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Feasibility study of composite skin reconstructed by mixing keratinocytes and acellular dermal matrix for wound repair

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Summary

Questions under study: Composite skin containing autologous keratinocytes is a new approach to solving the problem of extensive skin defects. We propose a new strategy to construct the composite skin by mixing autologous and non-autologous keratinocytes, so that the time and the cost for constructing the composite skin could be reduced.

Methods: Human keratinocytes were mixed with Balb/c mouse keratinocytes at appropriate proportions, seeded onto the surface of porcine acellular dermal matrix (ADM) and cultured in vitro to reconstruct composite skin, which was then transplanted to a Balb/c mouse skin defect. Quality of the wound healing as well as the homing of the non-autologous keratinocytes were observed.

Results: Wounds healed well with the transplanted composite skin containing two different keratinocytes. The take rate of the grafts ranged from 78.3% to 81.5%. Histological observation showed that both epidermal and dermal structures of the regenerated skin were perfect and that the basal membrane was obvious with the laminin and collagen IV positively stained. In the early grafting phase, there are many non-autologous keratinocytes in the new epidermis. With the lapse of time, non-autologous keratinocytes decreased gradually and were eventually replaced by the autologous keratinocytes.

Conclusion: A composite skin was reconstructed by mixing two different keratinocytes at a certain proportion and then co-culturing them with dermal scaffold, which can be used for repairing full thickness skin defect wounds. This new strategy could save the source of autologous skin effectively and shorten in vitro culture time of composite skin.

Key words: composite skin; keratinocyte; mixed culture; acellular dermal matrix

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Introduction

In vitro reconstruction of composite skin containing autologous keratinocytes and a dermal substitute is a new approach to solving the problem of extensive skin defects, such as providing wound repair material for extensive deep burn patients [1, 2]. Our previous studies demonstrated that composite skin reconstructed in vitro from autologous keratinocytes and porcine acellular dermal matrix (ADM) was effective for wound repair and able to form a complete basilar membrane after the graft survives [3]. But as the source of autologous keratinocytes is limited and in vitro proliferation often needs 3–4 weeks or longer, it is not flexible enough to meet clinical requirements of various pathological conditions and treatment programmes [4]. In the present study we proposed a new strategy to construct the composite skin by mixing autologous and non-autologous keratinocytes and co-culturing them in vitro with acellular dermal matrix (ADM), so that the time and the cost for construction of the composite skin could be reduced. The feasibility of this new strategy for a permanent skin substitute was also explored in this study.

Methods

Culture of autologous and non-autologous keratinocytes

All the animal experiments of the present study were approved by the Animal Care Committee. Balb/c mice at 2–3 days of age were used as donors of autologous keratinocytes, while the non-autologous keratinocytes were taken from the skin tissue disposed from circumcision of healthy persons.

Keratinocytes from Balb/c syngeneic donors or from human xenogeneic donors were cultured individually in the same condition. They were incubated at 37 °C in 5% CO_2. The culture medium consisted of a mixture of DMEM and Ham's F12 made up with 10% FCS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin, 5 μg/ml transferring, 2×10^{-9} M triiodothyronine, 8×10^{-4} M adenine and 10 ng /ml epidermal growth factor. The medium was changed every 2–3 days.

Preparation of ADM

Normal pig split-thickness skin, 0.2 mm thick, was washed and treated with Dispase (GIBCO BRL Life Technologies, Inc. USA) followed by Triton X-100 (Sigma Chemical Co, St Louis, MO, USA) to completely remove epidermis and cellular components from the dermis. Routine histological observation showed that the tissue cells and epidermal appendages were absent. However, the basic dermal architecture of collagen bundles remained unaltered [5].

In vitro reconstruction of composite skin

Autologous and non-autologous keratinocytes were cultured and amplified. The second generation cells were digested to single cell suspension and trypan blue stained. The viability was greater than 90%. The experiment was conducted in five groups: autologous keratinocytes group (group 1), where all keratinocytes were autologous; 1:1 autologous/non-autologous keratinocytes group (group 2), where 50% keratinocytes were autologous and 50% were non-autologous; 1:3 autologous/non-autologous keratinocytes group (group 3) where 25% keratinocytes were autologous and 75% were non-autologous; 1:5 autologous/non-autologous keratinocytes group (group 4), where 17% keratinocytes were autologous and 83% were non-autologous; and non-autologous keratinocytes group (group 5), where all keratinocytes were non-autologous. keratinocytes were seeded on the epidermal side of ADM at a density of 5×10^5 cells /cm^2, and then cultured in vitro. On the 7th day after culture, a small piece of specimen was harvested. Histological section hematoxylin and eosin (H&E) staining confirmed that the keratinocytes had grown into a confluent layer. The composite skin was ready for transplantation.

Composite skin transplantation

Sixty Balb/c mice (Shanghai Sippe-Bk Lab. Animal Co., Ltd) were equally randomised into five groups described above, the mice were anaesthetised and hair on the back was shaved. Full thickness skin on the lateral side of the spinal column near the abdomen was resected to the depth of deep fascia to create a wound about 1.5 cm × 1.5 cm, to which the above composite skin was transplanted. To prevent interference with observation due to shrinkage of the wound edge and creeping of the epidermal cells around the wound, a graft chamber was used to separate the wound from the surrounding skin [6].

Gross observation of the graft

Two weeks after transplantation, the dressings were removed and the status of wound healing was observed. The criterion for survival of the composite skin: macroscopic evidence of a pink or normal colour epidermal layer. The criteria for necrosis: the wound turned brown or dark, the epidermal layer fell off and ADM was exposed about 2 weeks after transplantation. The criteria for rejection: the composite skin graft survived for 2 weeks and the epidermal layer was visible, but with lapse of time the colour turned brown or dark gradually, the epidermis and the dermis separated, the necrotic area of epidermis reached 50% or more, and histological study showed degeneration and necrosis of the epidermal matrix, and lymphocyte infiltration in ADM and at the junction of ADM and epidermis.

Survival rate of the graft

The wound was examined 3 weeks after grafting to determine the borders of the graft and areas of necrosis or rejection. The percentage of graft survival was determined using the paper template technique [7]. The percentage of graft take was quantified as follows:

$$\frac{(\text{Total area of graft} - \text{area of necrotic tissue}) \times 100\%}{\text{Total area of graft}}$$

Histological observation of the graft

The mice were sacrificed, and the specimens of the graft were harvested on the appropriate day (7, 14 or 21 days) post grafting. Some specimens were paraffin embedded and sliced into 5 μm sections for H%E staining. Epithelial thickness was measured with an ocular micrometer (Nikon, Inc., Garden, N.Y.) as described previously [8]. Three measurements were performed at different sites, and the average was taken as the epithelial thickness. Some specimens were OCT embedded, snap frozen in liquid nitrogen, and stored at –80 °C for immunohistological observation of laminin and collagen IV. Rabbit anti-laminin (1:500) and rat anti-collagen IV (1:200) antibodies were purchased from DAKO, Glostrup Denmark. Histological immunostaining was performed on frozen tissue sections. Frozen sections were picked up on glass slides coated with 1% 3-aminopropyltriethoxysilane in acetone dried overnight at 38 °C and incubated with the specific antibody for 3 h at 4 °C. Slides were developed by avidin-biotin peroxidase complex method using the appropriate Vectastain kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with triple strength hematoxylin and coverslipped using Glycergel (Dako, Carpinteria, CA). The other specimens were embedded in epoxy resin, sliced into ultra-thin sections, and examined under a transmission electron microscope.

Homing of non-autologous keratinocytes

The transplanted composite skin was sampled periodically and sectioned to observe the change in the number of human keratinocytes immunohistologically by using anti-HLA-ABC (DAKO) as the primary antibody described above.

Results

In vitro reconstruction of composite skin

Autologous and non-autologous keratinocytes adhered on the surface of ADM, and grew into a single cell layer about 1 week after culture, and formed composite skin containing 2–4 keratinocyte layers about 2 weeks after culture. There were no differences between mouse and human keratinocytes in term of adhesion and proliferation (fig. 1).

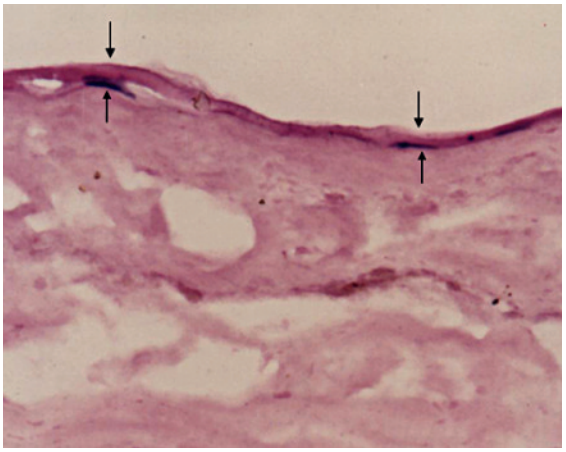

Figure 1

Mixed autologous keratinocytes at the ratio of 1:5 adhered on the surface of ADM, and grown into a single cell layer layer (arrow) about 1 week after culture in vitro (H.E. ×200).

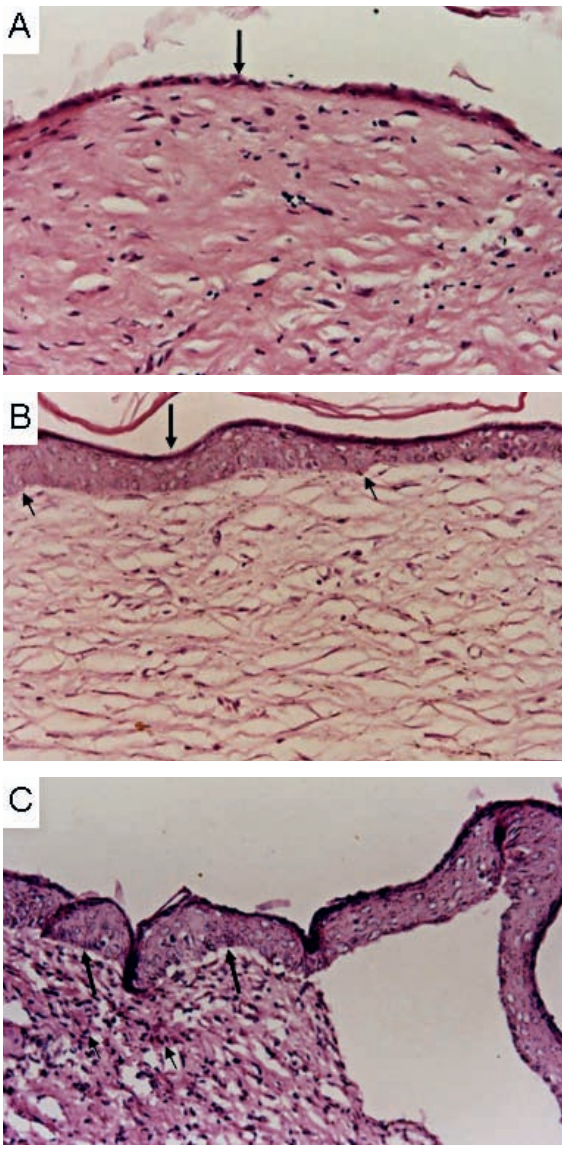

Figure 2

(A) By one week after composite skin transplantation using 1:5 mixed keratinocytes, the epidermis begins to form (arrow), and the epidermis-dermis junction is flat (H.E.×200). (B) Two weeks after grafting the epidermis becomes thicker (large arrow), the epidermal ridge begins to form (small arrow), and no evident infiltration of inflammatory cells is seen (H.E.×200). (C) At 3 weeks, the epidermis and the dermis separate (large arrow), and evident infiltration of inflammatory cells is seen in the non-autologous graft (small arrow) (H.E.×200).

Gross observation of the graft

Up to two weeks post-grafting, the gross appearances of the transplanted composite skin in group 1, 2, 3, and 4 were almost the same. The surfaces of the grafts looked pink and a very thin epidermal layer was seen. But in group 5, the epidermal layer of the grafts began to change in colour, became loose, and then became necrotic, representing an acute rejection reaction. At the third week post-grafting, the epidermis of the grafts separated from the dermis, and the necrotic graft of a large area was seen in group 5. But in the other groups, the colour of the composite skin reconstructed from mixed keratinocytes remained similar to that of the composite skin reconstructed from autologous keratinocytes only, where there was no evident colour change or epidermal separation from the dermis. Four weeks after grafting, the epidermal layer in group 5 was rejected completely, where ADM was exposed, vascularised and adhered closely with the wound. In group 2, 3 and 4, formation of the epidermal layer was seen, showing no significant difference from that of group 1 (fig. 5).

Survival rate of the graft

The skin-graft chamber prevented migration of the epithelium from the wound edge. Three weeks after transplantation, the survival rate of the graft was 80.4 ± 6.7%, 78.3 ± 7.1%, 79.4 ± 8.3% and 81.5 ± 5.7% for group 1, 2, 3 and 4 respectively. There was no significant difference in the survival rate between the groups. However, in group 5, a large area of the graft was rejected and became necrotic, so that it was difficult to evaluate the survival area in this group.

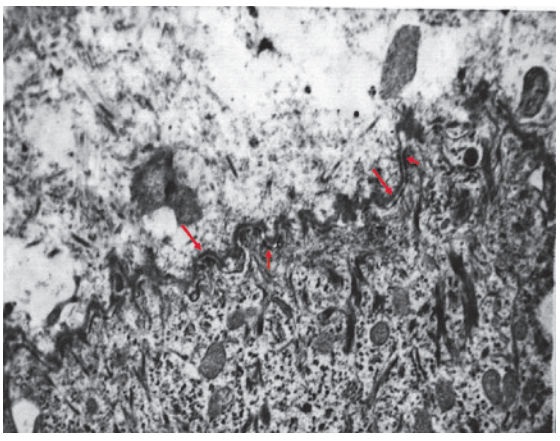

Figure 3

3 weeks after composite skin transplantation using 1:5 mixed keratinocytes, formation of a continuous basement membrane is seen at the dermal-epidermal junction (large arrow), and hemidesmosomes are seen along the junction (small arrow) (TEM×8,000).

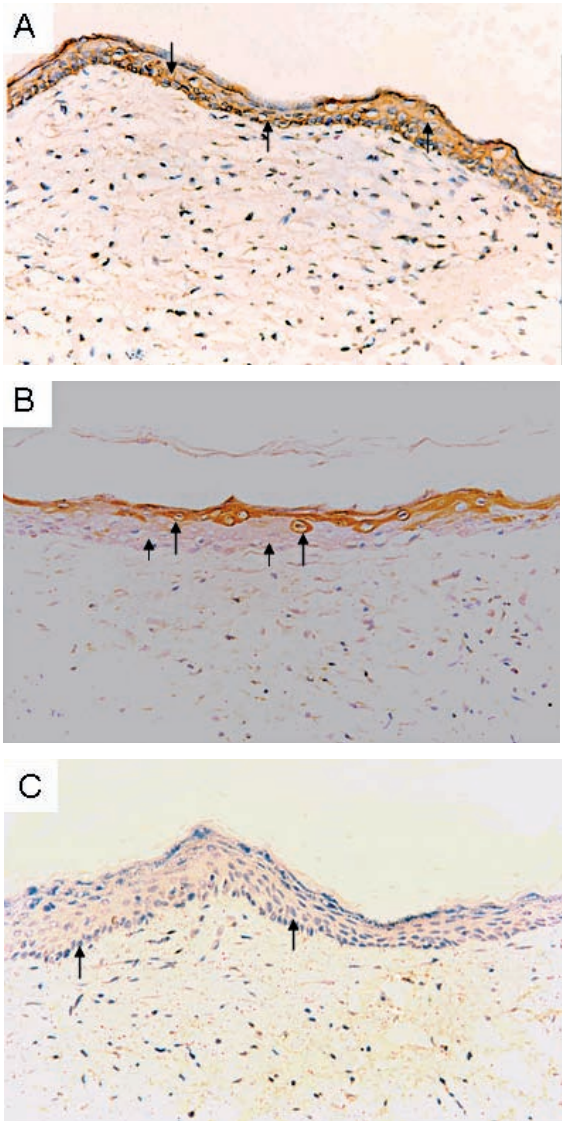

Figure 4

Anti HLA-ABC antigen immunohistochemical staining after composite skin transplantation: brown stained cells are positive cells. (A) At 2 weeks after composite skin transplantation using 1:5 mixed keratinocytes, anti HLA-ABC positive cells locate in the full epithelial layer (arrow). (B) At 3 weeks after composite skin transplantation using 1:5 mixed keratinocytes, anti HLA-ABC positive cells mainly locate the outer epithelial layer (large arrow), and there is no significant evidence of the presence of positive cells in the inner epithelial layer (small arrow), (C) At 3 weeks after composite skin transplantation using pure mouse (autologous) keratinocytes, anti HLA-ABC is negative (arrow) (immunohistology × 200).

Histological and immunological observation of the graft

About 1 week after transplantation, new blood vessels and host fibroblasts were seen filling in ADM. Collagen fibres in the dermis were arranged regularly parallel to the epidermis. In group 1, 2, 3 and 4, the epidermal structure began to form. The epidermis-dermis junction was flat. After 2–3 weeks grafting, the epidermis became thicker, and the basal layer, the suprabasal layer or even the stratum corneum were seen, and the epidermal ridge began to form.

Up to 1 week after transplantation of the composite skin, mild inflammatory cell infiltration was present in group 1, 2, 3 and 4, and two weeks after grafting, there was no evidence of inflammatory cell infiltration. However, in group 5 infiltration of a number of inflammatory cells was observed, forming an evident oedematous band between the epidermis and the dermis, indicating implant rejection. At the third week of transplantation, the epidermal layer separated from the dermis, and evident inflammatory cell infiltration of the non-autologous graft was seen (fig. 2).

Epithelial thickness of the graft

Light microscopy showed that the epithelial layer in group 1, 2, 3 and 4 thickened by 3.2, 2.7, 3.2 and 2.6 fold respectively at the third week of transplantation as compared with that at the first week, and showed no significant difference in epithelial thickness between group 1 and group 2, 3 and 4.

Immunohistological staining findings of laminin and collagen IV

Two weeks after transplantation, both laminin and collagen IV in group 1, 2, 3 and 4 expressed strongly and continuously at the epidermal and dermal junction without evident fragmentation.

Ultrastructure of the dermal-epidermal junction in the graft

As was shown by electron microscopic analysis, a continuous basement membrane was seen in the dermal-epidermal junction at the third week post-grafting. The hemidesmosomes were also seen along the junction in group 1, 2, 3 and 4 (fig. 3).

Homing of non-autologous keratinocytes after transplantation of composite skin

Two weeks after transplantation, the autologous keratinocytes of the composite skin graft located mainly in the epidermal inner layer, while the non-autologous keratinocytes located mostly in the upper part of the epidermal layer. At the fourth week after transplantation, no existence of the non-autologous keratinocytes was observed in group 2 and 3, and only small numbers of areatus and streak non-autologous keratinocytes were seen in group 4. By the fifth week post-grafting, there were no more non-autologous keratinocytes in group 4. In group 1 (autologous keratinocyte group), anti-HLA-ABC stain was always negative (fig. 4).

Discussion

The evolution of dermal matrix is relatively conserved across species. Porcine skin is structurally similar to mouse and human skin. A previous study [9] demonstrated that ADM prepared from porcine skin had satisfactory tissue compatibility and low immunogenicity for both mice and humans. It has been used as a dermal substitute in animal models and in clinical trials for deep burn wound management. Thus, we used porcine ADM as the scaffold for keratinocyte culture.

Allogeneic mouse keratinocytes might be a better choice for graft as the non-autologous

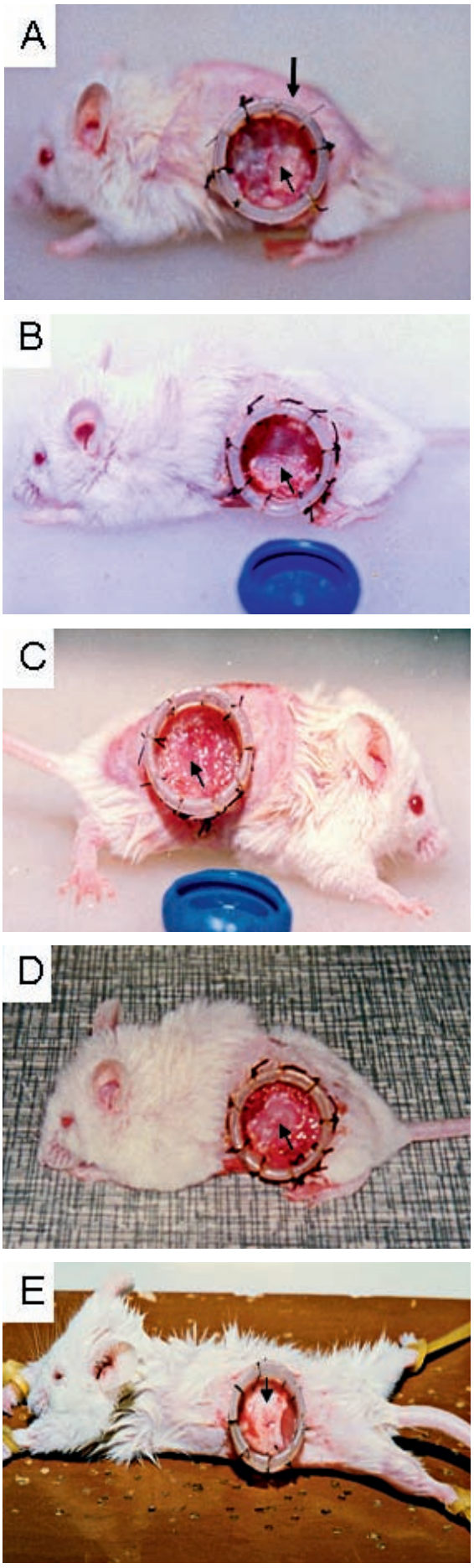

Figure 5

(A) At 4 weeks after composite skin transplantation, an epidermal layer is evident in group 1 (small arrow); the large arrow indicates the graft chamber. In group 2, 3 and 4 (B, C, D), an epidermal layer is also seen (arrow), which is not significantly different from that in group 1. In group 5 (E), the epidermal layer is completely rejected, where ADM is exposed, vascularised and closely adheres with the wound (arrow).

mixture in the present study. The reason why we used human keratinocytes as non-autologous keratinocytes is for the convenience of observing homing of the non-autologous keratinocytes. In our previous studies we mixed Wistar rat keratinocytes and F344 rat keratinocytes at a 1:1 ratio for suspension transplantation to save the source of autologous skin, and achieved good wound healing after transplantation [10]. But it was difficult to label and thus we were unable to observe homing of the non-autologous epidermal cells transplanted. As anti-HLA-ABC stain of human keratinocytes is positive, it can be used as a marker for trace observation. For this reason, we used Balb/c mice as the recipient, and human keratinocytes as non-autologous keratinocytes to mix with mouse autologous keratinocytes to reconstruct composite skin, so that it was possible to observe homing of the non-autologous keratinocytes after transplantation of the composite skin.

Non-autologous keratinocytes survived only a relatively short time and closed the wound temporarily. But with time lapsing and development of rejection reaction, the wound was exposed again [11]. Composite skin reconstructed by mixing autologous and non-autologous keratinocytes at an appropriate proportion was able to close the wound permanently and no acute rejection reaction was observed within the period of observation. The survival rate and epithelial thickness of composite skin reconstructed from the mixed keratinocytes at different proportions was similar to that reconstructed by pure autologous keratinocytes after transplantation. Histological observation showed that the epidermal layer contained basal layer, spinous layer and cornified layer. Formation of the basal membrane at the epidermal and dermal junction by 3 weeks indicated a complete connection between the epidermis and the dermis. These findings suggest that introduction of an appropriate proportion of heterogeneous keratinocytes into autologous keratinocytes does not affect closure of the wound and formation of the normal skin structure.

Heterogenous keratinocytes may induce growth of auto keratinocytes by multiple biological effects, including inter-cell contact, release of cytokines (such as transforming growth factor β1, IL-6 and IL-8), and synthesis of extracelluar matrix (such as fibronectin, laminin and collagen IV) [12]. Infiltration of a small number of inflammatory cells within the graft dermis in group 1, 2, 3 and 4 indicated that rejection of the recipient to mixed transplantation of non-autologous keratinocytes is weak and sluggish. Tracing observation of anti-HLA-ABC positively stained keratinocytes demonstrated that heterologous keratinocytes located in the full epithelial layer in the early phase of composite skin transplantation, and with the lapse of time, they located in the outer epithelial layer and finally disappeared, indicating that non-autologous keratinocytes are unable to

survive permanently. More experiments are needed to observe the mechanism of how non-autologous keratinocytes are rejected.

Compared with the composite skin reconstructed from pure autologous keratinocytes, the mixed culture reported here is advantageous in that it needs shorter time of in vitro culture, saves the source of autologous skin, and is more flexible in practical usage. The non-autologous keratinocytes could be readily available, so by mixing auto/non-autologous keratinocytes at a 1:1 ratio, culture time may be, theoretically, reduced and the source of autologous skin saved by 50%, as compared with pure autologous keratinocyte culture. By mixing auto/non-autologous keratinocytes at a 1 to 5 ratio, the source of autologous skin may be saved by 80%. This is of great significance for the treatment of extensive deep burn patients with a marked lack of autologous skin sources. Non-autologous keratinocytes include heterologous and heterogenous keratinocytes, and both can be mixed with autologous keratinocytes for transplantation. Although there are abundant sources of heterologous keratinocytes such as pig skin keratinocytes for use, they are from different species and may cause transmission of infectious diseases between different species. Compared with heterologous keratinocytes, heterogenous keratinocytes are from different individuals of the same species and therefore have relatively low immunogenicity and weak post transplantation rejection. For this reason, heterogenous keratinocytes seem more suitable for mixed transplantation with autologous keratinocytes.

In recent years, attempts of spraying non-cultivated keratinocytes onto a wound directly, or seeding them to the dermal matrix have shortened the time of in vitro culture [13, 14]. The existing problem is that without in vitro amplification the number of keratinocytes is limited and cannot meet the requirement of extensive burn patients who lack skin sources. The present study demonstrates a novel method of using a mixed grafting approach, which not only shortens the time of in vitro culture but saves skin resources.

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