

Clinical Application and Histological Properties of Autologous Tissue-engineered Skin Equivalents Using an Acellular Dermal Matrix

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Abstract

We developed a transplantable tissue-engineered skin equivalent composed of autologous cultured keratinocytes, fibroblasts, and a decellularized allogeneic dermis (acellular allogeneic dermal matrix; ADM) obtained from cadavers. In a process taking 3 weeks, cultured autologous keratinocytes from burn patients were expanded and then grown on ADMs. The tissue-engineered autologous skin equivalents (TESEs) were then transplanted in a one-stage procedure to the debrided third-degree burn wounds of 4 patients. The mean graft survival rate was 96%. Delayed graft loss and graft fragility were not observed. Histological and immunohistological findings indicated that the transplanted TESE had similar characteristics to normal human split-thickness skin grafts. These results suggest that the TESE using ADM can be used for permanent repair of full-thickness skin defects.

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Key words: tissue engineering, skin equivalent, acellular dermal matrix

Introduction

Cultured autologous keratinocytes are used to cover extensive skin defects, such as those after major burns¹. Keratinocytes are used in the following main transplantation methods: transplantation of cultured keratinocytes as epidermal sheets^{1–3} and use of cultured autologous keratinocytes in tissue-engineered skin equivalents (TESEs)^{4–6}. Epidermal sheets show improved survival after transplantation to extensive burn areas³ but are fragile and unstable because of the lack of a dermal component^{2,3}. A TESE, on the other hand, consists of an epidermal layer with a dermal matrix

layer embedded with fibroblasts^{4–6}. A dermal layer of bovine collagen matrix has commonly been used. Despite early successes, cultured skin substitutes have not been accepted widely because of variable rates of graft survival, low graft quality, and the complexity of postoperative care^{5,6}.

To resolve these problems, we developed transplantable autologous TESEs using an acellular allogeneic dermal matrix (ADM) derived from human cadaver skin as the dermal component. Because of its physiological properties, ADM has been used successfully for such indications as dermal regeneration, abdominal wall reconstruction, and breast reconstruction after mastectomy^{7,8}. However, the use of ADM as a dermal scaffold in

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cultured skin substitutes has been limited. We have previously reported preliminary clinical results of transplantation of TESEs using ADM^{9,10}. Here, we describe the clinical, histological and immunohistological features of the transplanted autologous TESE.

Materials and Methods

1 Preparation of ADM

Pieces of split-thickness (0.25–0.50 mm thick) cryopreserved cadaver skin obtained from the Japan Skin Bank Network (the official skin bank of Japan) were rapidly thawed at 37°C in phosphate-buffered saline (PBS) (Gibco-BRL, Grand Island, NY, USA). The skin was incubated with 1 M sodium chloride at 37°C for 12 hours to separate the epidermis from the dermis. The separated dermis was then incubated in PBS with continuous gentle agitation at room temperature for 7 to 10 days to remove all the cellular components, and then stored at 4°C.

2 Preparation of the TESE

To prepare autologous keratinocytes, small samples of healthy skin (2 cm square, 0.3 mm thick) were obtained from 4 patients with extensive burns. The epidermis and dermis were separated by exposing the skin to a neutral protease (Dispase, Godo Shusei Co. Ltd., Tokyo, Japan) for 3 hours at 37°C. The epidermis was treated with 0.25% trypsin (Gibco-BRL) for 15 minutes at 37°C to disaggregate keratinocytes. The separated dermis was cut into small pieces and placed in dishes to obtain cultures of dermal fibroblasts. After the pieces of dermis had become attached to the culture dishes, Dulbecco's modified Eagle's medium (Gibco-BRL) with 10% fetal calf serum (FCS) (Gibco-BRL) was added to the dishes, which were then incubated at 37°C in 5% CO₂. Keratinocytes were collected, centrifuged, and resuspended in a keratinocyte growth medium (KGM; Defined Keratinocyte-SFM, Gibco-BRL). The KGM did not contain bovine pituitary extract. To create the TESE, the subcultivated autologous fibroblasts ($5 \times 10^5/\text{cm}^2$) were seeded onto the reticular side of the ADM in 2% FCS and KGM. Two days later, subcultivated keratinocytes ($5 \times 10^5/$

cm^2) were seeded onto the basement membrane side (dermoepidermal junction side) of the ADM in the same medium. After 2 or 3 days of culture, the medium was changed to KGM with 10% FCS to induce keratinocyte differentiation. After an additional day of culture, the cultured TESE was transferred to an air-liquid interface to promote stratification of the epithelial layer. After 5 to 7 days of culture at the air-liquid interface, the TESE was ready for clinical use. The average time required to create a TESE from the recipient's skin was 21 days.

All procedures for preparation and clinical application of ADMs were approved by the ethics committee of Kyorin University (No. 07), Tokyo, Japan, and performed with the patient's informed consent.

3 Clinical Applications of Autologous TESEs

The autologous TESEs were washed thoroughly with Hanks's balanced salt solution (Gibco-BRL) before being transplanted onto the defects of the 4 patients with extensive burns. A sheet of TESE was placed onto a debrided deep burn wound and fixed to the wound bed with 4-0 Vicryl[®] sutures (Ethicon, Inc., Sommerville, NJ, USA). Then, the transplanted TESE was covered with a petroleum jelly (Vaseline, Unilever, Rotterdam, The Netherlands)-soaked, silicone-coated gauze and immobilized with a bolus gauze dressing. The transplant site was opened on the fifth postoperative day.

Patient 1 was a 36-year-old woman with third-degree burns to 46% of the body surface. Four 5×5-cm sheets of TESE were transplanted to the debrided third-degree burn wound of the right thigh. Patient 2 was a 63-year-old man with third-degree burns to 40% of the body surface. Seven sheets of TESE were transplanted to the debrided third-degree burn wound of the abdomen. Patient 3 was a 29-year-old woman with second- and third-degree burns to 75% of the body surface. One 5×2.5-cm TESE was transplanted to the debrided third-degree burn wound of the right thigh. Patient 4 was a 41-year-old woman with second- and third-degree burns to 98% of the body surface. Four 5×5-cm sheets of TESE were transplanted to the debrided third-

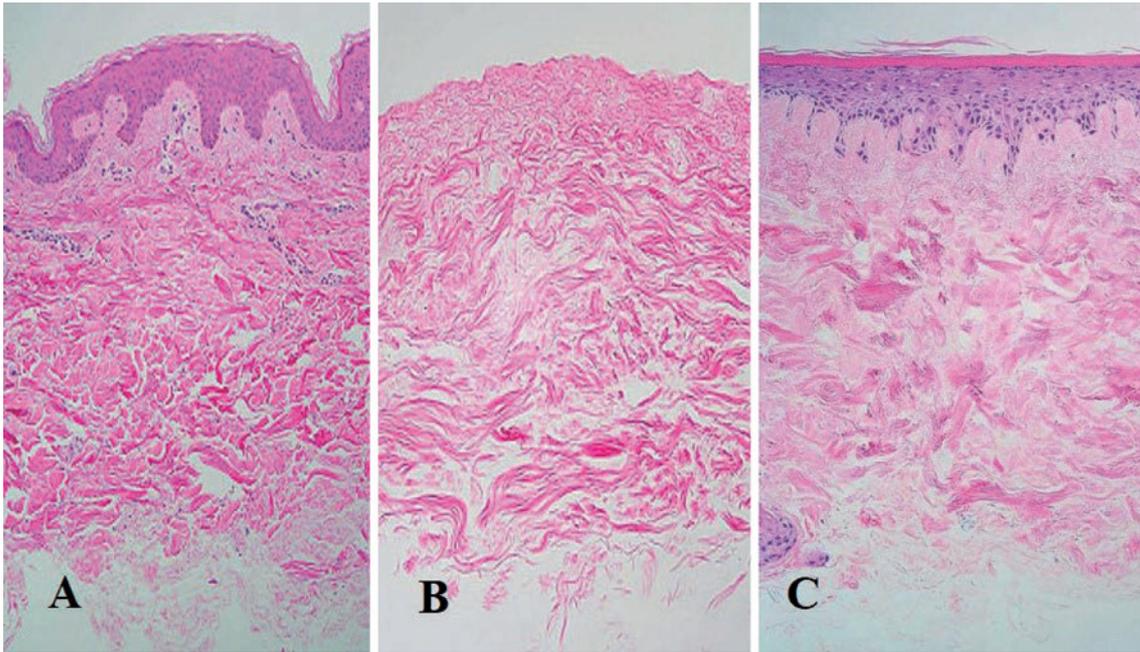


Fig. 1 Histologic features of ADM and TESE. (A) Cadaver allogeneic skin. (B) Acellular allogeneic dermal matrix (ADM) after decellularization. (C) Preoperative appearance of a tissue-engineered skin equivalent (TESE). Allogeneic cells were removed and replaced with patient's cells. (H&E staining, original magnification: 40 \times).

degree burn wound of the abdomen.

4 Histological Study

Samples of ADM and TESE were fixed in 10% formalin, and routine paraffin sections were stained with hematoxylin and eosin (H & E). They were also stained immunohistochemically with antibodies against the following: type IV collagen, laminin, involucrin (Sigma-Aldrich, St. Louis, MO, USA), Ki-67, and β 1 integrin (Dako, Glostrup, Denmark). The avidin-biotin-peroxide complex technique with diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) was used.

Results

1 Histologic Features of ADM

The ADM had no epidermal or dermal cells (**Fig. 1A, B**). The ADM showed dense collagen fibers of various sizes and had a similar biological structure to the native dermis, which included the bilayer dermal structure with papillary and reticular dermis. Immunohistological studies showed staining for type IV collagen and laminin and indicated that the

basement membrane was intact in the prepared ADM (**Fig. 2**).

2 Clinical Results

Graft survival was evaluated 14 days after transplantation (**Fig. 3, 4**). The survival rate of the transplanted TESE was 93% for patient 1, 90% for patient 2, 100% for patient 3, and 96% for patient 4. The areas covered by TESEs had completely re-epithelialized by 28 days after transplantation. Although various degrees of hypopigmentation were seen on the transplanted TESEs, there was no delayed graft loss or graft fragility during the observation period (42 to 270 days) (**Fig. 3, 4**). Patients 1 and 2 were discharged, whereas patients 3 and 4 died of multiple organ failure approximately 50 days after TESE transplantation. However, there was no evidence to suggest that the transplantation contributed to their deaths.

3 Histological Analysis of Autologous TESE Transplantation

Examination of H & E-stained slides showed that the seeded keratinocytes adhered to the ADM and

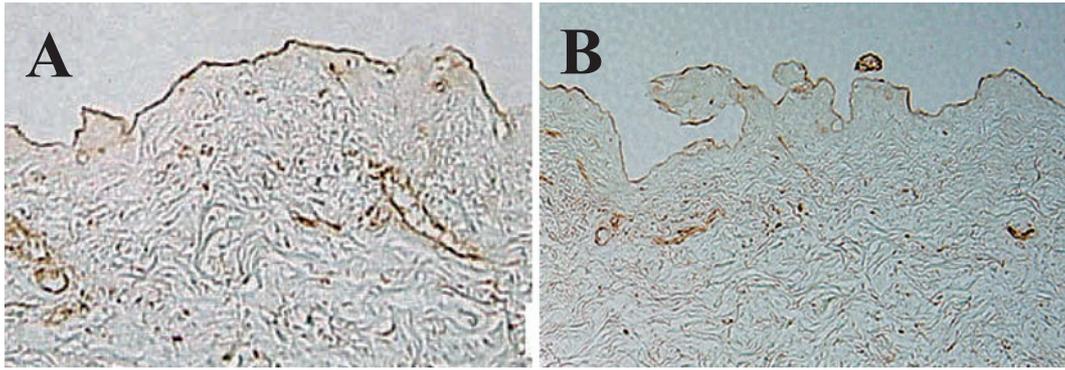


Fig. 2 Immunohistochemical staining of ADM. **A**, Immunohistochemical staining for type IV collagen. **B**, Immunohistochemical staining for laminin. (Original magnification: 100×).

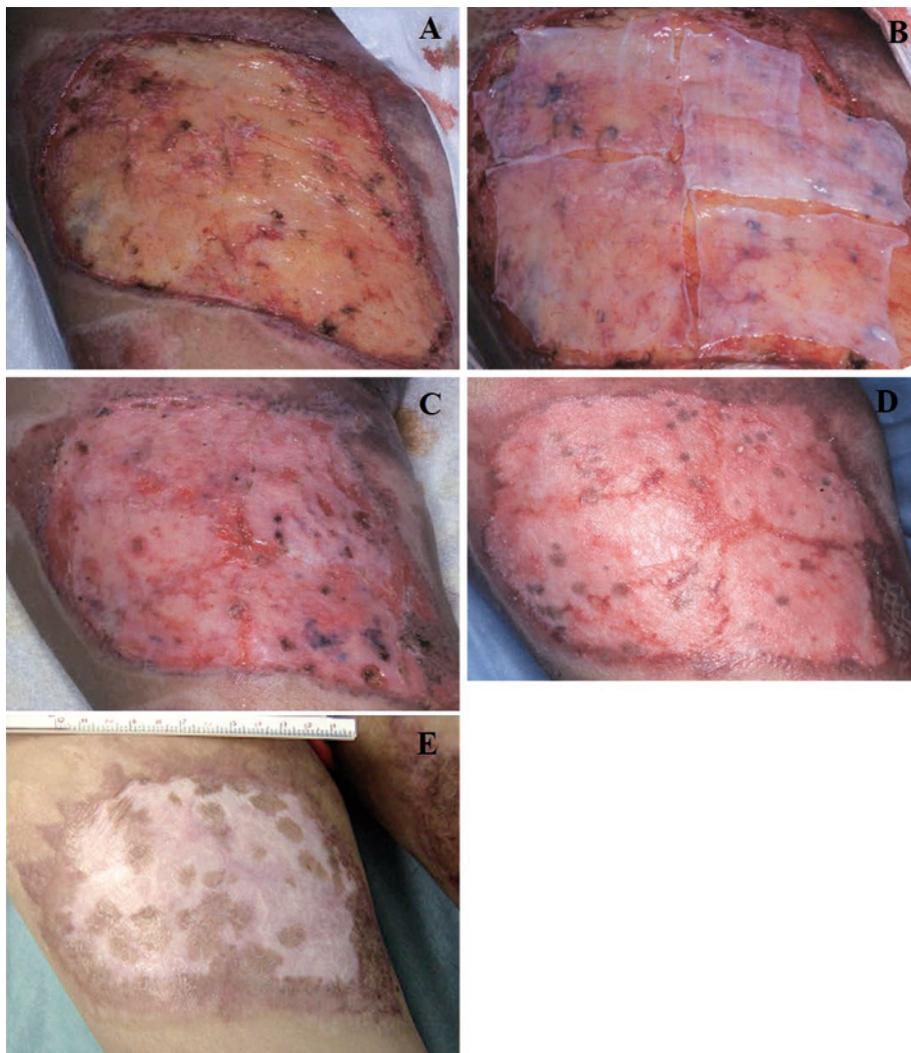


Fig. 3 Appearance of transplanted autologous TESE (Patient 1, a 36-year-old woman). **A**, Debrided deep burn wounds of the right thigh. **B**, TESEs (4 sheets of TESE, mean size: 5×5 cm) were transplanted onto the wounds. **C**, Fourteen days after transplantation. **D**, Forty-two days after transplantation. **E**, Nine months after transplantation. The transplanted TESEs survived well without delayed graft loss.

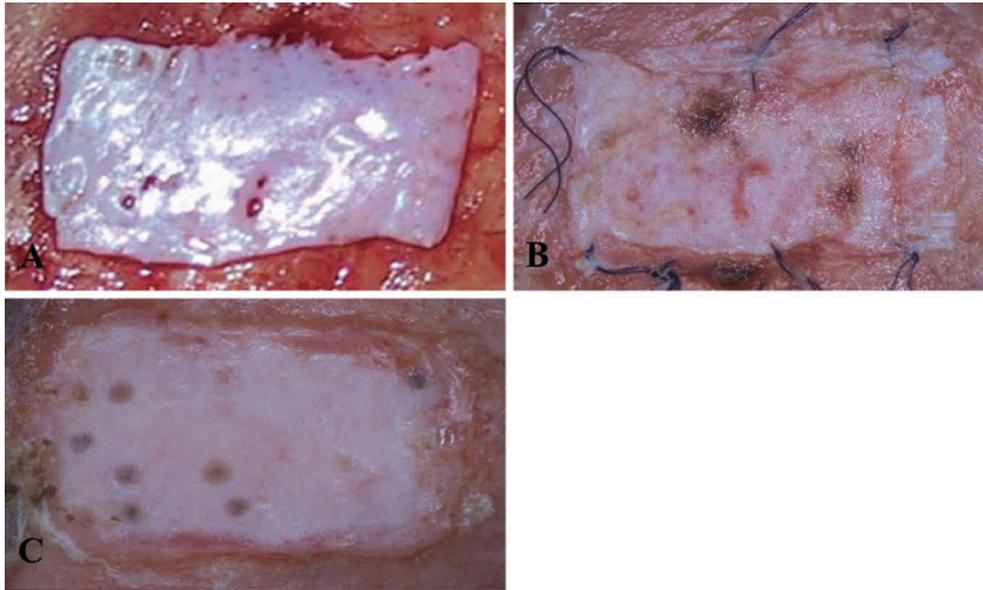


Fig. 4 Appearance of transplanted autologous TESE (Patient 3, a 29-year-old woman). **A**, One sheet of autologous TESE (5×2.5 cm) was placed onto a debrided deep burn wound of the right thigh. **B**, Seven days after transplantation. **C**, Forty-two days after transplantation. The TESE survived completely.

formed a confluent epithelial layer, which progressed to stratification and differentiation. Rete ridge structures were preserved (**Fig. 1C**, **Fig. 5A**).

Immunohistochemical studies showed staining for type IV collagen along the dermoepidermal junction (**Fig. 6A**) and staining for $\beta 1$ integrin in the basal layer of the epidermal cells (**Fig. 6C**). These findings indicated that the basement membrane structure was present and that cellular attachment to the ADM was consistent with normal dermoepidermal attachment. Expression of Ki-67 was more prominent in the TESE than in the normal epidermis but was located only in the basal epidermal layer of the TESE; this finding indicated that active mitosis was present in the basal layer without evidence of neoplastic changes (**Fig. 6E**). Involucrin was detected in a location similar to that in normal skin and suggested the formation of the keratinized envelope and epidermal polarity (**Fig. 6G**).

Forty-two days after transplantation, biopsy specimens were taken from patient 3. Staining with H & E (**Fig. 5B**) showed that the dermal part of the TESE (transplanted ADM) had a well-vascularized dermal matrix, normal collagen structures, and a remaining bilayer structure with papillary and

reticular dermis. No immunological rejection was noted. The transplanted TESE was incorporated well with the wound bed. A slightly hypertrophic epidermis with stable attachment of the epidermis to the ADM was noted. Staining for type IV collagen was seen along the dermoepidermal junction of the transplanted TESE (**Fig. 6B**). There were no significant differences in $\beta 1$ integrin and Ki-67 expression before and after transplantation (**Fig. 6D**, **F**). Staining for involucrin showed the keratinized envelope and epidermal polarity, as was seen before transplantation (**Fig. 6H**). These findings indicated that the transplanted TESE had characteristics similar to those of normal human split-thickness skin grafts (STSGs).

Discussion

We have created transplantable TESEs closely resembling normal STSGs by incubating cultured autologous keratinocytes and fibroblasts from burn patients with ADMs obtained from cadavers. The resulting TESE has a durable, well-keratinized epidermal layer firmly attached to the ADM. The ADM retains an intact basement membrane zone that helps keratinocytes adhere^{11,12}. The ADM also

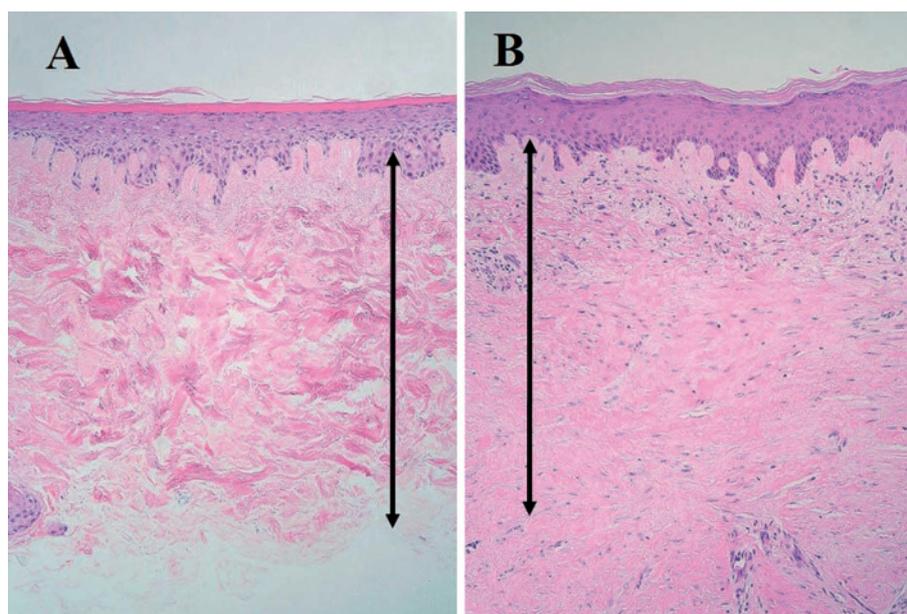


Fig. 5 Histologic features of transplanted TESE (Patient 3). **A**, Autologous TESE before transplantation. **B**, Forty-two days after transplantation. Arrows indicate the areas of ADM as dermal layers of TESEs. The transplanted TESE was incorporated well with the wound bed. (H & E staining; original magnification: $\times 40$).

offers an ideal environment for the growth of angiogenic cells because the physiological structures of the dermis, such as bilayer structures with papillary and reticular dermis, are better preserved in the ADM than in other types of artificial dermis^{7,13}.

Bovine collagen matrix has been widely used as a dermal component in TESEs. Boyce et al. have described clinical results of a TESE (cultured skin substitutes) that used a collagen sponge as the dermal component⁵⁶. They reported that the survival rate of the cultured skin substitute graft was 81.5% and that that of STSG was 94.7%. The number of cases of transplantation in our study was small, but the mean graft survival rate was 96%. This result highlights the advantage of using ADM as the dermal component in cultured skin equivalents.

Although several types of ADM-based cultured skin equivalents have been produced, the results of clinical studies have been far from satisfactory¹⁴⁻¹⁷. To improve clinical results, we prepared the ADM without protease, surfactants, or sterilizing procedures, which are commonly used for preparing ADM^{7,13,14}. Although our method of ADM preparation takes more time than methods using detergents, native dermal structures are better preserved and

the utility of the ADM as a scaffold for tissue-engineered skin is improved.

In the present study, the effects of fibroblasts seeded onto ADM were not elucidated. However, fibroblasts seeded onto TESEs have some beneficial effects on the transplantation of TESEs¹⁸⁻²⁰. Okazaki, et al. have reported that subepithelial fibroblasts enhance epithelial differentiation in cultured skin equivalents¹⁹. Eedag, et al. have reported that the addition of fibroblasts to ADM-based skin substitutes enhances early keratinocyte proliferation, epidermis formation, and dermal neovascularization²⁰. These findings suggest that fibroblasts seeded onto ADM-based TESEs contribute to epidermalization and improve the survival of the TESEs mainly from the preparation process to the early phase of TESE transplantation.

On the other hand, bone marrow-derived fibroblasts and myofibroblasts, as well as locally preexisting fibroblasts, have been suggested to promote dermal regeneration and wound healing²¹⁻²³. In our study using a burn injury model in rats, we have found that bone marrow-derived fibroblasts, which are recruited to the wounded dermis from 10 to 14 days after burning, may play an important role

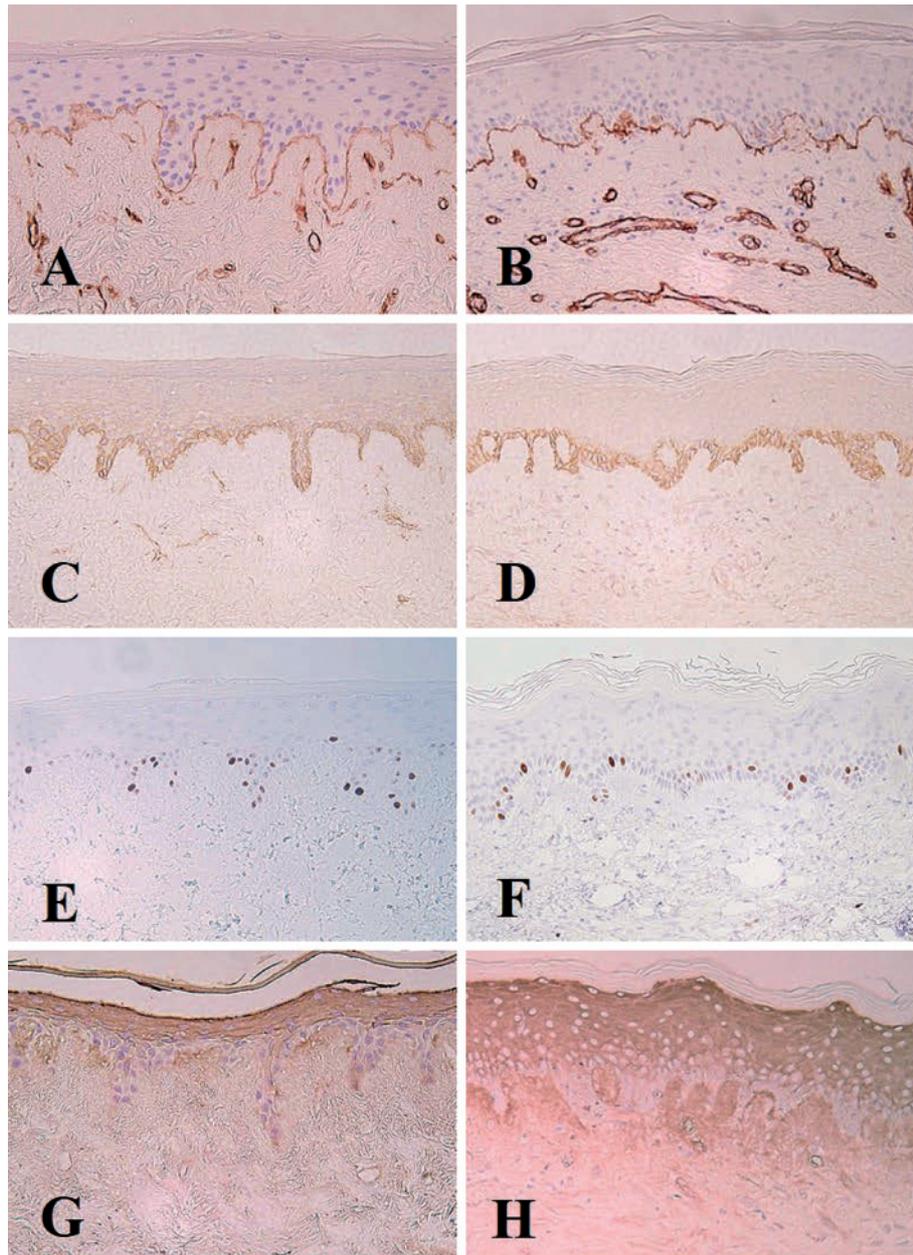


Fig. 6 Immunohistochemical staining of transplanted autologous TESE (Patient 3). The pictures on the right (A, C, E, G) are of the TESE before transplantation. The pictures on the left (B, D, F, H) are of the TESE 42 days after transplantation. A, B: type IV collagen. C, D: β 1 integrin. E, F: Ki-67. G, H: involucrin. (Original magnification: 100 \times).

in enhancing epithelialization and promoting dermal regeneration²². These findings suggest that recipient-derived fibroblasts, compared with fibroblasts seeded onto the TESE, may have more important effects on dermal regeneration, such as maintaining the bilayer dermal structure, in the proliferative and remodeling phase of wound healing after transplantation of ADM-based TESEs.

Major limitations of TESEs using ADM include a

risk of disease transmission due to allografts, the time and cost of TESE production, and hypopigmentation of transplanted sites. Every skin bank has the same problem of disease transmission; however, a stricter donor-selection process has helped address this problem. However, the problems of the time and cost of TESE production remain unresolved. Improvements in cultivation of keratinocytes and fibroblasts may be needed to

resolve these problems. Pigmentation disorders of transplanted sites are common in transplanted epidermal sheets and TESEs, because of the difficulty of regulating the number of melanocytes in the cultivation of keratinocytes¹⁶. Further study is needed to establish methods using cultured melanocytes for producing pigmentation-controlled TESEs.

In conclusion, our study results suggest that TESEs using ADM can be used for permanent repair of full-thickness skin defects.

Conflict of Interest: The authors declare that they have no conflict of interest in this study.

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