Mixed Suspension of Cultured Autologous and Allogenic Keratinocytes in Fibrin Glue for the Treatment of Full-thickness Burns

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Abstract: In-vitro cultured autologous and allogenic keratinocytes were mixed in single-cell suspension and transplanted for the treatment of full-thickness, third-degree burns. Ten patients were selected according to a protocol approved by the authors’ local Institutional Review Board. After epifascial necrectomy, the burns were temporarily covered with fresh allografts, and a mixed suspension of in-vitro cultured autologous and allogenic keratinocytes in fibrin glue with a density of $10^7$ cells/mL was transplanted to the wounds by injection with a syringe in multipoint underneath the allograft. The grafting density was $5 \times 10^6$ cells per 100 cm$^2$. The transplanted keratinocytes proliferated on the epifascial surface and grew into confluent epithelial layer 28 ± 7 days after transplantation. Complete re-epithelialization and stable skin conditions were achieved 62 ± 11 days after transplantation. This method decreased in-vitro cell culture time to 5 days and resulted in permanent re-epithelialization of third-degree burn wounds. To the authors’ knowledge, this is the first report where autologous and allogenic keratinocytes were mixed and transplanted together to treat third-degree burn wounds. The performance and density of the keratinocytes remain to be studied.

Timely and effective closure of wounds determines the clinical results of intensive burn treatment. Split-thickness skin autografts (STSG) are recognized as the gold standard for burn wound closure, but is limited due to a shortage of donor skin when the severe burn area exceeds 60% total body surface area (TBSA). Since 1975, when Rheinwald and Green established the reliable method of culturing human epidermal cells in stratified and confluent layers, in-vitro cultured epidermal cell sheets have been developed to complement autografts in treating deep and large burn wounds. Cultured epithelial autografts (CEA) can substitute a skin graft to some extent, but are restricted by time-consuming cultures, vulnerability to mechanical stimuli, and an unpredictable take rate.

An improved technique of transplanting cultured epidermal cells in single-cell suspension has been developed and achieved practical, clinical
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results. Kaiser and Green4 and Stark et al5 applied non-confluent single keratinocytes suspended in fibrin glue to burn wounds covered by allograft, and found that re-epithelialization was rapid. Keratinocyte suspension is beneficial in that it can lessen the application time compared to CEA, and enhance epithelialization to improve mortality, morbidity, and overall scar quality.6,7 Meanwhile, several studies exploited the function of cultured allogenic keratinocytes in treating skin wounds and showed promising results.8–10 The allogenic keratinocytes can stimulate wound healing by simultaneously secreting numerous growth factors (GFs), although much remains unknown about its mechanism of action.

The present study aimed at simultaneously transplanting both the in-vitro cultured autologous and allogenic keratinocytes in mixed single-cell suspension to epifascial surface of third-degree burn wounds. Proliferation of keratinocytes and the healing effect on third-degree burn wounds of 10 patients were observed and discussed.

Materials and Methods

Patient selection. The local Institutional Review Board approved the study protocol and all patients, or their family representative, gave informed consent. Ten cases were selected and included the following criteria: 1) adult patients who had a third-degree burn area of 400 cm\(^2\)–600 cm\(^2\) (3%–5% TBSA); 2) these third-degree burns were spatially isolated from other burns and located in positions without joints (ie, anterior and posterior trunk, thighs, and legs), so that cell transplantation and wound coverage could be easily achieved.

Burn wound treatment. Eschar excision to epifascial layer was performed and fresh allografts were sutured in place as temporary cover. The burns were not otherwise treated until it was time for the keratinocyte transplantation procedure.

In-vitro cultures. Keratinocyte cultures were performed according to methods reported by Rheinwald and Green7 and Stark et al.4 Healthy skin (2 cm × 3 cm) squares were harvested without site preference from non-burned areas of either the selected patient or an adult burn patient in the authors’ hospital who had meshed STSG transplantation. New wound caused by skin harvest operation healed, and did not result in an additional scar for patients. The harvested skin biopsy was washed three times in sterile phosphate buffered solution (PBS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin. Afterward, the biopsy was cut into 5 mm × 3 mm squares and digested in 0.2% solution of dispase (Roche Applied Science, Indianapolis, IN) for 16 hours at 4˚C to separate the epidermal layer from dermis. The isolated epidermal sheets were further digested in PBS, pH 7.4, containing 0.025% trypsin and 0.02% EDTA, for 20 minutes at 37˚C. After neutralization of trypsin by adding fetal calf serum, the solution was filtered through a 150-mesh sieve. Keratinocytes were collected by centrifugation (800 r/min, 10 minutes), and then diluted to a density of 2 × 10^6 cells/mL by Dulbecco’s modified eagle medium, DMEM (Gibco®, Invitrogen). Keratinocytes were seeded at a density of 5000 cells/cm\(^2\) into 75 cm\(^2\) culture flasks filled with 20 mL DMEM supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Culture flasks were incubated at an atmosphere of 37˚C, 5% CO\(_2\).

After cells reached a confluence of 70%–80% in 3 days, digestion and collection of free keratinocytes were performed as described previously. Both autologous and allogenic keratinocytes were cultured using the same method. After the first passage, autologous and allogenic keratinocytes were mixed at a cell ratio of 1:1, seeded together at a density of 5000 cells/cm\(^2\) into the 75 cm\(^2\) culture flask, and cultured to a > 80% confluence in about 2 days. The mixed keratinocytes were then digested and collected. Trypan blue exclusion test determined the keratinocyte cell viability was > 90%.11 Finally, a mixed suspension of autologous and allogenic keratinocytes in single-cell suspension was prepared by suspending the collected cells with fibrin glue (Hualan Biology, China) with a density of 1 × 10^7 cells/mL.

Transplantation. Mixed suspension of autologous and allogenic keratinocytes in fibrin glue was transplanted to the third-degree burn wound by injection with a
Table 1. Characteristics of the selected patients.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Burn origin</th>
<th>Burn area (%TBSA)</th>
<th>Burn location</th>
<th>Burn area (% TBSA)</th>
<th>Skin donor site</th>
<th>Time for complete wound re-epithelialization (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>21</td>
<td>Flame burn</td>
<td>46</td>
<td>Left arm</td>
<td>3</td>
<td>Abdomen</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>28</td>
<td>Flame burn</td>
<td>35</td>
<td>Abdomen</td>
<td>5</td>
<td>Right leg</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>36</td>
<td>Flame burn</td>
<td>25</td>
<td>Left leg</td>
<td>3</td>
<td>Abdomen</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>48</td>
<td>Molten steel burn</td>
<td>35</td>
<td>Back</td>
<td>4</td>
<td>Right thigh</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>18</td>
<td>Molten steel burn</td>
<td>40</td>
<td>Left thigh</td>
<td>3</td>
<td>Abdomen</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>23</td>
<td>Heat crush injury</td>
<td>25</td>
<td>Back</td>
<td>3</td>
<td>Right leg</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>25</td>
<td>Acid corrosion</td>
<td>46</td>
<td>Right leg</td>
<td>5</td>
<td>Abdomen</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>21</td>
<td>Molten steel burn</td>
<td>53</td>
<td>Abdomen</td>
<td>4</td>
<td>Abdomen</td>
<td>72</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>18</td>
<td>Acid corrosion</td>
<td>50</td>
<td>Abdomen</td>
<td>4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>24</td>
<td>Flame burn</td>
<td>60</td>
<td>Right thigh</td>
<td>4</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*Study was suspended for these patients due to premature allograft separation from the wounds. These wounds were treated with meshed STSG transplants.

Figure 1. A) Third-degree abdominal burn on admission. B) Burn wound after epifascial necrectomy on the eighth day after injury. C) Topical keratinocyte islands (arrows) formed 14 days after transplantation of mixed suspension of autologous and allogenic keratinocytes. D) Twenty-eight days after transplantation, keratinocytes grew into confluence (arrows).
syringe in multipoint underneath the allograft at a density of $1 \times 10^5$ cells/cm$^2$.

**Results**

Ten patients were selected under the protocol (Table 1). The allografts of 8 patients began to shed from the wound 28 ± 7 (mean ± SD) days after transplantation, and epidermal cell islands formed in the exposed burn areas. These islands expanded and grew into confluence, which created a new epithelialized layer. Complete re-epithelialization was achieved 62 ± 11 days after transplantation, at which point patients were discharged with stable skin conditions. Two patients’ wounds were healed by transplantation of meshed STSG since the allografts had prematurely separated from their wounds.

**Case 1:** A 28-year-old woman was injured by self-burning in a domestic conflict, resulting in third-degree burns of 35% TBSA (head 6%; upper extremities 15%; chest 9%; abdomen 5%). Five days after injury, eschar excision was performed on burns located at the head, extremities and chest, covered with meshed STSG from unburned thighs.

Epifascial necrectomy of abdomen burn was operated on the eighth day after injury (Figure 1A, B) and covered by fresh allograft. Meanwhile, a 2 cm × 3 cm split-thickness skin biopsy was harvested from the patient’s right leg for keratinocyte culture. Five days later, mixed suspension of autologous and allogenic keratinocytes in fibrin glue was transplanted underneath the allograft. Topical keratinocyte islands formed on the wound surface, 14 days after transplantation (Figure 1C), which suggested that keratinocytes in single-cell suspension started to proliferate, demonstrating potential to generate epidermal cell sheets *in situ*.

**Figure 2.** A) Third-degree burn before surgery. B) Burn wound after epifascial necrectomy. C) Mixed suspension of autologous and allogenic keratinocytes in fibrin glue was transplanted underneath the allograft. D) Complete re-epithelialization.
The allograft eroded and shed 27 days post transplantation. Epidermal cell islands further extended and grew into confluence on the wounds (Figure 1D). All of the wounds were completely healed and the patient was discharged with stable skin conditions after a 2-month hospitalization.

**Case 2:** A 36-year-old man sustained a 25% TBSA burn to his lower extremities and trunk, resulting from a traffic accident that involved a gasoline fire. A third-degree burn (3% TBSA) located in posterior of his left leg was selected. Four days post injury, epifascial necrectomy was performed and a fresh allograft was used as temporary cover after the wound was cleaned (Figure 2A, B). A 2 cm × 3 cm section of healthy skin was harvested from his abdomen.

Nine days after the injury, a mixed suspension of autologous and allogenic keratinocytes in fibrin glue was transplanted and placed beneath the allograft (Figure 2C). Thirty days after transplantation, the allograft began to shed from the wound revealing newly formed epidermal cell islands. Complete re-epithelialization and wound healing were observed 67 days after injury (Figure 2D). At the 3- and 6-week follow-up visits, the healed burn was stable and had a smooth appearance.

**Discussion**

In this study, a mixed suspension of *in-vitro* cultured autologous and allogenic keratinocytes in fibrin glue was prepared and transplanted to allograft covered third-degree burns. This technique was utilized to treat third-degree burns of 10 patients and found that keratinocytes could proliferate and expand on wound surfaces and eventually grow into confluent epithelial layers that completely covered and healed the burn wounds. The central innovative aspects of this study included: 1) time spent in culturing keratinocytes was shortened by combining autologous and allogenic keratinocytes; and 2) instead of enriching epidermal cells *in vitro*, keratinocytes were transplanted early and proliferated *in vivo* on epifascial surfaces.

Early eschar excision and wound coverage can substantially decrease the incidence of invasive infection, prevent post-burn deleterious inflammatory responses, and improve survival. In the present study, early excision was performed and the resulting wound was temporarily covered with a fresh allograft. Simultaneously, autologous and allogenic keratinocytes were cultured in 5 days, which is much less than autologous keratinocyte suspension alone (11 days) and CEA (23 days), as Stark et al have reported. The proliferation rate of mixed keratinocytes was accelerated by growth factors expressed by allogenic keratinocytes, which would decrease the period of time required for collecting enough cells and accelerate wound healing. By virtue of mixed autologous and allogenic keratinocytes, wound infection and healing efficiency can be improved. Different from CEA, which was cultured into epithelial sheet *in vitro* and taken by the wound, transplanted keratinocytes in single-cell suspension are cultured on the surfaces of fascia, which is more beneficial for keratinocytes to proliferate, and likely provides abundant nutrients. The fate of cultured allogenic keratinocytes has yet to be clearly identified, although several studies have suggested that *in-vitro* cultured allogenic keratinocytes can be activated, expressing basement membrane proteins to enhance cell migration; its lysates contain wound repair stimulating factors that promote proliferation and migration. The impact of allogenic keratinocytes on proliferation and re-epithelialization of autologous keratinocytes needs to be further studied.

The present study established a novel means of keratinocyte transplantation based on the method Stark et al developed. Complete re-epithelialization of third-degree burn wounds was achieved by virtue of both autologous and allogenic keratinocytes, which were transplanted to the wound early and facilitated *in-vivo* cell proliferation. Growth factors that allogenic keratinocytes secrete and the favorable growth environment fascia provides for autologous keratinocytes might accelerate wound healing.

**Keypoints**

- Complete re-epithelialization of third-degree burn wounds was achieved by virtue of both autologous and allogenic keratinocytes, which were transplanted to the wound early and facilitated *in-vivo* cell proliferation
- Combining autologous keratinocytes with allogenic keratinocytes in a mixed suspension reduced *in-vitro* culture time, and complete re-epithelialization of the wounds was achieved

**Conclusion**

To the authors’ knowledge, this is the first report on mixed suspension of autologous and allogenic keratinocytes in fibrin glue for the treatment of full-thickness burns. By combining autologous keratinocytes with allogenic keratinocytes, *in-vitro* culture time was reduced and complete re-epithelialization of the wounds
was achieved, which suggests this is a promising method of keratinocyte transplantation for the treatment of full-thickness burns.

References


