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Original Article

Cryopreserved cultured epithelial allografts for pediatric deep partial dermal burns: Early wound closure and suppression of scarring

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A B S T R A C T

Background: In deep partial thickness dermal burns (DDB) where greater than 50% of the dermis is lost, severe pain, scarring and contractures occur. Therefore, skin grafting may be required. In children, scar contracture occurs because scarred skin does not stretch with growth creating the need for additional scar-releasing or skin-grafting surgeries. In order to resolve this problem, we used cryopreserved cultured epithelial allograft (cryopreserved allo-CEG), which can be grafted shortly after sustaining a wound. We reevaluated the promotion of early wound closure of burns and suppression of scarring by this treatment.
Methods: Cryopreserved allo-CEGs were used to treat 50 cases of pediatric DDB from 1992 to 2000. These cases were reviewed with regard to the time until epithelialization, take percentage, and pain level. Also, in order to examine why cryopreserved allo-CEG promotes healing of burns and suppresses scarring, growth factors and cytokines in the cryopreserved allo-CEG were measured. Cryopreserved allo-CEG sheets were solubilized and concentrations of TGF-α, TGF-β1, IL-1α, IL-1β, PDGF-AA, VEGF, KGF, IL-6, b-FGF, as well as metalloprotease-1 (MMP-1) and HGF, which are noted to have scarring suppression effects, were measured before grafting.
Results: Grafting of cryopreserved allo-CEGs in 50 cases of childhood DDB resulted in early epithelialization (9.32 ± 3.63 days on the average) and an almost 100% take rate. Also, pain relief (pain reduction or elimination, reduced need for anesthetics) was seen in all cases. Although 15—23 years have now elapsed, adverse events have not been observed. Cryopreserved allo-CEG contains IL-1α, IL-1β, PDGF-AA, TGF-α, TGF-β1, VEGF, and IL-6 have wound healing effects. The concentration of IL-1α was higher than the concentrations of other components, and this was followed by TGF-α, TGF-β1, b-FGF and VEGF. Although the concentration of MMP-1, which has a scarring suppression effect, was high, HGF was not detected.
Conclusion: Cryopreserved allo-CEG contains growth factors that promote wound healing and factors that suppress scarring. Three effects, namely (1) early wound closure, (2) scarring suppression, and (3) pain relief were seen with grafts of cryopreserved allo-CEG in cases of childhood DDB. These observations show that cryopreserved allo-CEG is clinically useful and effective for the treatment of childhood DDB.
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1. Introduction

More than 100,000 pediatric burn cases occur each year around the world. Among such injuries, deep dermal partial-thickness scald burns remain one of the most common types in children. It is well known that when conservative therapy is used for cases of DDB, scarring and contracture occur over an extensive area because the burn takes 3—8 weeks to heal, sometimes even longer [1—4].

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Furthermore, in cases where infection occurs at the wound surface, migration of the infection to the deep burn can occur and may lead to serious complications or death [1–4]. Skin grafting is thus deemed to be preferable whenever possible [1–4]. However, harvesting donor skin forms a new wound surface on a patient's body and causes additional pain. Skin grafting may also lead to disfiguring scars at both the recipient and donor site. It is thus difficult to decide whether grafting or conservative therapy should be chosen. Another prognostic problem is that following extensive DDB, even when the wound heals, scar contracture occurs because scar tissue does not stretch with growth, and scar-release or skin graft surgery has to be performed again. Additionally, a DDB wound injures the layer in which sensory nerves are found and each daily dressing change is accompanied by severe pain so that treatment becomes an excruciating experience for these children [4,5]. Pain relief medication or an anesthetic must thus be administered during the daily dressing change. Because scarring may also result in poor body image or psychological damage, pediatric burn victims may experience mental distress over a long period of time.

In 2009, autologous cultured epithelial grafting (auto-CEG) was approved by the US Food and Drug Administration. In the same year, an auto-CEG product (product name: JEIS; made by J-TEC Co., Ltd.), was approved by the Ministry of Health, Labor, and Welfare of Japan and became available as a public health insurance benefit for patients with burns covering 30% or more of their body surface. Also in Japan, the new Regenerative Medicine Act was promulgated on November 25, 2014 to provide safe cell therapy treatment to citizens. We therefore take this opportunity to review pediatric DDB treatment using cryopreserved allo-CEG with the intent of promoting the usefulness of keratinocyte cell therapy.

A method for culturing human keratinocytes was established in 1975, and auto-CEG was applied clinically to a burn for the first time in 1981 [6]. Thereafter, from the 1980s to 1990s, auto-CEGs were used frequently for the treatment of burn patients [7–13]. However, even though auto-CEG was regarded as an ideal therapeutic method for extensive burns, the therapeutic outcomes were unpredictable. Auto-CEG cannot be prepared immediately after sustaining a burn, a waiting period of no less than 4 weeks is required until grafting is possible. Another major problem is that, during the waiting period, infection or granulation occurs on the wound surface of the burned skin, which causes the auto-CEG not to take [9–13].

On the other hand, allo-CEGs can be administered immediately after sustaining a burn. Hefton et al. applied allo-CEG to a DDB burn wound in 1983 and reported that it is an excellent biological dressing [14]. Also in 1986, Madden et al. used an allograft on a mixed DDB and DB burn wound and reported that wound healing was observed at an earlier stage than expected [15]. Although allo-CEG thus became the subject of great interest, it was found that it took to the wound surface only temporarily and was replaced gradually by autologous epidermal cells [16–19]. It was thus demonstrated that although allo-CEG cannot be used to treat a total skin defect wound, it is extremely effective as a temporary viable biological dressing in the treatment of DDBs, which are partial-thickness defect wounds [15,16]. Studies have demonstrated that the fresh-CEG and frozen-CEG were as effective as the freshly prepared CEG in treating partial-thickness wounds [20–22].

We have been using cryopreserved allo-CEGs for the treatment of pediatric DDB for 25 years and have reported that it is an excellent graft material that can be prepared rapidly without sacrifice on the part of the donor and can be cryopreserved for clinical use at any time [23,24].

In order to determine the long term viability of cryopreserved CEGs, we used FACS analysis and colony-forming efficiency to measure the cell survival rate at 1 month, 6 months, and 1 year after grafting, and reported a viability of approximately 60% after a year. Further, in agreement with Brain et al. [16], we confirmed by PCR analysis that the Y gene of the donor remains in the female recipient for 3 months [24]. As shown by Duinslaeger et al. [25], we also reported that a half-side test of the donor site wound clearly demonstrated the scarring suppression effect of cryopreserved epithelial grafts [24].

In the present study, we reevaluated 50 cases of pediatric DDB treatment using cryopreserved allo-CEGs from 1992 to 2000. From the postoperative monitoring, we noted that when cryopreserved allo-CEG is grafted shortly after sustaining a burn, it induces early closure of the wound and suppresses scarring. We wanted to determine why cryopreserved allo-CEG promotes wound healing and suppresses scarring. We hypothesized that the growth factors and cytokines contained in the cryopreserved allo-CEG promote the proliferation and migration of keratinocytes to promote earlier wound closure. We also examined whether or not any scar-suppression factors are present. We thus measured the concentrations of various growth factors and cytokines which are involved in wound healing and believed to be produced by keratinocytes [26–29], as well as MMP-1 [30–32] and HGF [33].

2. Patients and methods

This study was approved by Kurume University Hospital and its affiliated hospitals. During the years 1992–2000, cryopreserved allo-CEGs were grafted in 50 cases. The cryopreserved allo-CEGs were prepared from excessive epidermal cells obtained from patients for auto culture in the treatment of burns and associated scars. The donors were burn patients who received autologous cultured epithelial grafting. These patients gave their consent of their own will to use any excess skin cells collected for the treatment of other burn patients. After obtaining permission of the donor patients, these cells were prepared into cultured epidermal allografts and cryopreserved.

2.1. Culture method for preparing epithelial grafts

The keratinocytes were cultured according to the technique of Rheinwald and Green [6], and described in detail in a previous report [23,24]. When the keratinocytes were differentiated and formed into a sheet, the cell sheet was treated with enzymes (Dispase, Godoshusei, Tokyo, Japan), detached from the culture flask, and fixed on a carrier (collagen membrane, Meiji Seika KK, Tokyo). All patients were examined for HIV, HBC, HCV, HTLV-1 and bacterial infections, and all were confirmed to be unaffected. For HBV, HCV and HTLV-1, antigen–antibody complexes were measured. For HIV, antigen–antibody complex was qualitatively analyzed and examined by Western blotting. The culture medium was examined and confirmed to be free of contamination and bacterial infections once per week.

2.2. Cryopreservation and thawing procedure

The freezing mixture was made of the basic medium (DME and Ham's F-12) supplemented with 10% fetal calf serum and 10% glycerin (a cryoprotective agent). For cryopreservation, 150 cm^2 of cultured epithelium together with carrier gauze was placed in a 50 mL Falcon tube, soaked into the freezing mixture, left at 4 °C for 2 h, placed on a polystyrene box and kept at −80 °C overnight, and then frozen at −135 °C on the following day [23]. Cryopreserved cultured epithelial allografts were quickly thawed in heated water (37 °C) immediately before grafting (Fig. 1) as reported previously [24].
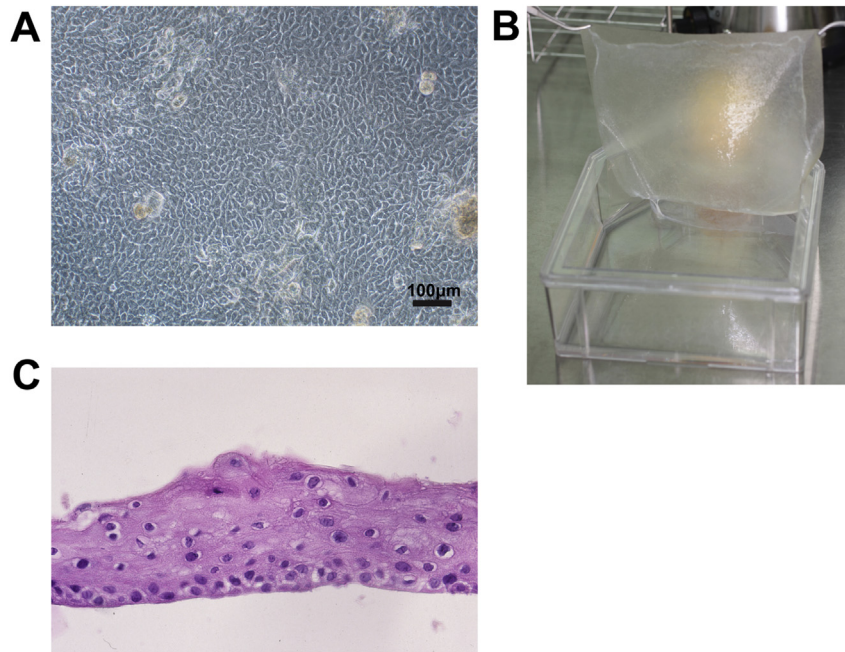
Fig. 1. Confluent keratinocytes and a cryopreserved-CEG sheet. A) Confluent keratinocytes are well-differentiated with clonal growth. B) Cryopreserved-CEG sheet with carrier. The CEG detached from the culture flask. C) Histology of CEG cryopreserved for (H.E. ×400). The structure of the epidermis is well-preserved.

2.3. ELISA assay of growth factors, cytokines, and MMPs in cryopreserved allo-CEG

Cryopreserved allo-CEGs were thawed in water heated to 37 °C, and the cryopreserved solution was washed repeatedly with physiological saline to eliminate the freezing mixture. To each epithelial cell sheet (150 cm^2), 2.0 mL of physiological saline was added. After solubilizing the cell sheets by ultrasonication and using a homogenizer, dilution to a total liquid volume of 2.5 mL was performed in a measuring cylinder and the resulting solution was used as the "solubilized solution." Nine types of growth factors and cytokines (IL-1α, IL-1β, PDGF-AA, TGF-α, TGF-β1, VEGF, KGF, b-FGF, IL-6, and HGF) and MMP-1 were measured with ELISA kits.

[ELISA Kits Used]: Human IL-1α: Life Technologies (IL-1α ELISA Kit), human IL-1β: Life Technologies (Human IL-1β ELISA Kit), human/mouse PDGF-AA: R&D Systems (Quantikine® Human/Mouse PDGF-AA Immunoassay), human TGF-α: R&D Systems (Quantikine® Human TGF-α Immunoassay), human TGF-β1: R&D Systems (Quantikine® Human TGF-β1 Immunoassay), human VEGF: Life Technologies (Human VEGF ELISA Kit), human KGF: R&D Systems (Quantikine® Human KGF Immunoassay), Human b-FGF: Life Technologies (Human FGF Basic ELISA Kit): R&D Systems® (Quantikine® Human FGF basic Immunoassay), human IL-6: R&D Systems (Quantikine® Human IL-6 Immunoassay), human Pro-MMP-1: R&D Systems (Quantikine® ELISA Human Pro-MMP-1), human HGF: Life Technologies (Human HGF ELISA Kit).

2.4. Histological analysis

Biopsy specimens were tissue samples from the same patient that were taken one year after the grafting from the site of the cryopreserved allo-CEG grafting and from a site where no grafting was performed. Hematoxylin and eosin and Masson trichrome staining were performed on the biopsy specimens obtained one year after cryopreserved allo-CEG grafting.

3. Surgical procedure

Before grafting, a tangential excision was performed to remove the eschar of the burn wound and debridement was performed to a depth where clean and bleeding wounds could be controlled. After preparing a graft bed on the burn wound and ulcer, the cryopreserved cultured epidermal allograft was grafted, a petrolatum-coated tulle gauze was placed over them, and they were sutured with the base at several points from above the petrolatum-coated tulle gauze with nylon thread. Next, a thick dry gauze applied with antibiotic ointment (gentamicin) was placed and elastic-taped, protected by an elastic bandage. For the treatment of large wounds, wet-to-dry dressing using gauze soaked in 0.5% silver nitrate solution and dry gauze was applied and antibiotic ointment was not used. For both cases, tie-over dressing was not performed. The first change of the dressing was done on the 4th day; after that, only the gauze on top was changed daily.

Table 1
Clinical outcome of cryopreserved allo-CE grafting.

Number of cases	50 (23 males, 27 females)
Age	7 months to 18 years
Total graft area	24,011.3 cm^2 (average graft area: 370.56 ± 459.79)
Period from sustaining of wound to grafting	4–7 days
Average period from graft surgery to epithelialization	9.32 ± 3.63 days
Pain after graft	Awareness of pain was reduced or eliminated
Complications	Cases of narcotic analgesic administration: 0
	Post-graft infection: 0
	Melting: 0
	Keloid: 0
	Hypertrophic scar: light partial 5
Follow-up-period	2–9 years (average 5 years 6 months)

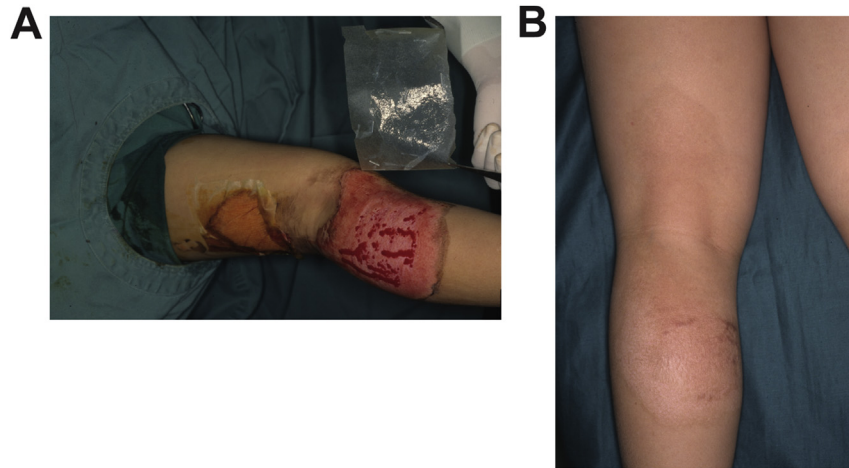Additional conventional grafting: 0.

Fig. 2. Clinical application, case 1. A) The patient is a 7-year-old-female on the day she sustained a scalding burn on her right leg (degree II DDB). After debridement of the burn eschar on the 6th day after the burn, two cryopreserved allo-CEG (300 cm^2) that were prepared as described in Materials and methods were grafted to the wound bed. B) One year after grafting, the cryopreserved-CEG graft site has smooth epithelialization and no contracture.

4. Results

4.1. Clinical application of cryopreserved allo-CEG grafting

The total area of grafts grafted in 50 cases was 24,011.3 cm^2, the average graft area was 370.56 ± 459.79 cm^2, and the period from sustaining the wound to grafting was 4–7 days. The average period from graft surgery to epithelialization was 9.32 ± 3.63 days, clearly faster than the 3–8 weeks it generally takes for epithelialization of ordinary DDB. Although partial, local infection was observed, epithelialization occurred quickly. There were no cases where the cryopreserved allo-CEG graft became detached from the recipient site. There were no occurrences of keloid, scarring and contractures were slight, and scar formation was reduced or suppressed. Pain at
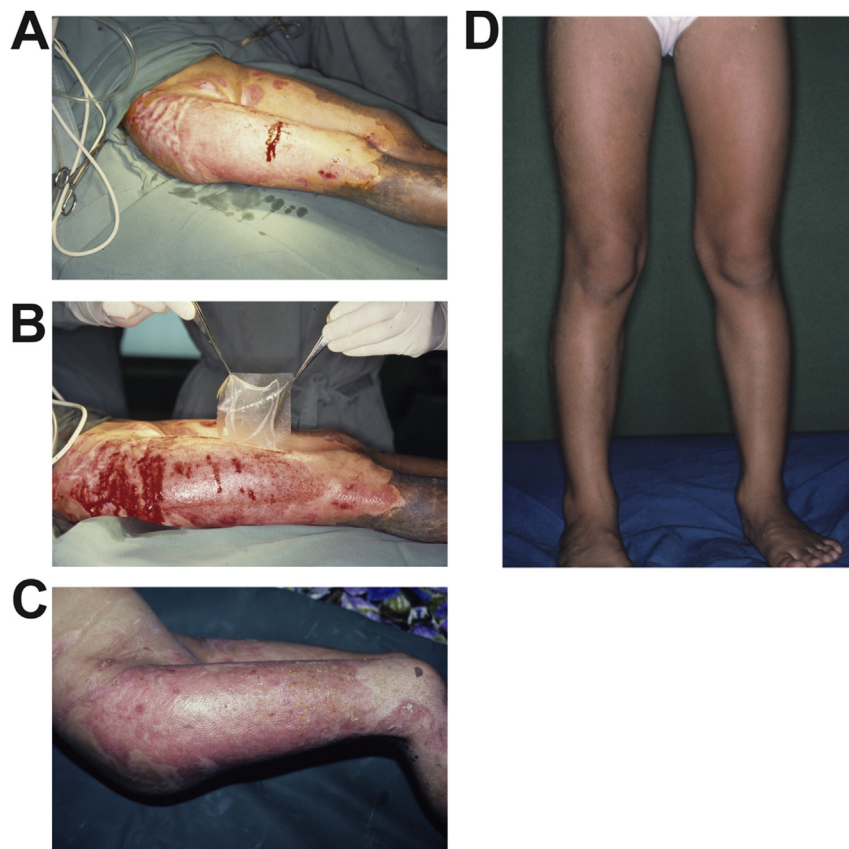

Fig. 3. Clinical application, case 2. A) A scalding burn is shown on a 10-year-old girl. She has degree II DDB to DB and the BSA was 35%. B) After debridement of the burn eschar on the 7th day after sustaining the wound, 8 cryopreserved cultured epithelial allograft sheets (1200 cm^2) were grafted to both sides. C) Seven days after grafting, the cryopreserved-CEG graft site shows clear epithelialization. The patient's hospitalization after operation was only 14 days. D) Five years after grafting, there are no scars and movement disorders.

the recipient site was reduced or eliminated and there were no cases of narcotic analgesic administration (Table 1). Pre- and postoperative photos are presented in Figs. 2–4.

The results for the three clinical applications presented in the figures were as follows:

Case 1: A 7 year old girl. She suffered scalding burns on her right leg from hot water from a pot at home (degree II DDB). After tangential excision of the burn eschar on the 6th day after the burn, two cryopreserved allo-CEG (300 cm^2) that were prepared as described in Materials and methods were grafted to the wound bed. Seven days after the grafting epithelialization had been completed and was able to leave the hospital. She showed excellent progress after one year with no scar contractures and smooth epithelialization had been achieved (Fig. 2).

Case 2: A 10 year old girl. She fell on a hot water bathtub and suffered scalding burns on both of her legs (degree II DDB). She was interned at the burns center after receiving primary care at an emergency care facility. After tangential excision of the burn eschar on the 7th day after sustaining the wound eight cryopreserved cultured epithelial allograft sheets (1200 cm^2) were grafted to both legs. Seven days after the graft epithelialization had been completed and she left the hospital 14 days after the surgery. Four years after grafting there are no scars or displacements (Fig. 3).

Case 3: A 10 year old boy. He jumped into a large public bath suffering scalding burns to his body (the BSA was 80%. Degree II, DDB to DB). He was hospitalized at the burns center after receiving care at an emergency facility. After tangential excision of the burn eschar on the 6th day after sustaining the wound, twenty cryopreserved allo-CEG sheets (1500 cm^2) were grafted to the left side and a conventional mesh skin graft was applied to the right side. Seven days after the graft epithelialization had been achieved and he was able to leave the hospital 14 days after the surgery. Four months after grafting a hypertrophic scar, redness and contracture were observed at the conventional mesh skin graft site on the right. Smooth epithelialization and no hypertrophic scars were observed on the left side where the cryopreserved allo-CEG graft was applied (Fig. 4).

4.2. Concentration of growth factors, cytokines and MMP-1 in cryopreserved allo-CEG

It is known that cryopreserved allo-CEG induce a paracrine effect, secreting growth factors onto the wound bed and affecting

Fig. 4. Clinical application, case 3. A) A 10-year-old boy who suffered from scald burns. At the time of injury, the BSA was 80%. On the day of the burn, the burn wound was of substantially the same depth at right and left sides (degree II DDB to DB). B) After debridement of the burn eschar on the 6th day after sustaining the wound, 20 cryopreserved all-CEGs (1500 cm^2) were grafted to the left side and a conventional mesh skin graft was applied to the right side. C) At the 4th month after grafting, hypertrophic scar, redness, and contracture were observed clearly at the conventional mesh skin graft site at the right side. At the cryopreserved-CEG graft site at the left side, smooth epithelialization was seen and a hypertrophic scar was not observed. D) Eight years after grafting. Although the scar at the conventional mesh skin graft site matured, contracture and disfigurement were observed. The cryopreserved-CEG graft site at the left side has no contracture, is flat, and is thus superior to the right side esthetically.

interactions among cells of the epidermis and the dermis. The cytokines and the growth factors contained in cryopreserved-CEG. In cryopreserved allo-CEG, the concentration of IL-1α was higher than the concentrations of other components (Fig. 5). IL-1α expression is followed by VEGF > TGF-β > TGF-α in cryopreserved-CEG. KGF, which is not produced by keratinocytes, was not detected (Fig. 5).

MMP-1 and HGF are known to play an important role in controlling over-production of collagen and thus reducing hypertrophic scars in vivo [32–35]. Although MMP-1, which suppresses scarring, was contained in cryopreserved-CEG, HGF was not detected (Fig. 5). Together, these results led us to believe that cryopreservation does not compromise the secretion of proteins involved in wound healing and reduced scar formation.

4.3. Histological analysis of biopsy specimens obtained from cryopreserved allo-CEG grafting

We compared the tissue in the area where the cryopreserved allo-CEG was performed and tissue from an area where no graft was performed for the same patient. HE staining showed the presence of a rete ridge in the epidermal layer of the tissue sample from the cryopreserved allo-CEG grafting site. The upper dermis layer was regenerated and remodeling of the collagen fibers was observed, the proliferation of fibroblasts and production of fiber were suppressed (Fig. 6). On the other hand, rete ridge formation was not found in the sample taken from the site where no cryopreserved allo-CEG grafting was performed. The epidermal layer was flat, the collagen fiber in the upper dermis layer was dense and fibrillated and it was found to be over abundant. Masson Staining also showed the formation of a rete ridge in the cryopreserved allo-CEG grafting

Fig. 5. Levels of growth factors, cytokines and MMP in cryopreserved-allo CEG. The mean values for cryopreserved allo-CEG, calculated from three independent experiments. In cryopreserved allo-CEG, the concentration of IL-1α was ten times the concentrations of other components (*P* > 0.01). Although IL-1α is followed by VEGF > TGF-β > TGF-α, there were higher levels in cryopreserved-CEG. KGF, which is not produced by keratinocytes, was not detected. Concentration of MMP-1 and HGF in cryopreserved allo-CEG: Although MMP-1, which suppresses scarring, was contained in cryopreserved allo-CEG. HGF, which is not produced by keratinocytes, was not detected.

site tissue sample. In the upper dermis layer fiber was sparse and sweat glands random presence was observed. In the sample from the site where there was no cryopreserved allo-CEG grafting the formation of a rete ridge could not be confirmed. The epidermal layer was flat, the collagen fiber in the upper dermis layer was dense and fibrillated and here too it showed to be over abundant (Fig. 6).

5. Discussion

Generally, deep dermal burns do not heal for 3–8 weeks, sometimes longer, and extensive scarring and contracture occur after conservative therapy [1–4]. Conventional autologous skin grafting is thus deemed to be preferable whenever possible. However, with conventional skin grafting, a new wound surface is formed at the skin donor site and a disfiguring scar is formed at both the recipient site and the donor layer − in addition healthy skin is also damaged. The use of cryopreserved allo-CEG allows us to cover a DDB wound without creating a donor site, avoiding the problem of additional damage. In the present study, we retrospectively reviewed the use of cryopreserved allo-CEGs for grafting in 50 cases of pediatric DDB wounds during the 8-year period from 1992 to 2000. The time from sustaining the wound to grafting is generally 4–7 days with conventional therapies, but the grafting of cryopreserved allo-CEG was performed shortly afterwards. Whereas healing of a DDB burn takes 3–8 weeks with conventional conservative therapy, in cases where cryopreserved allo-CEG was grafted, the average period until epithelialization was 9.32 ± 3.63 days. Thus, we found that when cryopreserved allo-CEG is used, fast wound healing is promoted and early epithelialization is achieved. It was also found that excellent healing effects in comparison to conventional skin grafting are obtained in esthetic terms as well (Fig. 2). It is extremely beneficial that scarring is suppressed, which reduces the amount of additional post-operative surgery (Fig. 3) and relieves some emotional distress of the patients. In case 3 the same patient suffered the same kind of burns on both sides of his body, burns caused by same temperature hot water and with the same depth. We were able to apply all the available cryopreserved allo-CEG to the left side of the patient but had to apply the mesh conventional skin graft to the right side. This case allowed us to compare the healing process of the wounds with both methods.

Fig. 4 shows that, at the conventional skin grafting site, scarring is not suppressed and a hypertrophic scar is formed. On the other hand, at the cryopreserved allo-CEG site, scarring was suppressed and smooth epithelialization is achieved.

Histology shows that at a conservatively epithelialized site, the epidermis is thickened and excessive fibrosis of the dermis is seen. However, at site at which cryopreserved allo-CEG was grafted, there was no thickening of the epidermis, rete ridges were observed, and over-fibrosis of the dermal portion was suppressed (Fig. 4).

Why is wound healing promoted and why are the effects of early epithelialization and scarring suppression obtained? It has been reported that cultured keratinocytes produce growth factors and cytokines that promote wound healing, including growth factors, such as b-FGF, TGF-α, TGF-β, EGF, VEGF and PDGF, cytokines, such as IL-1α, IL-6 and IL-8, and extracellular matrix proteins, such as collagen, laminin, tenacin and fibronectin [26–30].

On the other hand, it is also known that a cryopreserved allo-CEG sheet itself releases growth factors. Tamariz-Domínguez et al. [34] reported that frozen cultured sheets of human epidermal keratinocytes express growth factors (TGF-α, TGF-β, EGF, VEGF and PDGF) and extracellular matrix proteins (collagen, laminin, tenacin and fibronectin). Also, Auxenfans et al. reported that IL-1α, IL-8, and
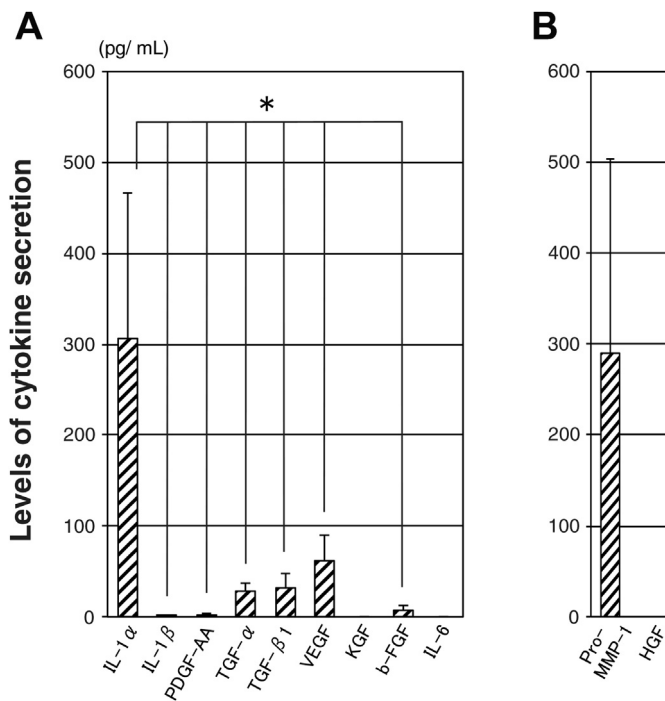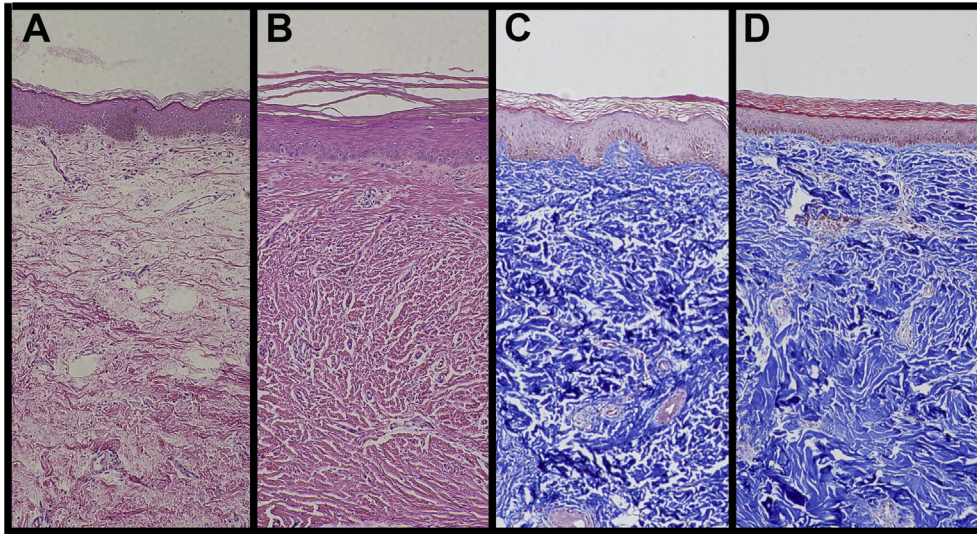
Fig. 6. H&E and Masson trichrome staining of cryopreserved allo-CEG and scar tissue. The histological findings after the cryopreserved allo-CEG are shown (A,C) When the cryopreserved allo-CEG is grafted, a rete ridge reforms and the proliferation of fibroblasts and production of fiber are suppressed. Then the dermis becomes softer. In contrast, the scar tissue is hard, fibrillated, and there is no rete ridge formation (B,D) original magnification ×100.

vascular endothelial growth factor (VEGF) were found in three samples of frozen allogenic epidermal sheets [35].

In the present study, we solubilized cryopreserved allo-CEG sheets before grafting and measured the respective concentrations of TGF-α, TGF-β1, IL-1α, IL-1β, PDGF-AA, VEGF, KGF, IL-6, b-FGF, as well as metalloprotease-1 (MMP-1) and HGF, which are known to have scarring suppression effects. We found that the amount of IL-1α was the highest and this was followed by VEGF > TGF-β > TGF-α > b-FGF. It was found that low amounts of IL-1β and PDGF-A are present and that there is very little IL-6. Also, KGF, which is not produced by keratinocytes, was not detected. The growth factors and cytokines contained in the cryopreserved allo-CEG itself.

What is the significance of the high levels of IL-1α? Keratinocytes are known to synthesize IL-1 in response to injury and IL-1 has been shown to stimulate fibroblast and keratinocyte growth, collagen synthesis by fibroblasts. Sauder et al. suggest that topical administration of IL-1α may be useful for the promotion of wound healing [36]. It was also reported that the maximal induction of KGF mRNA was observed when IL-1α was present [37] and that IL-1α induces KGF [38]. It has also been reported that a hypertrophic scar is not formed when the amount of IL-1α is high [39]. Clearly, high concentrations of IL-1α are beneficial for wound healing and scar suppression.

It is known that TGF-β, b-FGF, and VEGF are involved in angiogenesis, TGF-β1 and PDGF mediate fibroblast migration, and TGF-α stimulates the migration and proliferation of keratinocytes [40,41] and are also believed to promote wound healing.

Recent evidence shows that MMP-1 regulates wound healing and suppresses keloid formation and scarring [31–33]. The high concentration of MMP-1 contained in cryopreserved allo-CEG correlates with the clinically observed suppression of scarring after grafting of cryopreserved allo-CEG.

Our results suggest that the growth factors and cytokines contained in cryopreserved allo-CEG induce the proliferation and migration of keratinocytes to promote wound closure. In particular, ILα and MMP-1 are likely be involved in the scarring suppression by cryopreserved allo-CEG. The suppression of scarring after grafting is especially important clinically. Fig. 3 clearly shows that, at the conventional skin grafting site, scarring is not suppressed and a hypertrophic scar is formed. On the other hand, at the

cryopreserved allo-CEG site, scarring was suppressed and smooth epithelialization is achieved.

A basic advantage of cryopreserved allo-CEG is that although cryopreserved allo-CEG is a temporary treatment, it releases growth factors and cytokines to provide beneficial effects as a delivery system of wound-healing growth factors and cytokines. Another advantage of cryopreserved allo-CEG is that large quantities can be cryopreserved so that grafts can be supplied immediately when necessary for treatment.

Further, the extremely early epithelialization of a DDB wound and significant reduction of occurrence of scarring and disfigurement scar by use of cryopreserved allo-CEG are also beneficial in terms of the psychological aspects of patients. It is also important for the patient that there is no donor morbidity because there is no additional skin grafting or skin collection.

Burns and the associated wound care procedures can be extremely painful and anxiety-provoking for children. After the initial burn is sustained, procedural pain remains both the most intense and undertreated type of pain despite continual advancements in burn wound care [42,43]. Traditionally in order to prevent or reduce the pain associated with burn care intravenous anesthesia and sedatives such as Opioid and Ketamines have been used [42]. Importantly, pain reduction during burn wound care procedures has been linked with clinically significant improvement in wound healing (i.e., re-epithelialization) rates [42–44].

In the cases of the present study, intravenous administration of a sedative or anesthetic in the process of a post-operative dressing change was unnecessary and it was thus found that pain can be relieved by grafting cryopreserved allo-CEG at an early stage. This is extremely important for a pediatric patient and improves the motivation for rehabilitation and quality of life.

In general, it is known that an extensive wound to the skin, such as a burn, is accompanied by extremely intense pain and administration of a sedative or anesthetic is required in the process of daily dressing change process. Cryopreserved allo-CEG may be one solution to this problem.

6. Conclusion

We found that growth factors and cytokines released by cryopreserved allo-CEG promote re-epithelialization and suppression

of scarring. The removal of burn eschar from a wound surface by tangential excision of the burn eschar within 10 days during the early stage after sustaining of a wound is of primary importance in pediatric DDB treatment strategy. It is also important to graft cryopreserved allo-CEG to the refreshed wound bed to achieve early wound closure.

It was also found that cryopreserved allo-CEG has the effect of pain relief on a wound site after grafting. From the above, we conclude that treatment using cryopreserved allo-CEG is of benefit to a patient and contributes greatly to a reduction in long-term medical costs related to the series of treatments for burn wounds and post-burn-wound scarring.

Conflict of interest

All authors declare no conflict of interest associated with this manuscript.

Disclosure

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