Effect of Cultured Dermal Substitute Composed of Collagen Sponge Seeded with Fibroblasts in Simultaneous Skin Graft Overlay

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Abstract

An artificial dermal substitute composed of a xenogenous collagen sponge has been widely used to create a dermal component in a full-thickness skin wound. However, one of the disadvantages in using the dermal substitute is that it requires a 2-stage surgical procedure for final wound closure. In order to close the wound much more rapidly, another type of dermal substitute which enables a simultaneous skin graft overlay is needed. In the present study, we investigated the effect of a collagen sponge seeded with isogenic fibroblasts on a simultaneously overlaid skin graft in a rat model. Normal dermal fibroblasts of SD rat were seeded and cultured on a collagen sponge composed of cross-linked porcine type I collagen. Full-thickness skin wounds were created on the dorsum of SD rats. The collagen sponges with or without fibroblasts were placed in the wounds, then covered simultaneously with split-thickness skin graft (STSG). At 1 week and 4 weeks after grafting, overlaid STSG survival, TGF-β1 level, wound contraction, final re-epithelization and histology of the grafts were studied. The overlaid STSG survived moderately but the seeded fibroblasts did not enhance the STSG survival rate. Also, there were no significant differences in wound contraction and in the final wound epithelization between the collagen sponge seeded with and without fibroblasts. Both collagen sponges with and without fibroblasts developed neo-dermal structure without any specific immunological reaction. However, the fibroblast-seeded collagen sponge grafts showed significant re-epithelization along the junctional surface between the collagen sponge and the STSG by out-growing of the hair follicle cells. This epithelization seemed to result in formation of epithelial inclusion cysts in the neo-dermis. It was suggested that the seeded fibroblasts strongly induced such an epithelial outgrowth. The cultured collagen sponge may have beneficial effect on re-epithelization in a certain type of simultaneous skin graft overlay such as meshed STSG.

Introduction

It is known that the dermal component of the skin plays an important role in wound healing. Full-thickness skin defects lacking the dermal component tend to heal with wound contraction resulting in hypertrophic scar formation. When the full-thickness skin defects are wide such as an extensive burn injury, split-thickness skin grafts (STSG) or cultured epidermal autografts are performed to close the wounds. However, a thinner

Key words: cultured dermal substitute, fibroblast, skin graft overlay.
STSG results in poor cosmetic appearance and a thicker STSG leaves a hypertrophic scar on the donor site. Also, a lack of sufficient underlying dermal bed in the recipient site results in poor survival of cultured epidermal autografts\(^5\). In order to create dermal component on the full-thickness skin defects, a number of dermal substitutes have been produced\(^7\) and now they can be classified into 3 types. Any type of dermal substitute is expected to work as a template of dermal matrix and to be gradually replaced with the host collagen matrix to make a neo-dermis.

The first type of dermal substitute is the acellular allogenic dermal matrix composed of an acellularized allograft skin. This type of dermal matrix has been commercially available in western countries as a dermal substitute, which can be used with a simultaneous skin graft overlay\(^9\). However, the problems in shortage of allograft skin supply and potential risk of disease transmission from the donor skin have not been resolved\(^8\). The second type of the dermal substitute is a biodegradable matrix such as polyglactin mesh. These materials are usually used with cultured fibroblasts\(^6\). However, the survival and cosmetic result of simultaneously overlaid STSG with the cultured fibroblasts are not reliable or predictable\(^10\).

The third type is an artificial dermis composed of cross-linked animal collagen matrix. This type of dermal substitute can be mass-produced and has much safer behavior clinically. Originally, the artificial dermis was composed of 2 layers with outer silicone membrane, and has been used to create neo-dermis on the wounds. The representative products are Integra\(^10\) (Integra Life Sciences Corp., Plainsboro, NJ, USA\(^10\)), Terudermis\(^8\) (Terumo Co., Japan\(^10\)), and Pelna\(^10\) (Kowa, Co., Ltd., Japan\(^10\)). These materials need a two-stage surgical procedure for the final wound closure, because a waiting period of neovascularization after grafting is required. This is one of the great disadvantages of the artificial dermis\(^10\). To resolve this problem, authors have developed much thinner porous collagen sponge and have reported that the collagen sponge can be used with a simultaneous STSG overlay\(^10\). However, further improvement or modification of the collagen sponge is required for the clinical application. On the other hand, in recent years, artificial collagen sponges, which contain cultured fibroblasts, have been developed\(^13\). However, the major purpose of these cultured collagen sponges is to accelerate the granulation tissue formation and wound healing of chronic skin ulceration such as in a diabetic foot ulcer\(^13\). So the behavior of the cultured collagen sponge as a dermal substitute combined with skin grafting on a full-thickness skin defect has not been clarified. In the present study, we investigated the influence of the collagen sponge seeded with fibroblasts to the simultaneous STSG overlay in full-thickness skin defects of rats.

**Materials and Methods**

**Materials**

**Animals**

Six week-old male 15 Sprague-Dawley (SD) rats (Klea, Japan, Inc., Tokyo) were used for this study.

**Cultivation of fibroblasts**

Primary culture of fibroblasts was obtained from the skin of SD rat by biopsies, as described previously\(^17\). The skin was cut into small pieces and put on a petri dish. After the pieces of the skin were attached to the floor of the dish, they were incubated with Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL, U.S.A) supplemented with 10% fetal bovine serum (FBS; GibcoBRL, U.S.A.), at 37 °C, in 5% CO\(_2/95\)%air. The medium was changed 2 or 3 times a week. After a large enough number of fibroblasts were proliferated from the pieces of the skin, subcultivation of the cells was carried out with 0.25% trypsin/EDTA solution (GibcoBRL, U.S.A.). The third and fourth passages of the cultured fibroblasts were used for the further studies.

**Preparation of collagen sponge**

The collagen sponges composed of cross-linked porcine type I collagen were supplied from Kowa Co., Ltd., Tokyo, Japan. The pore size was between 70-120 μm and the thickness was 1 mm\(^14\). The collagen sponges were placed into 6 well culture dishes (Iwaki, Asahi Thecno Glass CO., Japan) under sterile condition. The collagen sponges were incubated with 1.5 ml DMEM 6 hours

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**Abbreviations**: DMEM, Dulbecco’s modified eagle medium; EDTA, ethylene diamine tetra-acetate; FCS, fetal bovine serum; SD, Sprague-Dawley; STSG, split-thickness skin graft; TGF-β1, transforming growth factor β1.
before seeding fibroblasts at 37 °C, in 5% CO2/95% air.

Preparation of fibroblast-seeded collagen sponge

In order to evaluate the attachment efficacy of fibroblasts onto a collagen sponge, following study was performed. Fibroblasts were seeded on a collagen sponge which was placed on a culture well. Twenty-four hours later, the well was washed 3 times with phosphate buffered saline (PBS), then the attached cells were detached from the collagen sponge with trypsin/EDTA treatment. Following testing with trypan blue, the number of viable cells was counted using a hemocytometer. The cellular growth on a collagen sponge was studied 3 and 5 days after seeding of cells by the same method above. According to these preliminary studies about cellular attachment and growth on the collagen sponge, fibroblasts were seeded on a cultured collagen sponge at the cell density of 1.0 × 10⁶ cells/cm², 2 or 3 days prior to the transplantation, when the cells were in a good proliferating stage. For the histological study, a collagen sponge seeded with fibroblasts was fixed with 10% formalin on the day of transplantation and the routine paraffin sections were stained with haematoxylin and eosin (H&E). Each study was triplicated.

In vitro evaluation of cultured collagen sponge overlaid by STSG skin.

In order to evaluate the stability of seeded fibroblasts when overlaid by STSG, a piece of SD rat's split-thickness skin was placed onto a fibroblast-seeded collagen sponge in a petri dish. Five days after incubation in DMEM with 10% FBS at 37 °C, in 5% CO2/95% air, the histology of the collagen sponge was studied using H&E staining. The study was triplicated.

Transplantation of Collagen sponge and STSG overlay

Before the surgical procedure, complete anesthesia of rats was obtained using ketamine. Two full-thickness skin wounds (2 × 2 cm square each) were created on the dorsum of a SD rat. A collagen sponge with fibroblasts was placed on one wound, and a collagen sponge without fibroblasts was placed on the other wound. Then the transplanted collagen sponge was covered with a simultaneous STSG isograft of another SD rat (0.015-0.018 inch thick).

Evaluation of wound healing

One week after grafting, the STSG survival rate was determined macroscopically using the paper template measuring technique by the method of Takami et al. The percentage of survival rate was determined using the following formula:

\[
\text{(the total area of skin graft — the area of necrotic tissue)/ the total area of skin graft} \times 100
\]

Four weeks after grafting, the size of grafts and the final appearance of epithelization of grafts were measured macroscopically. The percentage of the wound contracture rate was determined using the following formula:

\[
100 — \text{(the area of the wound at 4 weeks/the area of the wound at the operation day)} \times 100
\]

The percentage of the epithelization area of wound was quantified as follows:

\[
\text{the epithelization area at 4 weeks/the area of the wound on the operation day} \times 100
\]

The grafted wounds were biopsied at 1 and 4 weeks after grafting, and the routine H&E sections were evaluated. Enzyme-linked immunosorbent assays for TGF-β₁ of transplanted collagen sponge

One week after grafting, the grafted wound was excised and the transplanted collagen sponge was gently dissected to avoid contamination of blood. The dissected collagen sponge was homogenized in PBS and treated with an ultrasonic bath, then centrifuged. The TGF-β₁ level in the supernatant was measured by enzyme-linked immunosorbent assays (ELISA) using the commercially available TGF-β₁ assay kit (TGF-β₁ Human ANALYZA immunoassay kit, Wako Pure Chemical Industries, Ltd., Japan). The cross reactivity between TGF-β₁ of human and rats has been noticed.

Statistical analysis

Paired T test was applied for all quantitative analysis of the results. The significant criterion for the test was set at P values less than 0.05.

Results

Preparation of collagen sponge seeded with fibroblasts

The attachment of fibroblasts onto the collagen sponge was significantly lower than the attachment to normal culture dishes. The percentage of the attachment efficacy was 46 ± 12% (mean ± SD) onto the collagen sponge, and 68 ± 17% onto the normal dishes (Fig. 1). Three days after seeding fibroblasts, The cells proliferated well. There were no significant differences in
Fig. 1 Attachment and growth of fibroblasts seeded on collagen sponge and on normal culture dish. The attachment of fibroblasts onto the collagen sponge was significantly lower than the attachment to normal culture dish (\( \ast \) indicates \( p < 0.05 \)). There were no significant difference in proliferation of fibroblasts between the collagen sponge and the normal dish on 3 days after seeding. However, five days after seeding, the proliferation of fibroblasts onto the collagen sponge was significantly lower than the cells onto the normal dish (\( \ast \) indicates \( p < 0.05 \)). (\( \bullet \) ) indicates the values obtained from collagen sponge; (■), those from normal dish. Bars at symbols indicate S.D.

Fig. 2 Histology of fibroblast-seeded collagen sponge. Seeded fibroblasts are seen on the uppermost part of the collagen sponge (arrow). The Matrix of collagen sponge is porous. (H & E staining, \( \times \) 200)

Fig. 3 Appearance of overlaid STSG isograft 1 week after grafting. A) STSG on the collagen sponge with cultured fibroblasts. B) STSG on the collagen sponge without fibroblasts. Both grafts reveal good survival.
Table 1 The findings of collagen sponge transplantation with simultaneous STSG overlay

<table>
<thead>
<tr>
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<th>STSG survival rate, 1 week after transplantation (%)</th>
<th>Junctional epithelization rate, 1 week after transplantation (%)</th>
<th>Contracture rate, 4 weeks after transplantation (%)</th>
<th>Final epithelization rate, 4 weeks after transplantation (%)</th>
<th>Epithelial cyst formation, 4 weeks after transplantation</th>
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<tbody>
<tr>
<td>With fibroblasts</td>
<td>69.2 ± 23.1</td>
<td>71.3 ± 26.2</td>
<td>46.3 ± 18.9</td>
<td>98.2 ± 2.3</td>
<td>+</td>
</tr>
<tr>
<td>Without fibroblasts</td>
<td>66.4 ± 14.3</td>
<td>12.3 ± 8.2</td>
<td>39.2 ± 14.3</td>
<td>85.9 ± 17.6</td>
<td>-</td>
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* All of the values were expressed by mean ± SD
* *P < 0.05 vs. collagen sponge without fibroblasts.

cellular proliferation between the cells on the collagen sponge and on the normal dish (Fig. 1). However, five days after seeding, the proliferation of fibroblasts on the collagen sponge was reduced as compared to the cells on the normal dish. There were significant differences between the two (Fig. 1). Histologically, seeded fibroblasts were seen on the uppermost part of the collagen sponge (Fig. 2). Five days after incubation of collagen sponge and overlaid split-thickness skin in a culture medium, both skin and collagen sponge were histologically stable and no apparent morphological changes of the seeded fibroblasts were observed.

Graft survival rate and stability

One week after grafting, all STSG isografts were fully adhered to the wound bed. The survived area and the necrotic area of the grafted skin were easily identified macroscopically (Fig. 3). The survival rate of the overlaid STSG was 69.2 ± 23.1% on the collagen sponge with fibroblasts, and 66.4 ± 14.3% on that without fibroblasts (n=7 for each group). There were no significant differences between the two (Table 1).

Contracture and final epithelization of grafts four weeks after grafting

The grafted wounds revealed contracture and shrinkage of the grafts 4 weeks after grafting (Fig. 4). The percent contracture rate of the grafts was 46.3 ± 18.9% on collagen sponge with fibroblasts, and 39.2 ± 14.3% on the collagen sponge without fibroblasts (n=5 for each group). There were no significant differences between the two

Fig. 4 Appearance of overlaid STSG isograft 4 weeks after grafting. A) STSG on the collagen sponge with cultured fibroblasts, B) STSG on the collagen sponge without fibroblasts. Wound contraction and partial ulceration were seen on both grafts. Most of the grafted area have been epithelialized. Dashed lines indicate the margin of the grafts.
(Table 1).

Because of the wound contracture and re-epithelization from the wound margin, most of the necrotic area of the STSG healed and only small area of ulceration was observed. The final average epithelization area of the grafted wounds was 98.2 ± 2.3% on the collagen sponge with fibroblasts, and 85.9 ± 17.6% on that without fibroblasts (n=5 for each group). There were no significant differences in the final epithelization of both grafts (Table 1).

**Measurement of TGF-β1 in transplanted collagen sponge**

At 1 week after the transplantation with STSG overlay, TGF-β1 level in both sponge with and without fibroblasts (n=3 for each group) was below the detectable level of the assay. There was no apparent increase of TGF-β1 in the transplanted collagen sponge with fibroblasts.

**Histological appearance of grafts one week after grafting**

One week after grafting, the transplanted collagen sponge revealed marked inflammatory cell infiltration. Abundant neovascularization was also found especially in a lower half of the collagen sponge. The pores of the collagen sponge still remained partially. These findings were equivalently seen in both the collagen sponge with and without cultured fibroblasts (Fig. 5).

In the transplanted collagen sponge with fibroblasts, marked epithelial cell outgrowth from the edge of hair follicles in contact with the surface of the transplanted collagen sponge was observed. The new epithelial cells were growing along the junctional surface between the STSG skin and the collagen sponge (Fig. 6) and formed a new epithelium. So this phenomenon may be called as the "junctional epithelization". For quantitative evaluation of the "junctional epithelization", we measured the length of the epithelialized surface of the collagen sponge using microscopic measurement. The percentage of the "junctional epithelization" rate was determined as follows.

\[
\text{the length of the epithelialized surface} / \text{the total surface length of collagen sponge} \times 100
\]

The "junctional epithelization" rate was 71.3 ± 26.2% on the collagen sponge with fibroblasts, and 123 ± 8.2% on the collagen sponge without fibroblasts (n=5 for each group). The difference was statistically significant (Table 1).

**Histological appearance of grafts 4 weeks after grafting**

Four weeks after grafting, both collagen sponge with and without cultured fibroblasts were replaced by host collagen and formed neo-dermis. The neo-dermis was similar to the normal dermal collagen matrix without
Effect of Cultured Dermal Substitute in Simultaneous skin

Fig. 6  Histological appearance of STSG on the collagen sponge with cultured fibroblasts 1 week after grafting. As the result of the "junctional epithelization" (arrow), 2 or 3 hair follicles became united on the surface of the collagen sponge. SG: STSG, CS: Collagen Sponge. (H & E staining, A: × 40, B: × 100, C: × 200)

appearance of granulation tissue. There was very minor foreign body reaction such as appearance of giant cells. No apparent immunological rejection to the collagen sponge was observed. In the area where the overlaid STSG survived well, both STSG and "neo-dermis" were clearly observed (Fig. 7 A, B). In the area where the STSG did not survive, STSG disappeared and the neo-dermis was directly covered by re-epithelization from the wound edge.

In the transplanted collagen sponge with cultured fibroblasts, epithelial inclusion cyst formation was observed in the junctional area of the STSG and the neo-dermis(Fig. 7 B,C). The epithelial cyst formation was not observed in the collagen sponge without fibroblasts.

Discussion

Creating dermal component has been a topic of interest in the past decade. Among the produced dermal substitutes, collagen sponge has been most widely applied for full-thickness skin wounds such as in deep burn injuries, because of its availability and clinical safety. Recent advance in tissue engineering has allowed us to produce new transplantable cultured bio-matrix. Cultured artificial dermis is one of the representative materials in tissue engineered skin substitute. It has been reported that during the culture period, the seeded
fibroblasts secrete extracellular matrix components such as collagen and glycosaminoglycan, and some kinds of growth factors and cytokines such as TGF-β1. In the present study, we investigated the characteristics of a porcine type I collagen sponge seeded with isogenic dermal fibroblasts in simultaneous STSG isograft overlay models. The results showed that fibroblast-seeded collagen sponge survived with overlaid STSG but there were no significant differences in the STSG survival rate between the collagen sponge with or without fibroblasts seeding. This result indicated that fibroblasts might not be essential as far as the simultaneous skin graft survival is concerned. The survival of the grafts was not so stable and the average survival rate was about 70% of the grafted area. So further modification of the collagen sponge structure will improve the graft survival. Also, a
further study about the effect of other dermal component cells such as vascular endothelial cells to the graft survival should be done.

The fate of the seeded fibroblasts has not been clarified. Since inflammatory cells rapidly infiltrated into the grafted dermal substitute after grafting, it was too difficult to identify the seeded fibroblasts from the other inflammatory cells histologically. Tanaka et al. reported that the presence of the complex matrix of dermal components secreted by the fibroblasts might be more important than the fibroblasts viability. Our result showed that 4 weeks after grafting, there were no significant differences in wound contracture and in the final re-epithelization between the collagen sponge graft with or without fibroblasts seeding. This result may suggest that the seeded fibroblasts cannot preserve the biological effects for a longer period after grafting. Lamme et al. compared acellular dermal substitute with dermal substitutes seeded with fibroblasts at different densities. Their results showed that the improved wound contraction and cosmetic appearance were correlated with the higher number of fibroblasts present in dermal substitute at the moment of grafting. So it was suggested that the difference in cell density may lead us to different results. Further studies are needed to confirm this.

The grafted collagen sponge was rapidly vascularized and finally replaced with host collagen. The created neo-dermis was similar to the normal dermal structure without extensive fibrosis. Inflammatory cell infiltration was marked at 1 week post-grafting but completely subsided at 4 weeks after grafting. Appearance of giant cells was minimum and there was no evidence of immunological rejection to the grafts. These results indicate that the collagen sponge seeded with fibroblasts is immunologically inert. These features were equivalently seen in both the transplanted collagen sponges with and without fibroblasts. Immunological reaction is linked to the presence of class I and class II MHC antigens on the surface of the donor cell. It has been known that after in vitro cultivation, the fibroblast's expression of both classes I and class II MHC antigens is in an insufficient condition to initiate an immune response. It has also been shown that allogenic fibroblasts are accepted by the host with no evidence of immune reaction over a long time, permitting prior preparation of the dermal equivalent in a tissue bank. Because we used isogenic fibroblasts derived from SD rats in the present study, it seems that antigenicity of the seeded fibroblasts is totally negligible.

The most significant change in the fibroblast seeded collagen sponge after the transplantation was that the grafts showed significant re-epithelization along the junctional surface between the fibroblast-seeded collagen sponge and the overlaid STSG. This type of re-epithelization, the "junctional epithelization", was due to epithelial cell outgrowth from the edge of hair follicles in contact with the surface of transplanted collagen sponge. Since most of the seeded fibroblasts were found to be located on the surface of the collagen sponge histologically, it is understood that the hair follicle cells of the STSG edge were located very near to or in contact with the seeded fibroblasts after grafting. It has been known that fibroblast's feeder layer such as 3T3 cells enhance the efficient cultivation of epidermal cells. Yamada et al. similarly reported that the cultured dermal substitute containing fibroblast is capable of promoting epithelization on full-thickness defect wounds from the margin of the grafts. Therefore, it was suggested that the seeded fibroblasts on the cultured collagen sponge might promote the "junctional epithelization". On the other hand, it is known that fibroblasts can produce growth factors and cytokines to modulate epithelial growth and differentiation by paracrine action. Among them, TGF-β, secreted from fibroblasts has been reported to inhibit epidermal cell proliferation. However, in this study, an increase in tissue TGF-β level was not observed in the transplanted fibroblast-seeded collagen sponge. These findings may lead us to the hypothesis that seeded fibroblasts enhanced the re-epithelization from the edge of hair follicles. However, further studies about the ability of seeded fibroblasts to produce TGF-β and the other type of cytokines are needed to confirm the role of the cells in the re-epithelization process.

Hansbrough et al. reported a similar experiment using dermal substitute with fibroblasts with meshed STSG overlay without pointing out the fact of "junctional epithelization". One possible reason for this may be due to the different property of the dermal matrix, because they used a biodegradable matrix as a dermal substitute.
The other possible reason is because they used a meshed STSG as overlaid grafts. In the present study, we applied a sheet STSG for the coverage on the artificial dermis. Meshed STSG has many interstitial openings in order to extend its size. So it was suggested that meshed STSG overlay may induce re-epithelization from the skin appendages in a different manner from the "junctional epithelization" in the sheet STSG overlay. The re-epithelization in meshed STSG induced by seeded fibroblasts may accelerate the closure of the meshed interstitial. This may be an important possibility for fibroblast-seeded cultured dermal substitute in future clinical applications.

The fate of the "junctional epithelization" is not apparent yet. However, it may be possible that the epithelial inclusion cyst formation which was found only in the wounds transplanted with fibroblast-seeded collagen sponge at 4 weeks after the transplantation, is one of the final outcomes of the "junctional epithelization." Since there were no significant difference in the final epithelization rate between the grafts with and without fibroblasts, it was suggested that the "junctional epithelization" did not affect the STSG survival. Further studies about the effects of the seeded fibroblasts for the epithelial growth will clarify this question.

Conclusion

The cultured collagen sponge fibroblasts-seeded survived with overlaid STSG in an experimental rat model. The seeded-fibroblasts did not enhance STSG survival rate, wound contraction or the final wound epithelization. However, it was suggested that the seeded fibroblasts induced re-epithelization on the STSG-dermal matrix junction by promoting outgrowth of hair follicle cells. The cultured collagen sponge may have a beneficial action for re-epithelization in a certain type of simultaneous skin graft overlay.

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Reference