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Cultured Human Epidermis Combined With Meshed Skin Autografts Accelerates Epithelialization and Granulation Tissue Formation in a Rat Model

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Introduction: As the take rate of cultured epidermal autografts in burn wound treatment is variable, widely expanded meshed auto skin grafts are often used in combination with cultured epidermal autograft to increase the take rate and achieve definitive wound coverage. However, a long time (3–4 weeks) required to prepare a cultured epidermis sheet is a disadvantage. Allogeneic cultured epidermis can be prepared in advance and cryopreserved to be used in combination with auto meshed skin grafts for treating third-degree burns. Nevertheless, the human cultured epidermis (hCE) has not been proved to accelerate wound healing after meshed skin grafting. Here, we investigated the effect of hCE on wound healing in a rat model of meshed skin grafting.

Materials and Methods: Human cultured epidermis was prepared from human neonatal foreskin and assessed by the release of growth factors into the culture medium using enzyme-linked immunosorbent assay. Skin wounds were inflicted on male F344 rats and treated by the application of widely meshed (6:1 ratio) autogenous skin grafts with or without hCE (n = 8 rats per group). Wound area, neoepithelium length, granulation tissue formation, and neovascularization were evaluated on day 7 postgrafting.

Results: Human cultured epidermis secreted IL-1α, Basic fibroblast growth factor, platelet-derived growth factor-AA, TGF-α, TGF-β1, and vascular endothelial growth factor in vitro. In rats, hCE accelerated wound closure (P = 0.003), neoepithelium growth (P = 0.019), and granulation tissue formation (P = 0.043), and increased the number of capillaries (P = 0.0003) and gross neovascularization area (P = 0.008) compared with the control group.

Conclusions: The application of hCE with meshed grafts promoted wound closure, possibly via secretion of growth factors critical for cell proliferation and migration, suggesting that hCE can enhance the healing effect of widely expanded skin autografts.

Key Words: cultured epidermis, wound healing, meshed skin graft, rat model, growth factor, ELISA

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In the treatment of severe burn injuries, the shortage of donor sites for split-thickness skin grafts is a significant problem and various methods of wound coverage have been investigated to overcome it.[1] Cultured epidermal autografts (CEAs) have been considered as a significant milestone in the treatment of extensive burn wounds. However, the clinical use of CEA has limitations because of lower take rate, especially on an infected bed,[2] and mechanical fragility and frequent spontaneous blistering, particularly at the early stages.[3] To circumvent these problems, alternative methods of CEA application have been explored. A combination technique using widely expanded split-thickness autografts overlaid by the CEA is now recognized as a useful approach which contributes to prompt epithelialization and provides reliable stability of the resultant epithelium.[4,5] As a consequence, definitive wound coverage can be achieved; however, a delay of 3 to 4 weeks required for the generation of a CEA from the patient's skin creates a fundamental clinical problem because it occurs during the life-threatening phase in patients with severe burns.

Meanwhile, a similar technique combining widely expanded split-thickness autografts and allogeneic cultured epidermis (allo-CE) has been reported.[6] Although allo-CE is not expected to survive on the wound for a long time after application, it can release a number of growth factors that stimulate the activity of patient's cells at the application site, promoting wound healing.[7,8] In large third-degree burn wounds, all epithelial stem cells are damaged and epithelialization cannot be achieved with allo-CE alone; however, combined with widely expanded split-thickness autografts, allo-CE can enhance reepithelialization in the interstices of the meshed graft.[6] Allo-CE can be prepared in advance and cryopreserved so that it is ready to use for the treatment of severely burned patients in the acute phase while CEA is being prepared. Therefore, allo-CE can improve burn wound treatment in the acute phase, making up for the shortcomings of CEA.

Nevertheless, the effectiveness of the method based on the combined application of allo-CE and meshed autogenous skin grafts has not been yet established either in an appropriate controlled study or in an animal model.

In this study, we evaluated the effect of human (h)CE used in combination with a meshed skin graft on wound healing in a xenograft model. We used immunocompetent rats and no immunosuppressive agent to evaluate the wound healing effect of hCE in the condition which would be immunologically rejected similar to the clinical situation. Therefore, it is reasonable to extend the results obtained with a xenograft model in this study to an allogeneic transplantation in clinical use. First, hCE was assessed for the secretion of growth factors in vitro, and then applied with widely meshed (6:1) skin grafts on wounds inflicted on F344 rats. Our results indicate that hCE accelerated wound closure, and induced granulated tissue formation and neovascularization, suggesting the enhancement of the wound healing effect of widely expanded autografts.

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MATERIALS AND METHODS

Preparation of hCE

Human cultured epidermis was prepared by Japan Tissue Engineering Co., Ltd. using Green's method described previously[9,10] with some modifications. Briefly, cryopreserved keratinocytes cultured from human neonatal foreskin (C-001-5C; Life Technologies Corporation, Tokyo, Japan) were thawed and disseminated on irradiated 3T3-J2 cells used as a feeder layer. Keratinocytes were cultured in Dulbecco Modified Eagle Medium and Ham's F12 medium mixed 3:1 and supplemented with 5% fetal calf serum, insulin, hydrocortisone, cholera toxin, triiodothyronine, epidermal growth factor, and antibiotics in an atmosphere of 10% CO_2 at 37°C.

To distinguish human and rat keratinocytes on histological sections, the former were labeled with nontoxic lipophilic fluorescent dye PKH26 (Sigma-Aldrich Japan, Tokyo, Japan) according to the manufacturer's protocol. PKH26 is a nontoxic lipophilic fluorescent dye (emission at 567 nm) that stains the membranes of viable cells and is distributed between cells at mitosis; it has a half-life of over 100 days.[11–13] For PKH26 staining, keratinocytes cultured for 2 passages were washed with serum-free medium and treated with 4 mL of 2 μM PKH26 solution for 4 minutes at room temperature. Then, the cells were rinsed to remove the dye and further cultured with fresh medium until the next day. Human cultured epidermis was obtained as keratinocyte sheets which were detached after treatment with dispase and aseptically packaged for transportation.

Growth Factor Release By hCE

The secretion of growth factors by hCE sheets into culture medium was evaluated by enzyme-linked immunosorbent assay (ELISA). Briefly, after 2 passages, human keratinocytes were cultured with 30 mL of conditioned medium in a T150-flask for 24 hours and the medium was then harvested and stored at −80°C.

After thawing, the samples were centrifuged at 18,800*g* for 10 minutes to remove cell debris and analyzed for the release of growth factors. Basic fibroblast growth factor (bFGF), platelet-derived growth factor-AA (PDGF-AA), TGF-α, TGF-β1, and keratinocyte growth factor (KGF) were measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, Minn), and interleukin-1α (IL-1α), IL-1β, and vascular endothelial growth factor (VEGF) were measured using the Invitrogen ELISA kit (Invitrogen Corp., Camarillo, Calif) according to the manufacturers' instructions. The results were expressed as the amount of a growth factor released by 1 hCE sheet after 24-hour incubation compared with the fresh medium of the same composition as control.

Because irradiated 3T3 feeder cells used for keratinocyte culture may also release growth factors, confluent lethally irradiated 3T3 cells were incubated as above without human keratinocytes for 24 hours and their conditioned medium was analyzed by ELISA as described.

Experimental Animals

F344 8-week-old male (CLEA, Japan) rats were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The number of animals used in this study was kept to a minimum, and all possible efforts were made to reduce suffering in compliance with the protocols established by the Animal Research Committee of Kyoto University. Our experimental protocol was approved by the Animal Research Committee (Permit Number: Med Kyo 14570).

Combination Therapy Using hCE With Meshed Skin Grafting

A total of 16 inbred rats were acclimatized in individual cages for 1 week before treatment. They were randomly assigned to the control group and the hCE group according to their body weight. After intraperitoneal injection of sodium pentobarbital (30 mg/kg, Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan), the entire dorsum of the animals was clipped and depilated with a depilation cream. General anesthesia (inhalation of 1.5% isoflurane; Wako Pure Chemical Industries Ltd., Osaka, Japan) was also applied when needed.

A 3 × 3-cm full-thickness skin defect was created on the dorsum of each rat. We resected the dorsum skin with a scalpel and scissors leaving pannicles carnosus to prepare a full-thickness skin defect. A piece of split-thickness skin (0.4-mm thick) was harvested from the resected skin using a Padgett drum dermatome (KD-110; Keisei Medical Industrial Co., Ltd., Tokyo, Japan) and expanded at a ratio of 6:1 to prepare meshed skin grafts using a skin graft mesher (MD-11; Keisei Medical Industrial Co., Ltd.). The meshed skin graft was returned to the skin defect area and carefully attached at 8 points at the edges of the area with 5-0 nylon suture to produce the graft of a uniform shape and size on each rat.

After grafting, the wounds in the control group (n = 8) were covered with polyethylene films containing absorbent cotton (Derma-Aid; ALCARE Co., Ltd., Tokyo, Japan), whereas in the hCE group (n = 8), hCE sheets were applied onto the meshed skin grafts and covered with the same dressing (Fig. 1). Then, the site was secured with a surgical bandage (Silkytex; ALCARE Co., Ltd.).

Seven days later, the animals were sacrificed by CO_2 inhalation and photographed with a digital camera. Skin specimens were harvested, fixed in 10% neutral-buffered formalin solution, and cut at the center of each wound to obtain cranial and caudal halves. The cranial part was embedded in O.C.T. compound (Tissue-Tek; Sakura Finetek USA, Inc., Calif) and frozen in ethanol-dry ice, whereas the caudal part was embedded in paraffin to prepare 10-μm frozen sections and 6-μm paraffin sections, respectively. The paraffin-embedded sections were then stained with hematoxylin-eosin (HE) and azocarmine and aniline blue (AZAN).

Assessment of the Wound Area

The area where epithelialization was not observed macroscopically in the interstices of the meshed skin graft at day 7 was measured in the photographs using the ImageJ software program, ver. 1.45 (NIH, Bethesda, MD, USA). The results were used to calculate the total nonepithelialized area in each specimen.

Assessment of the Remaining hCE

To confirm the fluorescence of PKH26-labeled hCE before the application to the wound, a piece of hCE was embedded in O.C.T. compound and frozen in ethanol-dry ice at the time of operation (day 0). Frozen sections of hCE alone (day 0) and of wound specimens from the hCE group (day 7) were observed under a fluorescence microscope (KEYENCE BZ-9000 and BZ-II Analyzer ver. 1.42; KEYENCE Japan, Osaka, Japan) using a tetramethylrhodamine isothiocyanate filter. For the comparison, the sections were stained with HE and observed under an optical microscope.

Histological Assessment of Neoepithelium Growth

The length of the neoepithelium was measured starting from the edge of the meshed graft on HE-stained sections under an optical microscope (KEYENCE BZ-9000 and BZ-II Analyzer ver. 1.42). The measurements were performed on both sides of the two strips of grafted skin in the inner part of the wound (4 points in each section), and the total sum was used for the analysis.

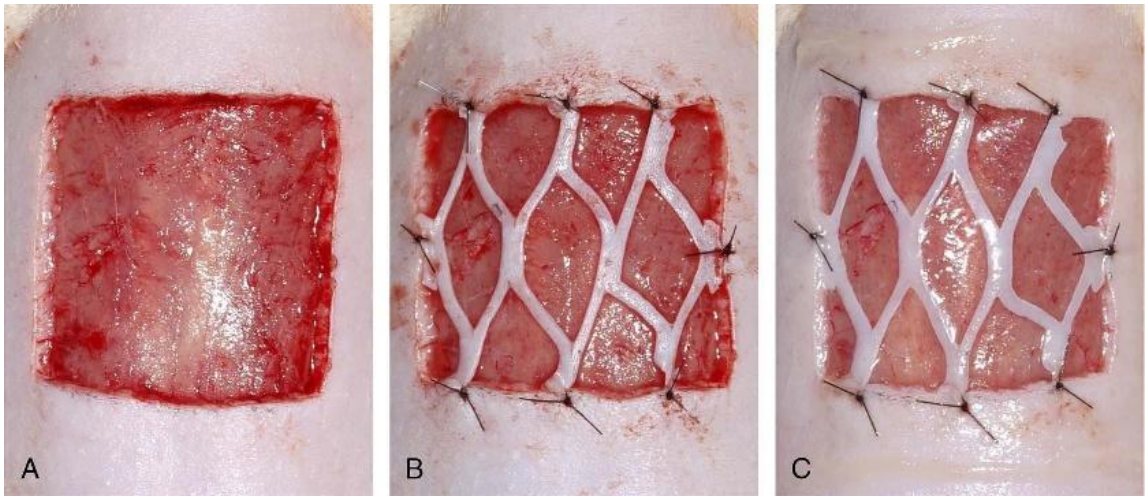


FIGURE 1. Meshed skin graft model. A, A 3 × 3-cm full-thickness skin defect was created on the dorsum of each rat. B, A 0.4-mm split-thickness meshed skin graft (6:1 ratio) was returned to the skin defect area and sutured in eight points. C, The grafted wound was covered with hCE in the hCE group.

Granulation Tissue Formation

To assess the formation of regenerated tissue, the thickness of granulation tissue newly formed in the interstices of the meshed skin graft was evaluated using AZAN-stained sections. In each section, there were 3 areas of granulation tissue; therefore, we measured the distance between the wound surface (excluding the epithelium) and the deep fascia at the center of each granulation area and calculated the mean value.

Immunohistochemical Staining and Evaluation of Newly Formed Capillaries

Immunohistochemical staining with von Willebrand factor (vWF) was used to detect newly formed capillaries. For this, 6-μm sections were dewaxed, rehydrated, and incubated with proteinase K (S3020; Dako Japan, Tokyo, Japan) for 5 minutes at room temperature for antigen retrieval. Anti-vWF rabbit polyclonal antibodies (1:5000, Code No. A0082; Dako) were used as the primary antibody followed by the secondary antibody (K4003, EnVision; Dako). The staining was visualized using 3-3'-diaminobenzidine-4HCl (DAB, Code 725191; Nichirei Biosciences Inc., Tokyo, Japan); the sections were then counterstained with hematoxylin and micrographs were taken under an optical microscope.

In each section, a rectangle of 500 × 300 μm was selected at the center of the granulation area beyond the muscle layer, and the number of newly formed capillaries in the rectangle and the cross-sectioned area of neovascularization were measured using the BZ-II Analyzer imaging software program (version 1.42; KEYENCE Co.).

Measurements and Statistical Analysis

All measurements in this study were performed by 3 plastic surgeons blinded to the group allocation. All data were expressed as the mean ± SD, and statistical significance of the difference was evaluated by Student *t* test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Growth Factor Release by hCE

The secretion of growth factors (bFGF, IL-1α, IL-1β, IL-6, PDGF-AA, TGF-α, TGF- β1, VEGF, and KGF) by hCE is shown in Figure 2.

The conditioned medium of human keratinocytes contained bFGF, IL-1α, PDGF-AA, TGF-α, TGF-β1, and VEGF, whereas IL-1β, IL-6, and KGF were not detected by ELISA (the detection sensitivity was 1.0, 0.7, and 15 pg/mL, respectively, which corresponded to 0.03, 0.02, and 0.45 ng/24 hours per sheet, respectively). The highest secretion was observed for VEGF.

The release of growth factors could not be attributed to 3T3 feeder cells which secreted only TGF-β1 (3.3 ng/24 hours per flask), whereas none of the other growth factors were detected.

Wound Area

Figure 3A shows representative macroscopic images of the wounds 7 days after grafting. The results indicate that the wound area in the hCE group was significantly smaller than that in the control group ($P = 0.003$; Fig. 3B), suggesting the enhancement of the healing effect by hCE.

Identification of the Epithelium

Fluorescent microphotographs of hCE before application (day 0) and of the wound in the hCE group on day 7 are shown in

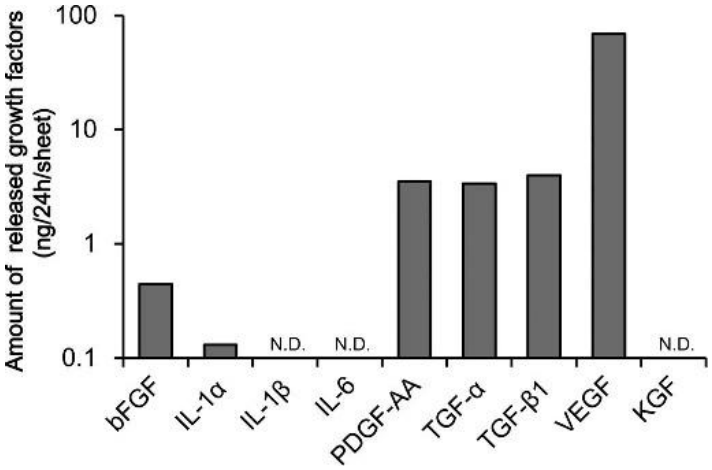


FIGURE 2. Growth factor release by hCE. The release of bFGF, IL-1α, IL-1β, IL-6, PDGF-AA, TGF-α, TGF-β1, VEGF, and KGF by hCE into medium after 24-hour culture. The vertical axis represents logarithmic scale. N.D., not detected.

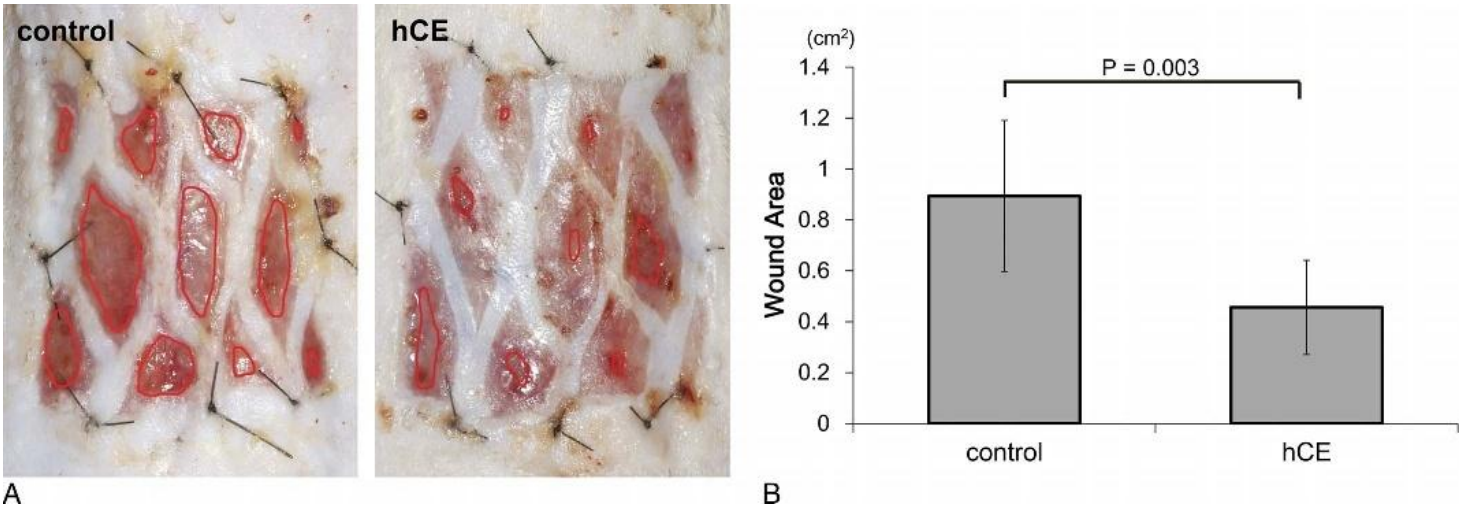


FIGURE 3. Wound area. A, Representative macroscopic images of the wounds in the control and hCE groups on day 7 postgrafting. The remaining wound areas are marked by red lines. B, Quantification of wound areas. The data are expressed as the mean ± SD (n = 8). The wound area in the hCE group was significantly smaller than that in the control group ($P = 0.003$).

Figure 4. Fluorescence could be clearly observed in the hCE sheet labeled with PKH26 dye (day 0; Figs. 4A, B). However, no fluorescence was detected in the wound tissue on day 7 postgrafting (Figs. 4C and D), indicating the absence of labeled human keratinocytes in the epithelium covering the wound surface.

Neoepithelium Formation

Histopathological evaluation of the HE-stained sections of the wound areas at day 7 postgrafting revealed that a separate sheet-shaped structure which would be seen in case of low hCE integration

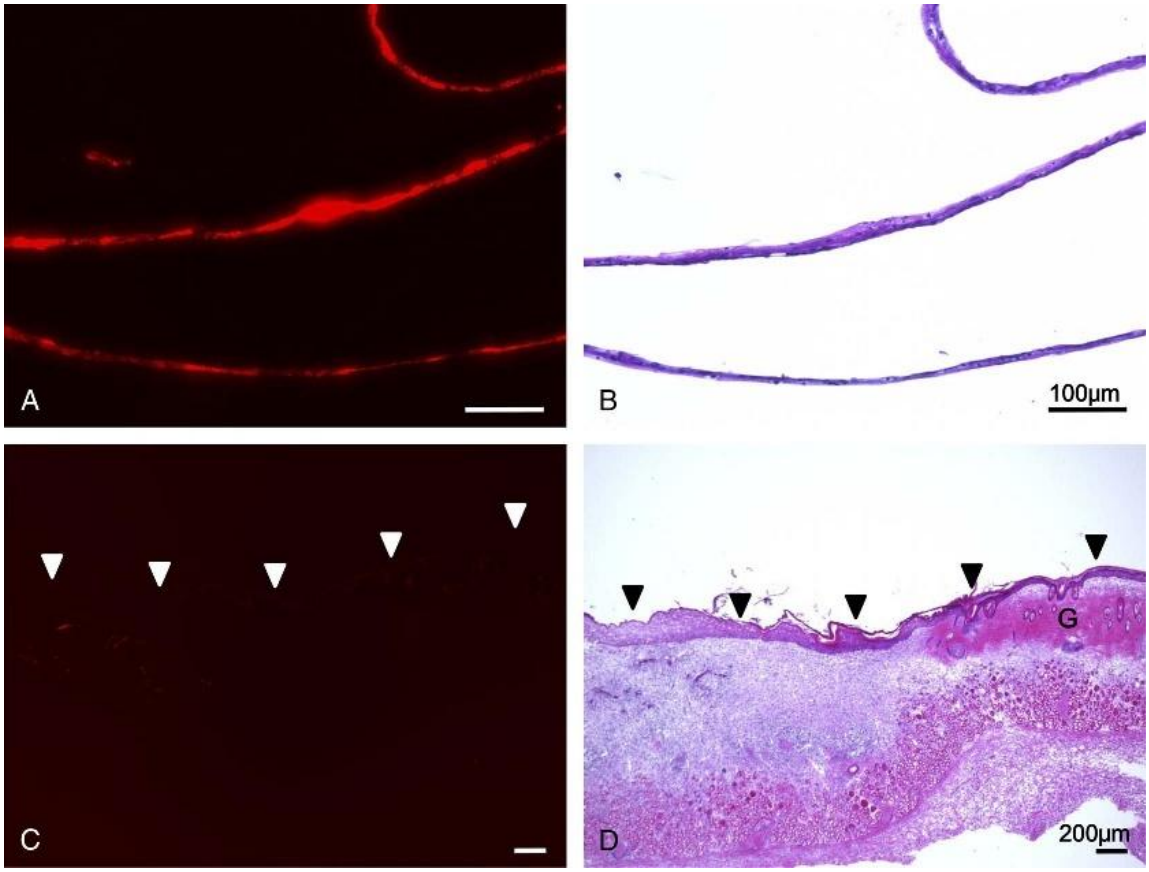
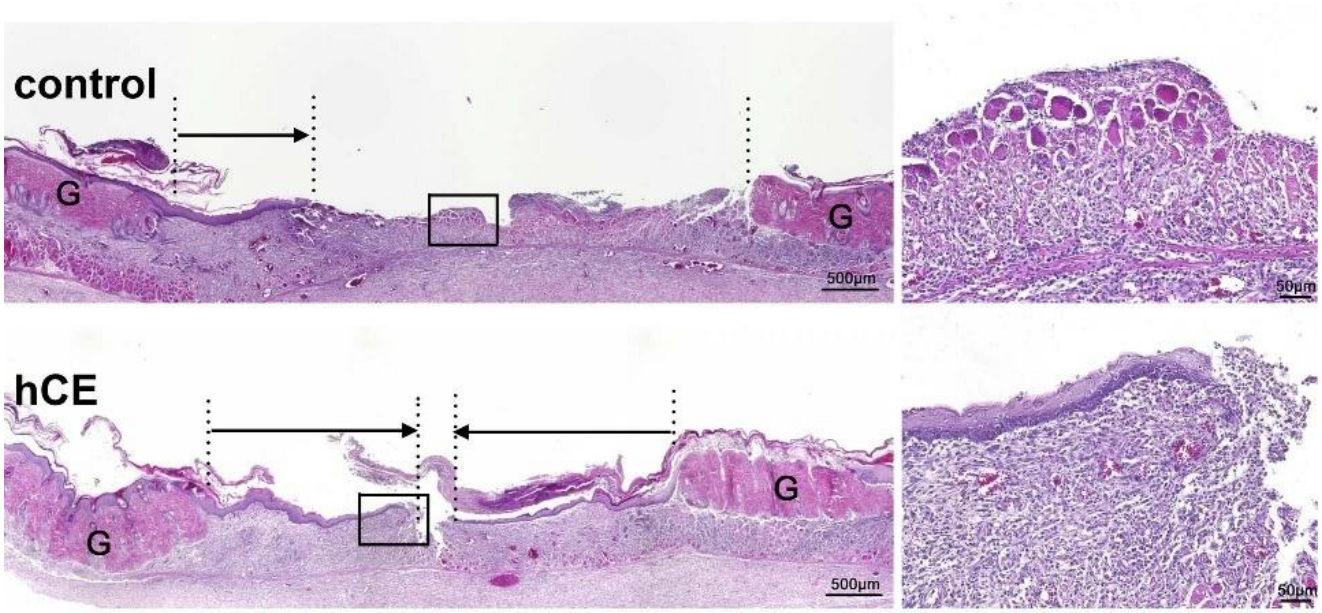


FIGURE 4. Identification of the neoepithelium. Frozen sections of hCE before the grafting (A, B) and of the wound tissue in the hCE group on day 7 postgrafting (C, D). Fluorescence was observed under a fluorescence microscope using a tetramethylrhodamine isothiocyanate filter (A, C) and HE staining was monitored under an optical microscope (B, D). Arrowheads indicate the epithelium. No PKH26-labeled hCE keratinocytes were observed in the epithelium which covered the wound surface on day 7.



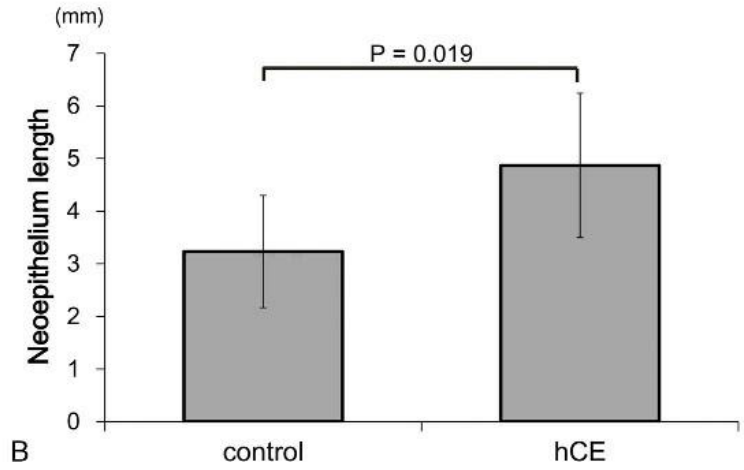


FIGURE 5. Neoepithelium length. A, HE-stained sections of the wounds in the control and hCE group on day 7 postgrafting (left panel: low magnification; right panel: high magnification of the area marked by a rectangle). Black arrows indicate the neoepithelium. G: meshed skin graft. B, Quantification of neoepithelium formation. The data are expressed as the mean ± SD (n = 8). The neoepithelium was significantly longer in the hCE group compared to the control group ($P = 0.019$).

was not observed on the wound surface in the hCE group (Fig. 5A). In the control group, a considerable portion of the graft did not take and some epithelium of the skin graft was necrotized and detached. The neoepithelium in the hCE group was significantly longer than that observed in the control group ($P = 0.019$; Fig. 5B), indicating that hCE promoted epithelialization in the grafted wound.

Granulation Tissue Thickness

Granulation tissue development in the control and hCE-treated wounds was analyzed in the AZAN-stained sections which showed that the formation of new connective tissue was intensified in the hCE group (Fig. 6A). Granulation tissue was generated on the exposed panniculus carnosus in the interstices of the meshed skin grafts, and its thickness in the hCE-covered wounds was significantly greater than that observed in the control group ($P = 0.043$; Fig. 6B).

Neovascularization

The formation of new blood vessels in the healing wound areas was evaluated by immunostaining with vWF on day 7 posttreatment (Fig. 7A). The number of new capillaries and gross area of neovascularization were significantly greater in the hCE group compared with the control group ($P = 0.0003$ and 0.008, respectively; Figs. 7B, C), which is consistent with the enhanced formation of the connective tissue in the wound (Fig. 6).

DISCUSSION

The results of this study indicate that hCE released a number of growth factors and accelerated wound healing in combination with extended meshed grafts.

It has been established that hCE promotes wound healing when used as allografts in deep dermal burns,[7,14–17] donor sites,[18–21] and chronic ulcers.[22–24] The beneficial effect can be attributed to the production of basement membrane proteins (collagen types IV–VII, laminin, and fibronectin) which enhance cell migration, creating a favorable environment for the ingrowth of keratinocytes from the wound bed and edges.[14,16] Moreover, keratinocytes in the epidermis can release a number of growth factors,[8,25,26] such as bFGF, IL-1α, IL-1β, IL-6, PDGF-AA, TGF-α, TGF-β1, and VEGF[27,28]; however, there has been no

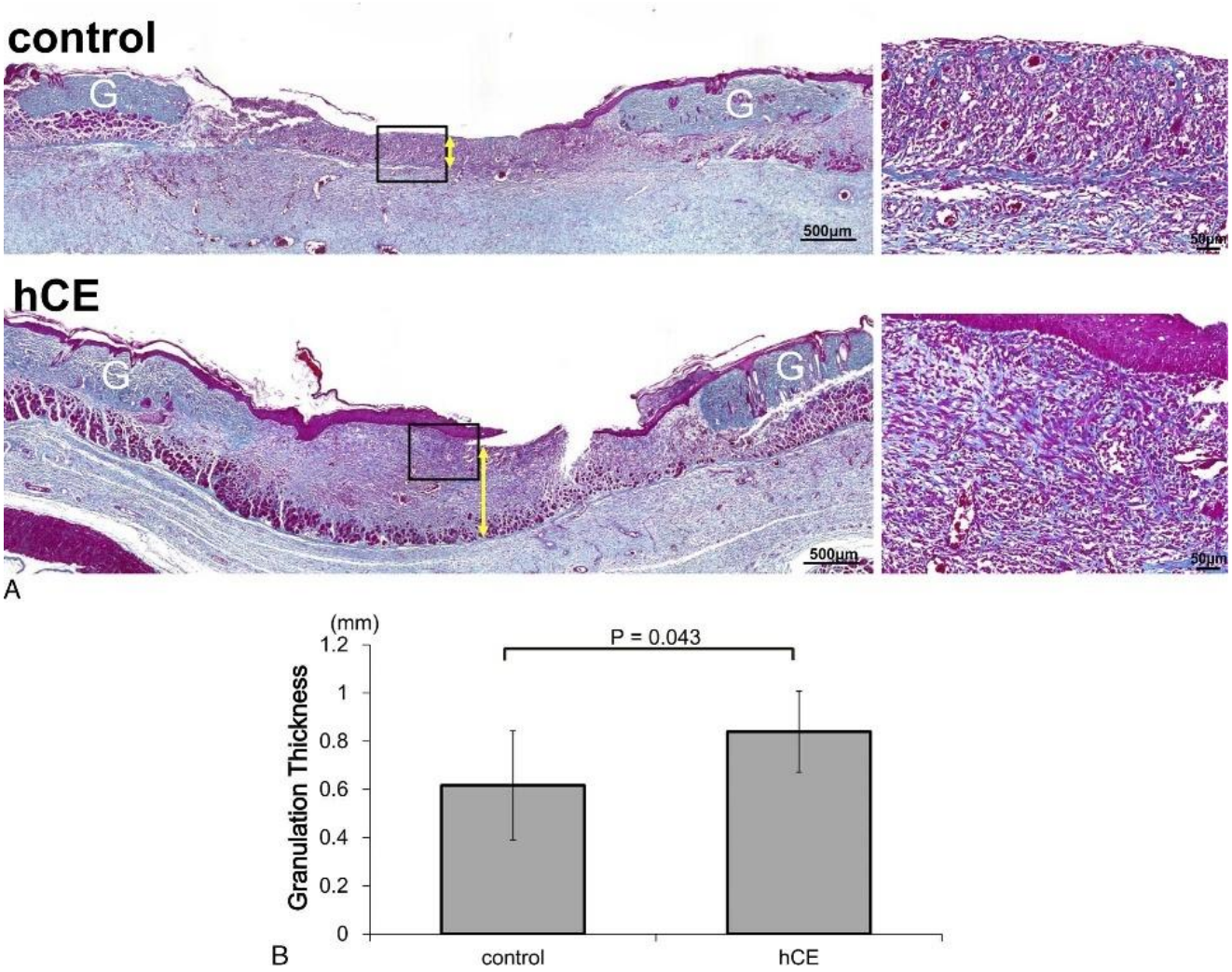


FIGURE 6. Granulation thickness. A, Representative AZAN trichrome-stained sections of the wounds in the control and hCE groups on day 7 postgrafting. Yellow arrows indicate the thickness of granulation tissue generated in the interstices of the meshed skin graft. G: meshed skin graft (left panel: low magnification; right panel: high magnification of the area indicated by a rectangle). B, Quantification of granulation tissue thickness. The data are expressed as the mean ± SD (n = 8). Granulation tissue formation was significantly enhanced in the hCE group compared to that in the control group ($P = 0.043$).

quantitative evaluation of growth factor secretion by keratinocyte sheets cultured on 3T3 feeder cells.

As it is difficult to assess growth factor release by hCE after its application to the wound surface, we performed in vitro analysis of keratinocyte conditioned medium before hCE grafting and detected bFGF, IL-1α, PDGF-AA, TGF-α, TGF-β1, and VEGF. As growth factors have a pivotal role in cell proliferation, differentiation, and metabolism during wound healing stages,[13] these results suggest that the released growth factors could account for one of the reasons of the accelerated wound healing with hCE. Besides, keratinocytes interact with fibroblasts in the dermal tissue via a paracrine loop,[29] suggesting that hCE-released growth factors may promote intercellular cooperation during wound closure.

The 3T3 feeder cells alone also produced TGF-β1. However, the feeder cells were detached and almost completely removed after human keratinocyte proliferation and the formation of confluent cell sheets; therefore, the contribution of 3T3 cells to the amount of growth factors in hCE conditioned medium was negligible.

Although animal models of meshed skin grafted wounds have been reported,[30–33] the variability in size and shape of used skin grafts made them unsuitable for the accurate evaluation of the wound healing process. In this study, we established a standardized model by using uniform skin grafts of identical thickness and shape, wounds of fixed size and location, and raw surfaces in the interstices of controlled size. In this model, the wound area was gradually reduced by epithelialization and wound contraction, and completely healed in 2 weeks, so we could not find any differences between the two groups at that time in a preliminary examination. Therefore, we evaluated wound healing on day 7 postgrafting and found that hCE accelerated epithelialization and granulation tissue formation.

Because of its flexibility, hCE tightly adheres to the uneven surface of the wound and coats the exposed cells and extracellular matrix immediately after application. The interspaces in the expanded graft are covered by the hCE sheet and are filled with plasma from the wound surface. Furthermore, as hCE keratinocytes survive on the wound for some time, they secrete growth factors and basement membrane proteins, creating an ideal environment for cell migration and proliferation. Importantly, hCE produced VEGF which, as a factor critical for angiogenesis in the wound bed, should stimulate the formation of granulation tissue.

However, the absence of PKH26-labeled hCE keratinocytes on day 7 indicates that hCE did not take to the rat wound and may have been detached and lost during sampling, possibly because our xenograft model was based on immunocompetent rats.

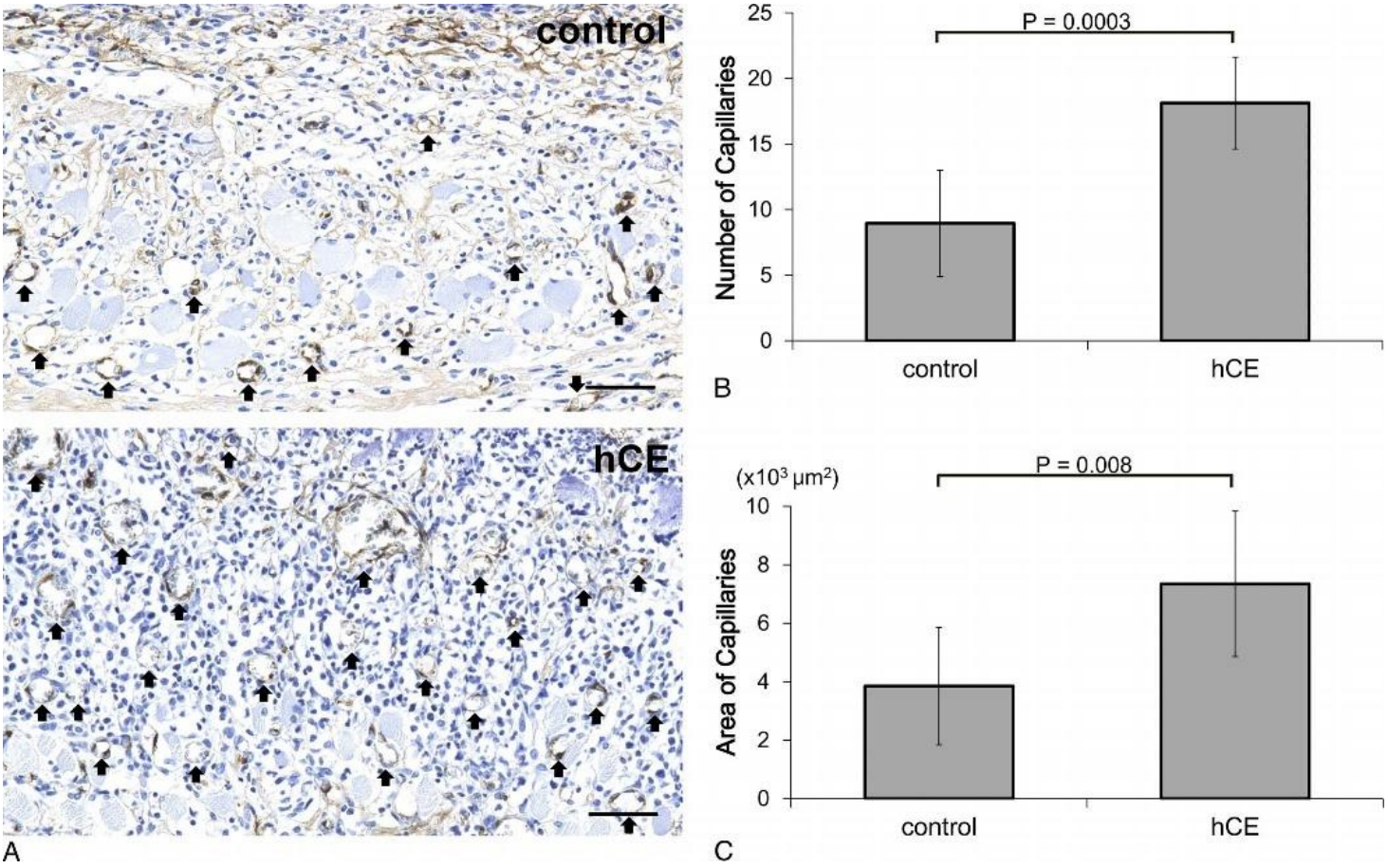


FIGURE 7. Neovascularization in the healing wound. A, Immunohistological staining with vWF to reveal neovascularization in the wounds of the control and hCE groups on day 7 postgrafting. Black arrows indicate newly formed capillaries. Scale bar: 50 μm. B, Quantification of new capillaries. The number of capillaries in the hCE group was significantly higher than that in the control group ($P = 0.0003$). C, Gross neovascularization area was significantly larger in the hCE group than in the control group ($P = 0.008$). The data are expressed as the mean ± SD (n = 8).

Previous studies have also used immunocompetent animals to study hCE effects on wound healing. Thus, it has been shown that the frozen hCE accelerates re-epithelialization and promotes early formation of granulation tissue in a full-thickness skin defect model based on immunocompetent mice.[34] Moreover, cultured human keratinocytes enhanced epidermal wound healing in a skin defect model based on pigs.[35] Our results are consistent with these data, suggesting that cultured human keratinocytes can exert beneficial effects on the wound healing process in different species by secreting growth factors and other biologically active proteins.

In clinical settings, the approach based on the combination of the allo-CE and meshed autogenous grafts can be very effective in treating patients with severe burns. The beneficial wound healing effect of the combination technique of the allo-CE and autografts may achieve a comparable result to that of the combination method of the CEA and autografts. This combination method of the allo-CE and autografts that can be performed while the CEA is being prepared may promote wound healing and reduce the mortality of severe burn patients.

CONCLUSIONS

Our results indicate that hCE produced growth factors (bFGF, IL-1α, PDGF-AA, TGF-α, TGF-β1, and VEGF) and significantly reduced wound area by accelerating granulation tissue formation and angiogenesis if combined with the meshed skin graft in rats. The beneficial effect of hCE in an experimental model suggests that hCE can be potentially used to promote the grafting of widely expanded meshed autografts during the treatment of severe burns. Future studies are required to investigate the destiny of the transplanted keratinocytes when used as allograft.

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