells and tissue fragments were resuspended in keratinocyte media before plating in tissue culture flasks (T174, Costar, Corning Life Sciences, USA) seeded with 3×10^6 lethally irradiated 3T3-J2 cells. Cells were grown at 37°C in 7.5% CO₂ (4). On day 21, the silicone sheets were removed from the Integra and either an autologous keratinocyte suspension or cultured epithelial sheets were applied (Figure 2).

Histological assessment

Histopathological studies were performed on formalin-fixed, alcohol-dehydrated, xylene-cleared, paraffin-embedded, 5 µm sections stained by hematoxylin and eosin, trichome, and Sirius Red or Herovici using conventional microscopy with an Olympus BX61. Digital Images were captured using an Olympus DP70, 12 megapixel camera (Olympus, USA).

RESULTS

Macroscopic assessment

The wounds appeared to heal well in each group with minimal swelling or erythema. All the grafts had contact with the wound beds. There was no evidence of wound infection. The Integra size was not quantified in the present study.

Histological analysis

Hematoxylin and eosin stains were performed on the punch biopsies obtained on days 7, 14, 21, 29 and 35. Sirius Red and

Figure 3) Day 7. **A** Integra (Integra Life Sciences Corporation, USA) with fibrin glue showing early incorporation. **B** Integra without fibrin glue showing no incorporation. GT Granulation tissue

Herovici differential collagen stains were performed on days 21 and 29. Herovici stained the Integra collagen red and the glycosaminoglycans within the pores sky blue. On day 7, early granulation tissue ingrowth into the Integra with fibrin glue was observed. Integra without fibrin glue showed no incorporation (Figure 3). The wound beds treated with fibrin glue showed evidence of accelerated dermal repair at postgraft day 14 to postgraft day 21 (Figure 4). Collagen fibres from the Integra matrix with fibrin glue on day 14 were recognized on higher magnification. Giant histiocytes (foreign body reaction) were also seen with Integra and fibrin glue on day 14 with granulomas within the Integra pores. One of the eight day 21 wounds appeared to have delayed incorporation. The presence of these cells indicated an inflammatory process. Collagen in these wounds appear to be a function of remodelling of dense spicules of Integra collagen and new collagen deposition following ingrowth of fibrocytes into the Integra pores. This appeared as a wave phenomenon; at each time point there was a greater amount of new collagen at the base with less Integra visible (Figure 5). One curiosity is that the Integra, initially 1 mm in thickness, expanded as granulation tissue grew into the wound space and yet remained visible near the surface even after the 8 mm to 10 mm thick wound defect was completely filled. The initial inflammatory response preceded and was replaced by vascular and collagen fibre ingrowth. The refractile, dense Integra collagen was expanded by the inflammatory cells, mainly histiocytes, initially, filling

Figure 4) Day 14. **A** Integra (Integra Life Sciences Corporation, USA) with fibrin glue. **B** Integra without fibrin glue. GT Granulation tissue

what other authors have described as 'pores'. A wave of capillaries and fibrocytes followed and Integra collagen became incorporated into the pericapillary structure. Simultaneously, new less refractile collagen was being deposited into the 'pores'. What originated as 1 mm of Integra eventually expanded into a 9 mm to 10 mm area of granulation tissue, akin to a flat compressed sponge expanding in water. This was due initially to fluid expansion, then inflammatory cell migration from vascular and collagen fibre ingrowth, and finally by collagen secretion and cross-linking. Previous descriptions of Integra healing (12) have not described this expansion or feature due to the great depth and expansion of the wounds. Gradually, the Integra became more difficult to visualize, apparently incorporated into the perivascular collagen scaffolding. Some Integra collagen spicules were less readily incorporated and remained visible for an additional one to two weeks, surrounded by foreign body-type multinucleated histiocytes. For example, in one of eight day 21 wounds, the histiocytes around Integra spicules predominated, yet disappeared by day 35. Qualitative analysis of collagen by polarized Sirius Red staining performed on day 21 revealed that dense collagen was present in the specimen with Integra and fibrin glue. In addition, there was more granulation tissue present in this specimen. On day 35, the Integra with and

Figure 5) Day 21 wound with Integra (Integra Life Sciences Corporation, USA) and fibrin glue. 'Wave' phenomenon of Integra incorporation and progression of new collagen deposition. Sirius Red stain demonstrates pattern of early incorporation near surface, while deep illustrates the progressive collagen deposition around capillaries and in Integra 'pores' near base

without fibrin glue was totally incorporated and unrecognizable by any stain. Re-epithelization was noted from the wound edges onto the incorporated Integra predominately in the wound beds with fibrin glue.

DISCUSSION

The development of an artificial dermal matrix has been a great advance in the treatment of full-thickness wounds. These products have been successfully used not only in burns, but also as soft tissue replacements. A recent product, Integra is a bilayer membrane designed to provide immediate wound closure and permanent regeneration of the dermis. The use of a material like Integra allows for the harvest of an extremely thin autograft (0.006 mm), thereby reducing morbidity at the harvest site. Nonetheless, a new wound must be made to treat the old wound and one is limited by the finite amount of available normal skin. The larger the total body surface area of the burn, the less skin available for grafting. In the present study, we sought to investigate whether fibrin glue would accelerate the integration of the dermal regeneration template (Integra) in a 10 cm × 5 cm excised rectangular wound bed and if so, if it would allow for earlier incorporation of cultured keratinocytes, either as sheets or aerosolized in fibrin glue suspension. The histological assessment of the punch biopsies (day 7 to day 21) revealed that there is an acceleration of early phase dermal incorporation with fibrin glue. In addition, gross observation of the wounds revealed evidence of peripheral epithelialization suggesting successful incorporation of Integra. Peripheral epithelialization was not evident on day 21 but was on the day 29 dressing change, with increased collagen, increased granulation tissue and fewer residual Integra fibres. This suggests that applying cultured keratinocytes either as a suspension or sheets on day 21 was too early, even in the face of earlier take of the Integra with fibrin glue. It has been shown by Currie et al (12) that fibrin glue increases the shear adhesive strength. It also provides an immediate, highly cross-linked fibrin network that stabilizes the graft and facilitates graft nutrition by serum imbibition followed by ingrowth of vascular beds. A fibrin scaffold encourages an environment for the influx of fibroblasts and blood vessels. In our study, the use of fibrin glue at the wound base led to a temporary

and transient increase in migration of inflammatory cells and granulation tissue. By day 21, clinically and histopathologically, the wounds appeared to be identical.

Achieving an ideal, permanent, replacement of skin in full and deep partial thickness burn injuries and chronic wounds remains a significant surgical problem. There have been many studies over the past several years investigating the efficacy of various skin substitutes to treat acute and chronic skin defects (burns, scars, cutaneous ulcers and congenital cutaneous anomalies) (13-17). These studies, however, used cultured keratinocytes with and without fibrin glue to determine neoepithelialization. Presently, there are no studies in the literature that used Integra in combination with fibrin glue followed by application of cultured epithelium as a sheet or suspension. Keratinocytes can be applied in a solid form as a thin flexible transparent film (18,19). They can also be sprayed onto the wound as a keratinocyte-fibrin glue suspension. However, keratinocyte sheets have a relatively unpredictable take rate and produce an unstable epidermis. Delivery of autologous keratinocytes to the wound surface in suspension has been used as an alternative method that is easier to perform and can be frequently repeated. Fraulin et al (20) demonstrated the use of aerosolized spray to deliver viable keratinocytes to a porcine wound bed without autograft. The wounds receiving the sprayed cells re-epithelialized faster than the unsprayed. Navarro et al (21) studied this combination with split-thickness skin grafts. Keratinocytes can remain viable as a suspension in commercially available fibrin glue for approximately five days. Fibrin glue is an excellent template for cellular migration, and can be modified to create an ideal pore size for cells such as keratinocytes. It has been shown to improve the percentage of skin graft take as well as hemostasis, and has a bacterial protective effect. In a recent paper by Potter et al (22), fibrin was identified as a better proangiogenic scaffold than collagen for use in synthetic dermal replacement. Cohen et al (23) demonstrated that the combination of fibrin glue and an aerosol of noncultured epidermal cells significantly enhanced the epithelialization of wounds. In the present

study, keratinocytes ($1 \times 10^6 \text{ cm}^{-2}$) were applied too early to the Integra bed, thus limiting our outcome. Theoretically, application of cultured keratinocytes at day 29 may have enhanced the survival of the keratinocytes, improving our results because they may have been applied too early. Furthermore, increasing the number of swine used in the study could provide a better comparative analysis between the wounds with Integra with and without fibrin glue and cultured keratinocytes as sheets versus suspension.

The interplay of Integra, fibrin glue and keratinocytes in early dermal repair is complex. If we can optimize this relationship, open wounds will eventually be able to be covered with minimal donor site morbidity by using such a permanent skin replacement. Further analysis of collagen deposition and their upward ingrowth with fibrocytes into Integra pores may lead to a better understanding of the wave phenomenon we demonstrated. A follow-up study is currently underway, in which we will be comparing Integra with and without fibrin glue to split-thickness skin grafts. If we find that Integra with sprayed cells/fibrin sealant or cultured skin cell combinations helps wounds heal as effectively as or better than those treated with the gold standard, this would provide for a new tissue engineered skin.

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