INTRODUCTION

Skin cell isolation techniques are of interest for regenerative therapy development for burns. While partial-thickness (Grade IIa; mid dermal) burns are treated conservatively, and split skin or mesh-grafting is a routine indication in third-degree burns, the indication to grafting in deep second-degree (Grade IIb) wound defects in some patients exhibiting a borderline wound severity can be controversial. These patients are sometimes not treated with grafting...
to avoid overgrafting, for example, on the face and hands. After deciding not to treat this specific patient group with grafting, most of the patients start sufficient spontaneous healing within the first week under observation and intense wound care, but some patients in this population develop delayed wound healing over several months with complications, including infections. In these specific cases the long-term outcome, often with scar formation, skin discoloration and skin hypertrophy, and sometimes contractions is unsatisfactory (1). While such a development can typically be predicted 10 to 14 days after non mesh-grafting of the burn injury if the wound is not dry and reepithelialized, performing split skin grafting at this point is problematic. Single skin cell spray grafting of cultured cells was recently introduced (2), and we already implemented the method by using a processor controlled pneumatic cell sprayer for better controlled cell deposition for cultured autologous keratinocytes (3). Avoiding in vitro cell expansion in culture, by which the basal keratinocytes, considered to be important for wound regeneration, differentiate and lose their progenitor character, appears to be promising (4-7). Autologous skin cell transplantation in an intraoperative setting is based on removing healthy skin in the dermal layer from a non-prominent healthy skin area using a dermatom, followed by cell isolation at the point of care and immediate cell spray grafting to the burn site during the same operating session. Gravante et al used the Avita ReCell kit (Nedlands, Australia) and compared cell spray transplantation with mesh grafting in partial-thickness wounds and concluded that both methods produce similar results, while cell grafting was described as a well tolerated, safe and reliable technique (8).

Considering these results, we did not depart from the usual mesh grafting treatment, but considered this method only for patients in the aforementioned situation. We believe that the method described, involving trypsin digestion only and without cell washing by centrifugation, can be improved by: a) adding cell washing with a centrifuge to better remove remaining enzymes; and b) an initial step of dispase application to first separate the dermis from the epidermis prior to the trypsin application, so that the trypsin can easier reach the cells of the basal keratinocyte layer in the epidermal interface of the epidermis.

We describe the use of this modified two-enzymatical step isolation technique involving dispase, trypsin, and cell washing by centrifugation and we report our clinical implementation for the initial eight patients.

MATERIALS AND METHODS

Patients

Autologous skin cell transplantation was performed in our center on eight patients between 2007 and 2008. After preparing the logistics for the therapy, all patients were treated who met the inclusion or exclusion criteria described below and presented with the following clinical indications: partial-thickness wounds (Grade Iib) with remaining dermal structures of moderate size that were initially not treated with split mesh grafting to avoid over-grafting, but experienced delayed wound healing with no significant re-epithelialization within two weeks after injury. The interval between initial trauma and cell application was in all cases at least 14 days. The interventions were performed one time only in all cases. Some treatments were performed in areas exhibiting critical wound healing and often unsatisfying cosmetic results: two patients with burns on the face, and two patients with burns on the hands. Eligibility criteria for cell spray grafting included partial-thickness burn injuries that required initial surgical debridement under anesthesia and follow-up wound treatment with enzymatic debridement. Exclusion criteria were pre-existing local and systemic infections that required antibiotic treatment for more than 2 days prior to grafting; hypersensitivity to trypsin or other enzymatic wound treatments; a high anesthesiology risk. Patients received routine pre-operative investigations and a second final revision of the surgical indications/decisions.

The patients included 6 males and 2 females; the mean age was 30.3 years (9 months-58 years). The patients initially exhibited a mean burn total body surface area (TBSA) of 14% (3-40%). The mean Abbreviated Burn Severity Index (ABSI) (9) was 5 points (3-8 points). Details on the patients and wound locations are summarized in Table I.

Informed consent was obtained in all cases, and the individual's own decision after providing detailed information was respected in any case. We explained to the patients that their decision would have no impact on potential alternative conservative treatment. No patient to whom we suggested the procedure declined the proposal; see below for Institutional Review Board (IRB) approval.

All procedures were performed in a burn center operating room and under general anesthesia.
The biopsy treatment includes existing methods of enzymatic tissue digestion with dispase and trypsin, and the washing away of the connective tissue is performed using conventional patient cell centrifugation methods; all solutions and containers are sterile commercial products. If not otherwise indicated, all solutions were supplied by Biochrom AG, Berlin, Germany; and disposables by BD Biosciences, Bedford, MA, USA.

Specifically, the following steps for autologous skin cell-spray transplantation were performed. According to individual enzyme incubation times, this procedure requires around 70 to 90 minutes.

A) A 2-3 cm x 3-4 cm split skin biopsy (0.2 mm deep) is taken using a dermatom (Humeca D 42; Asclepios, Gutach, Germany). The biopsy is performed in a routine way, as known from split mesh skin grafting. The biopsy is transferred into a sterile disposable 100 mm Petri-dish (BD) and is cut into 4 x 4 mm pieces by the surgeon with a surgical disposable scalpel (the specimen must not dry out).

B) The cell separation, performed by a specialized laboratory technician, involves:

1) initial separation of dermis and epidermis over 25 to 40 minutes in a 2.5 unit/mL Dispase II (#4942078; Roche Diagnostics, Mannheim, Germany) containing phosphate buffered saline (PBS 1x without Ca²⁺, Mg²⁺) solution at 37°C in Petri-dishes;

2) The mechanical separation of the dermis and epidermis of each skin piece using forceps and scalpel, performed in a dry Petri-dish (the specimen must not dry out), with

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**TABLE I - PATIENT, TRAUMA AND WOUND CHARACTERISTICS OF CELL SPRAY TRANSPLANTATION PATIENTS**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Cause of trauma</th>
<th>Initial burn regions</th>
<th>Initial TBSA burn (%)</th>
<th>Initial ABSI (points)</th>
<th>Treated burn regions</th>
<th>Treated BSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>35 years</td>
<td>Blaze</td>
<td>Both hands, arms, thighs, backside</td>
<td>40</td>
<td>8</td>
<td>Right thigh, right knee</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>18 years</td>
<td>Scald</td>
<td>Penis, scrotum, both thighs, belly</td>
<td>15</td>
<td>4</td>
<td>Penis</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>4 years</td>
<td>Scald</td>
<td>Left leg</td>
<td>5</td>
<td>4</td>
<td>Left leg</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>42 years</td>
<td>Explosion</td>
<td>Right hand, both hips, legs; penis</td>
<td>5</td>
<td>5</td>
<td>Penis</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>39 years</td>
<td>Blaze</td>
<td>Face, forehead, right shoulder, right hand</td>
<td>10</td>
<td>5</td>
<td>Face, forehead</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>58 years</td>
<td>Explosion</td>
<td>Face, both hands</td>
<td>15</td>
<td>7</td>
<td>Face, left hand</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>9 month</td>
<td>Scald</td>
<td>Both arms and dorsal thigh</td>
<td>20</td>
<td>3</td>
<td>Arm circular left</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>46 years</td>
<td>Electro burn</td>
<td>Both hands, head, neck</td>
<td>3</td>
<td>5</td>
<td>Hand left</td>
<td>1</td>
</tr>
</tbody>
</table>

TBSA = total body surface area; ABSI = Abbreviated Burn Severity Index; BSA = body surface area; “Initial” refers to the diagnosis at the day of the initial trauma.

**Initial wound treatment after trauma**

Patients presented to our burn center initially received an escharectomy with tangential excision using a curette, Guillian knife, or a hydrourgical system (Versajet®; Smith & Nephew, London, UK) when the wounds exhibited a thick fibrin coating. Daily wound inspection included enzymatic debridement using collagenase gel (Iruxol®; Smith & Nephew, Hull, UK) in combination with fatty gauze (Jelonet®, Smith & Nephew).

**Cell spray grafting method**

Intra-operative treatment included wound disinfection with chlorhexidine-cetrimide solution. Immediately before cell application we performed sharp surgical spoon debridement under general anesthesia. Hemostasis was performed with regular gauze.

The biopsy for the skin cell transplantation was obtained from a non-involved region, as known from split thickness graft harvesting, and the tissue was procured into a single-cell suspension for cell spray transplantation. This cell isolation procedure is performed on site in the operating room immediately prior to cell transplantation during the same anesthesia session. Immediately following the biopsy, the tissue is procured under sterile conditions until application; the tissue is maintained under the direct supervision of the surgeon who performs the procedure using routine clinical devices, solutions and disposables available in skin burn centers.
Autologous skin cell isolation

no further use of the dermis parts;
3) The epidermal pieces are transferred into a 15 mL Falcon tube with PBS without Ca\(^{2+}\), Mg\(^{2+}\), to wash away the enzyme.
4) The epidermal pieces are then transferred into 15 mL Falcon tubes for 10 to 15 minutes at 37°C containing a single cell suspension and a 5 mL 0.05% Trypsin / 0.02% EDTA (# L2143; Biochrom AG, Berlin, Germany) solution, to allow for cell isolation from the epidermis, including from the interface layer of epidermis and regenerative basal keratinocyte containing dermis; followed by stopping with 7.5 mL Ringer lactate (B Braun, Melsungen, Germany) containing 10% of the patient’s own serum, taken previously, over 30 seconds;
5) The cell suspension is sieved through a 70 micrometer 50 mL Falcon tube sieve (all BD);
6) seven minutes of centrifugation at 100 g for cell washing to remove the enzymes;
7) The cells are put into clinical grade Ringer’s lactate solution (B. Braun, Melsungen, Germany) which is used as a carrier for cells during the spraying process. At this stage, cell counting in a Neubauer chamber showed a yield between 2 to 10 x 10\(^6\). Using a sterile disposable pipette (BD Biosciences), the cells are transferred into sterile disposable syringes (BD Biosciences) for cell deposition by spraying. We typically resuspend the cells in a 1 x 10\(^6\) cell/ml suspension, which we divided into one to five 2 mL syringes (BD Biosciences) for cell spraying; the trypan-blue viability of the cells was between 96% and 98%.
C) One syringe at a time, the syringes are placed in a processor-controlled pneumatic cell spray device through which the suspended cells are immediately spray-transplanted onto the burn site of the patient by spray deposition, as described earlier (3), allowing an even distribution of the cell suspension across a larger surface. Donor site area to transplant site “expansion” ratios were 1:20 to 1:60, with a mean of 1:25.

After aspirating the cells for cell spraying from the centrifugation tubes used for cell washing, few cells remained. To obtain control cultures of the cells sprayed on each patient, we rinsed the remaining cells left in the container with standard culture medium (Epilife; Gibco, Carlsbad, CA, USA; with Penicillin/Streptomycin; Biochrom, Berlin, Germany) and cultured them under standard conditions (Falcon Primary 25 cm, 2 flasks; BD, Franklin Lake, NJ, USA) in 5% CO\(_2\) culture incubators. For all patients treated, the cells pipetted into culture flasks successfully attached, spread, and remained viable. In all cases, we were able to produce follow-up cultures with typical passage behavior.

Wound treatment and followup

After cell spray grafting, the wounds were covered with polylactid sheets (Suprathel\(^{TM}\); PolyMedics Innovations, Denkendorf, Germany) combined with fatty gauze (Jelonek\(^{\circ}\)) cotton and elastic bandage. This dressing was used for one week. Thereafter we used Adaptic\(^{TM}\) (Johnson and Johnson, Eticon, Norderstedt, Germany) sheets under gauze dressings. After the initial days, the wounds were treated in the same way as after split mesh skin transplantation. The patient followup is also the same as for split mesh skin transplantation. To assess burn scars for clinical evaluation we used the Vancouver Scar Scale (10) (Tab. II).

Innovative practice versus clinical study

The skin cell transplantation treatment was planned solely to enhance the well-being of this unique patient group as described above; patients with these injuries appear relatively seldom. The method used is innovative, but given the cited results of Wood et al and Gravante (2, 8) it is not radically new. Skin cell isolation is well known to provide cells for medical cell applications, a technique already used in the past in some larger burn centers. Also, the tissue is stored under sterile operating room conditions throughout the entire procedure until the final cell transplantation procedure takes place; the tissue is maintained under the direct supervision of the surgeon who performs the procedure; and the cells undergo minimal manipulation only with a well characterized and widely accepted enzymatic cell isolation not involving in vitro culture. Therefore, the patients were not exposed to radically new procedures or considerable additional risks.

Considering the comparably low patient number per year we did not plan a clinical study. We therefore choose to consider this treatment as the introduction of an innovative practice. We planned to apply the practice on eight patients, and report to the responsible local Innovative Practice Advisory Committee of the IRB for re-evaluation. The procedure was only suggested to individual patients that had a reasonable expectation of success. Treatment was suggested to enable wound closure within 2 to 3 more weeks and reduce the development of long-term wound healing complications over several months. All patients re-
required outpatient followup for observation, and we planned to immediately report any potential adverse events to the Institutional Patient Safety Committee.

RESULTS

Although we did not perform a clinical study, we agreed to primary “endpoints” for the patient follow-up characterization: a) time to complete re-epithelialization of the treated area; and b) the esthetic and functional quality of re-epithelialization at six months’ followup. As secondary “endpoints”, we assessed for infections, inflammation, or any adverse effects or complications. The mean treatment area was 3% (0.5-6%) body surface area. Some patients received additional split skin mesh grafting prior to cell spray grafting, but only to wound areas unrelated to our analysis. These additional treatments are listed in Tables I

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treated II°b BSA (%)</th>
<th>Region treated and amount of sprayed cells</th>
<th>Full re-epithelialization of treated regions after day</th>
<th>Wound healing results at 6 months</th>
<th>Vancouver Scar-Scale Score at six months post cell spray grafting, Range from 0 - 14 points (worst)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Right thigh, right knee 9.8x10^5</td>
<td>14</td>
<td>Patient did not appear for followup, report on phone: Good clinical followup, occasional tickling, dry skin, esthetically good picture, no scars at regions treated</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>Penis 5.8x10^5</td>
<td>12</td>
<td>Slight hyper and hypopigmentation at region treated</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>Left leg 3.7 x10^6</td>
<td>16</td>
<td>Entire area: no functional findings, occasional tickling, dry skin Central area (1%): 7 Central (1%): hyperthrophic scar formation Peripheral (4%): hypopigmentation</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>Penis 4.0x10^5</td>
<td>21</td>
<td>Hypopigmentation at region treated</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>Face, forehead 5.0x10^6</td>
<td>10</td>
<td>Patient did not appear for followup, report on phone: good wound healing, no functional or esthetic concerns, no scar</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>Face, left hand 9.8x10^6</td>
<td>12</td>
<td>All areas: no functional or cosmetic restrictions, except hand: dry skin face: partially reddening</td>
<td>Hand: 1 Face: 1</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>Arm circular left 1.2x10^5</td>
<td>20</td>
<td>All areas: no functional or cosmetic restrictions, except upper arm: reddening, partial slight hypertrophy lower arm: reddening, partial hypertrophy</td>
<td>Upper arm: 4 Lower arm: 3</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>Hand left 2.4x10^5</td>
<td>16</td>
<td>Hand distal between 2nd/3rd/4th finger: Hyper- and hypopigmentation and scar (fingers can be spread apart about 1cm in palmar position with distinct sensation of tension, also itching and dryness) Hand proximal (0.5%): Pink skin Hand distal (0.5%): rough skin, hyperthrophic sanguine scar with partial strand formation</td>
<td>Hand distal (0.5%): 9 Hand proximal (0.5%): 4</td>
</tr>
</tbody>
</table>

All patients were treated with primary debridement and debridement prior to cell application; the interval between initial trauma and cell application was in all cases at least 14 days.
and II, but they were not considered in the evaluation described here. The localization of the cell spray transplantation is summarized in Table II, which also summarizes the yield of the cell isolation, in other words, the therapeutic cell amount.

The mean interval between trauma and cell spray transplantation was 16 ±4 days. Using single spray transplantation grafting, the ratio of skin biopsy to treatment surface could be enlarged from the typical 1:2 to 1:4 in mesh grafting to 1:20 to 1:60 with a mean of 1:25. Figures 1 to 3 show examples of patients prior to and post cell spray transplantation. The mean time to full re-epithelialization was 12.6 days (10-21 days). For clinical result evaluation at six months, we used the Vancouver Scar Scale (10) to assess the scar development. The evaluated parameters are described in Table II. All patients exhibited a constant improvement of the clinical picture, with at least 12 months followup. No patient showed side effects or complications.

Fig. 1 - Male patient, 42 years old (Tab. I, Patient 4) presenting areas of deep dermal burns after propane gas explosion trauma. A) During cell spray grafting, B) wound at follow up after four weeks and C) after six months.

Fig. 2 - 58-year-old male patient (Tab. I, Patient 6) presenting areas of deep dermal burns after explosion trauma. A) Patient 2 days after trauma during observation time without mesh grafting or cell grafting; B) five days after cell spray grafting; C) after 12 days, 16 days, and six months at followup.

Fig. 3 - 46-year-old male patient (Tab. I, Patient 8) presenting areas of deep dermal burns after electro-burn injury. A) during cell spray grafting; B) wound at followup after five days; and C) after six months. The cell spray device in action is visible in A).
Considering the clinical picture of the cases described prior to application, we believe that the functional and cosmetic results were excellent after cell spray transplantation. At this time, however, our information is limited to the feasibility of the cell isolation method.

DISCUSSION

The use of enzymatically isolated (11) and in vitro expanded cells cultured into skin cell-sheets (12) was introduced around the turn of the century. This application of in vitro cultured cells grown to sheet grafts was described to reduce mortality and pain (13). The availability and exclusive use of differentiated keratinocytes, often result in cosmetically unsatisfactory regeneration (14). The take rates are not consistent due to limitations of the method, for example, blister formation and the loss of some skin cell populations during in vitro expansion. The application of single sprayed cells after expansion in culture was therefore introduced (3, 15).

Single skin cell transplantation application follows a historic development of burn treatment methods. In the early 1900s, autologous skin 1:1 transplantation was introduced; in the mid-1900s, split skin and mesh grafting was established. Mesh grafting results in wound closure after a small “skin lattice” is placed on the wound; cells grow in the open spaces, thereby filling in the gaps and closing the wound. While the split ratio is typically 1:4, larger ratios are often associated with unsatisfactory results. In the 1960s the Meek technique was introduced (16), in which the split skin is cut mechanically into even smaller tissue pieces of around 1 x 1 mm, enabling split ratios of 1:4 to 1:6. The concept of non-cultured single cell spray grafting follows this development path, which is based on the application of single cells sprayed into the wound, where they continue to grow and regenerate skin in the wound. Here, the cells grow to close the wound from multiple starting points, thus allowing a larger split ratio of typically more than 1:20 to be used. Some authors describe a reduction of time to wound closure, which is generally known to contribute to a positive outcome (17). Gravante et al, however, pointed out that mesh grafting and cell spray grafting enable comparable results and re-epithelialization times (8) and report that melanocyte grafting may not be sufficient. On the other hand, the cosmetic aspects of moderate Grade IIb burn area treatment results are of special interest for burns on face and hands, a potentially interesting application for cell-grafting. The 1:20 ratio with cell-grafting allows comparably small biopsy sizes to be taken from non-prominent body areas, while the typical mesh pattern of mesh grafting on the burn site can be avoided.

The method we describe here enables skin cell spray transplantation using a modified cell isolation involving a two-enzyme approach and cell washing. While cell washing removes remaining enzymes prior to grafting, the application of dispase enables the dermis to be separated from the epidermis; this allows the subsequently used trypsin to isolate the cells from the epidermis-dermis interface, specifically the otherwise enclosed layer of the epidermis that contains regenerative basal keratinocytes. These skin progenitors are not selectively reached by conventional techniques. The assessment of a reproducible content and typical amount of basal keratinocytes is the subject of ongoing studies. We introduced this method in our center to provide a therapy to individuals with a rare medical condition. For these individuals the decision not to perform mesh grafting to avoid overgrafting resulted in delayed wound healing, which could be addressed by our method. Interestingly, our health care provider, BG Germany, reimburses the amount for this procedure that is otherwise received for mesh grafting surgery.

Our experience confirms positive results using a processor-controlled cell spray device, reported previously with in vitro expanded cells (3). The clinical case reports presented in this paper show that surgical debridement and consecutive application of sprayed autologous cells in suspension in this patient group results in re-epithelialization within an acceptable time and acceptable cosmetic outcome (Figs. 1-3). Since we did not include controls, for example, patients sprayed with cells isolated in a standard way, the specific benefits of the procedure cannot be assessed. Other limitations of our study include the fact that our results also summarize clinical evaluation data with subjective assessments of the burn depth only; a comparison to the normal healing time without the cell spray transplantation cannot be given; and a debridement treatment alone may have contributed to improved healing.

We present, however, a limited number of cases to provide information on the feasibility of the cell isolation method. Larger future clinical studies would be of interest and we are available to provide training on the method to interested centers.

In conclusion, we describe a novel method for skin cell
isolation in an intra-operative setting at the point of care and a treatment option with non-cultured cells for patients with Grade IIb burns that did not receive grafting to avoid overgrafting and did not heal in the appropriate time. We believe that our initial clinical data show interesting results while complications were not seen. For future clinical studies, we propose to consider multicenter studies on a patient group exhibiting all of the following clinical aspects: a) acute burns with Grade IIb areas of less than 15% body surface; b) no previous split skin transplantation on the wound to avoid overgrafting; c) treated over two weeks conservatively with intense wound care including enzymatical debridement; d) wound closure only up to 50% of the initial Grade IIb burn area within these two weeks.

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