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Original Article

# External administration of moon jellyfish collagen solution accelerates physiological wound healing and improves delayed wound closure in diabetic model mice

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ABSTRACT

*Introduction:* Artificial dermis is an effective therapeutic method for full-thickness dermal defects. However, the currently available artificial dermis made of porcine or bovine type I collagen has several limitations such as incomplete epithelialization and delayed migration of fibrogenic and angiogenic cells into the graft. We previously developed a composite dermal graft containing a mixture of moon jellyfish collagen and porcine type I collagen, and reported its stimulatory effect on both the re-epithelialization of the epidermis and the migration of fibrogenic and angiogenic cells into the graft. In the present study, we examined whether the same effect was observed by administering jellyfish collagen solution externally onto an artificial dermal graft made of bovine type I collagen.

*Methods:* We used a 6 mm full-thickness wound defect model. Moon jellyfish collagen was prepared as a concentrated 0.5% solution and dripped externally onto a transplanted artificial dermal graft made of bovine type I collagen. Wound repair and long-term dermal tissue remodeling were compared between mice administered jellyfish collagen solution on the bovine collagen graft and those transplanted with a composite dermal graft containing the same amounts of jellyfish and bovine collagens. The stimulatory effect of jellyfish collagen solution was also evaluated using diabetic dB/dB mice.

*Results:* External administration of jellyfish collagen solution onto the bovine collagen graft significantly accelerated wound closure compared to control saline. It also decreased the number of inflammatory cells infiltrating the wound and suppressed absorption of the transplanted graft, as well as reduced subsequent scar formation. Furthermore, external administration of jellyfish collagen solution onto the bovine collagen graft improved the delayed wound healing in diabetic model mice, and this effect was superior to that of the currently used basic fibroblast growth factor.

*Conclusions:* External administration of moon jellyfish collagen solution onto a bovine collagen graft significantly accelerated physiological wound healing and prevented excessive scar formation. It also improved wound closure in diabetic model mice, confirming its therapeutic application for intractable skin ulcers caused by impaired wound healing.



*Abbreviations:* b-FGF, basic fibroblast growth factor; H-E, hematoxylin-eosin; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

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## 1. Introduction

Dermal wound healing is a complex physiological process orchestrated by distinct cellular components present within the skin tissue or infiltrated from the circulating blood [1]. Proper wound healing can be achieved by re-epithelialization of the epidermis and granulation tissue formation in the dermis. Keratinocyte migration in the re-epithelialization process and infiltration of fibrogenic and angiogenic cells into the granulation tissue are the principal and indispensable factors for skin regeneration [2,3].

Impaired wound healing is frequently observed in aged and diabetic patients. It has become a serious medical issue in today's aging society [4]. The artificial dermis made of porcine or bovine type I collagen has been widely used in the treatment of full-thickness skin defects caused by deep burns [5–7]. Such conventional artificial dermis exerts a certain effect on granulation tissue formation in the dermis by providing a scaffold for migrating fibrogenic and angiogenic cells. However, it is particularly inefficient in re-epithelialization and requires subsequent epithelial transplantation from another part of the patient's body [8]. Open dermal wounds are susceptible to bleeding and infection, which may lead to life-threatening complications [9]. This makes artificial dermis treatment more precarious in patients with intractable skin ulcers with impaired regeneration. Novel therapeutic approaches for proper re-epithelialization are urgently needed.

We have previously reported on a novel artificial dermis that may overcome the limitations of the currently used dermal grafts. We developed a composite biomaterial graft containing a mixture of moon jellyfish collagen and porcine type I collagen [10]. When an artificial dermis made of this composite biomaterial was transplanted onto a full-thickness dermal defect, the re-epithelialization process was substantially faster than the control graft made of porcine type I collagen alone. The moon jellyfish collagen possesses a unique water solubility that is not found in other jellyfish species [11]. This characteristic together with the fact that it rapidly denatures and degrades at body temperature, renders moon jellyfish collagen suitable for an external medicine. Moreover, external administration may minimize the risk of any potential unexpected immune reactions caused by the unidentified invertebrate collagen component(s). These findings encouraged us to develop a novel therapeutic method to promote skin regeneration by externally applying jellyfish collagen onto the transplanted conventional dermal graft.

In the present study, we conducted a series of experiments to examine whether external administration of a moon jellyfish collagen solution onto the transplanted bovine type I collagen graft may exert a stimulating effect on the wound healing process. The results indicated that the external administration of jellyfish collagen solution onto the bovine collagen graft significantly accelerated physiological wound healing and prevented excessive scar formation. It also improved wound closure in diabetic model mice. These results provide new therapeutic insights into the management of otherwise intractable skin ulcers.

## 2. Methods

### 2.1. Preparation of artificial dermal grafts

Artificial dermal grafts used in the present study were prepared by a freeze-drying method using *t*-butyl alcohol as previously reported [10], except that they were made of type I bovine collagen instead of porcine collagen. The artificial dermis was punched out to a diameter of 6 mm for transplantation onto a dermal defect, which contained 167 μg of bovine type I collagen. The moon jellyfish collagen was purchased from the Jellyfish Research Laboratories (Yokohama, Japan). It was heat-denatured to prepare a 0.5% solution for external application. To prepare a composite biomaterial, jellyfish collagen was added to bovine collagen at a ratio of 13:87. The resultant artificial dermis with a diameter of 6 mm contained 25 μg of jellyfish collagen and the same amount (167 μg) of bovine type I collagen as a graft made of bovine collagen alone.

### 2.2. Mice

C57BL/6 J wild-type mice and BKS.Cg-+ *Lepr*$^{db}$/+ *Lepr*$^{db}$/Jcl mice (dB/dB mice) [12] at 7 weeks of age were purchased from CLEA Japan Inc. (Tokyo, Japan). All animals received humane care, and the experiments were approved by the Animal Experiment Committee of Tokai University.

### 2.3. Wounding and dermal graft transplantation

Mice were anesthetized and underwent full-thickness excisional wounding on their dorsum using a 6-mm biopsy punch (Kai Medical, Tokyo, Japan) as reported previously [10] Immediately after wounding, a dermal graft made of bovine type I collagen was transplanted onto the wound. It was followed by dripping 5 μL of 0.5% jellyfish collagen solution or basic fibroblast growth factor (b-FGF) solution (Kaken Pharmaceutical Co., Tokyo, Japan) onto the graft. The b-FGF solution is currently used for intractable skin ulcers [13]. Saline was used as control. The wounds were then covered with dressing tape and sutured to the surrounding skin. No external medication was applied when a composite graft containing a mixture of jellyfish collagen and bovine collagen was transplanted. The total amount of jellyfish collagen administered externally in the form of a solution and that contained in the composite dermal graft was set to be equivalent (25 μg/wound). Mice were housed individually in sterilized cages and provided with autoclaved food and sterilized water to prevent bacterial infection.

### 2.4. Estimation of wound closure

At day 6 or 10 after transplantation, the wound tissue was excised and fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde. Epithelial wound healing was estimated macroscopically by measuring the open wound areas. To easily recognize the edge of the regenerating epithelium, we poured a mixed solution of 10% May-Grunwald and 10% Giemsa (Merck, Darmstadt, Germany) on the wounds, as reported previously [10]. Photo pictures of each stained sample were analyzed using image J software (National Institutes of Health, Bethesda, MD), and the area inside the outline of regenerating epithelial edge was defined as the open wound area. The wound closure rate was calculated from the relative ratio of the epithelialized area (the original wound area subtracted by the open wound area) to the original wound area.

### 2.5. Histological and immunohistological examination

The excised graft tissues were fixed with 10% buffered formalin for regular hematoxylin-eosin (H-E) staining, or with 4% paraformaldehyde for immunohistological examination. Immunofluorescent staining was performed as previously described [10] with anti-F4/80 antibodies (Bio-Rad AbD Serotec, Kidlington, United Kingdom), followed by incubation with appropriate fluorescent secondary antibodies. After nuclear staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich), tissue

samples were examined under a confocal laser-scanning microscope (LSM 880, Carl Zeiss, Oberkochen, Germany). The number of F4/80-positive cells was counted in the area within 200 μm from the wound edge of each section, and the percentage of macrophages was calculated relative to the number of DAPI-stained nuclei per unit area as previously described [10].

### 2.6. Statistical analysis

Values are expressed as the mean ± SD. The Mann-Whitney U test was used to evaluate the significance of the differences among the groups examined. *P* values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. External administration of jellyfish collagen solution onto a bovine collagen graft accelerated re-epithelialization after wounding

First, we compared the effects on re-epithelialization after wounding between jellyfish collagen solution dripped on the bovine collagen graft versus the jellyfish collagen transplanted in a form of composite dermal graft. External administration of jellyfish collagen onto the transplanted bovine type I collagen graft significantly accelerated wound closure compared to the control saline at day 6 after wounding (Fig. 1). In contrast, when the same amount of jellyfish collagen was transplanted in a composite dermal graft, the wound closure ratios varied from one mouse to another, and the result was not significantly different from that of the control mice which had saline on the bovine collagen graft (Fig. 1).

Histological analysis using cross-sections of the excised wounds showed that the extension of regenerating epithelial cells toward the wound center was significantly promoted by external administration of the jellyfish collagen solution onto the bovine collagen graft but not by transplantation of the jellyfish collagen-containing composite dermal graft, compared with control saline dripped onto the bovine collagen graft (Fig. 2). In contrast to the thickened epithelial cell layer observed in the control mice given saline, the regenerating epithelial cell layer stimulated by external administration of jellyfish collagen onto the bovine collagen graft exhibited the same characteristic thin morphological features (Fig. 2) as previously reported [10].

### 3.2. Externally applied jellyfish collagen suppressed excessive inflammatory cell infiltration in the transplanted graft

We also compared the effects of jellyfish collagen administered in two different forms on cell infiltration in the transplanted dermal graft. For this purpose, we counted the number of cells observed in the wound marginal area at 0–200 μm from the margin of the transplanted graft. At day 6 after external administration of the jellyfish collagen solution onto the bovine collagen graft, the number of infiltrating cells was similar to that in the control saline group and significantly lower than that in the transplanted composite dermal graft (Fig. 3A). Immunohistochemical staining indicated that approximately 30% of infiltrating cells were F4/80-positive activated monocytes or macrophages in all three groups of mice (Fig. 3B), suggesting that the infiltration of monocytes/macrophages was suppressed at this time point in mice that were administered the jellyfish collagen solution externally compared to those with jellyfish collagen transplanted in the composite dermal graft.

### 3.3. External administration of jellyfish collagen solution suppressed degradation and absorption of the transplanted dermal graft

In the next set of experiments, we examined the long-term effect of jellyfish collagen administered in the two different forms on dermal tissue remodeling. While most mice transplanted with a bovine type I collagen graft followed by external administration of jellyfish collagen exhibited complete wound closure within 14 days after wounding (Fig. 4A), three weeks were

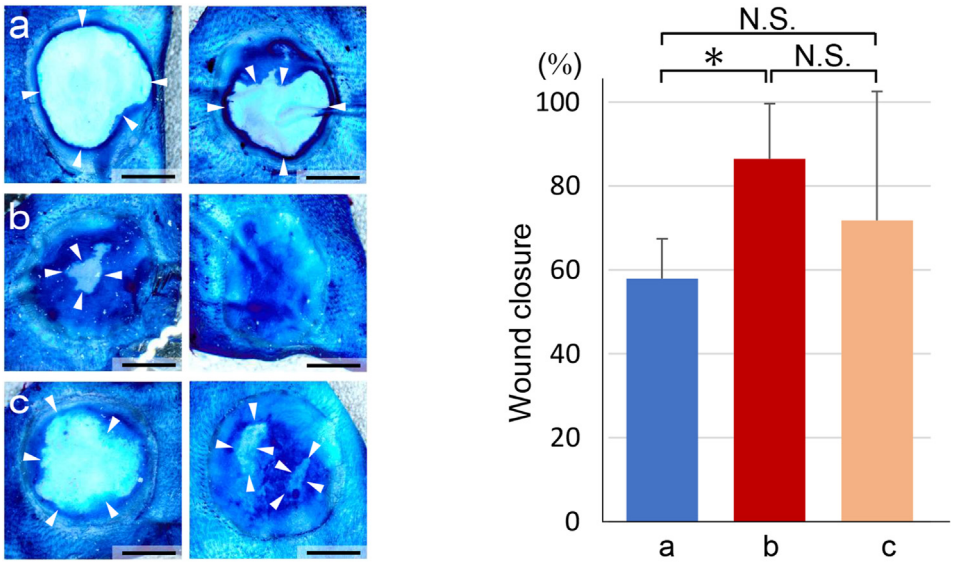


**Fig. 1. Effects of externally applied jellyfish collagen on physiological wound closure**. Wound pictures were taken at day 6 after transplantation of a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*) or 0.5% jellyfish collagen solution (*b*), or transplantation of a composite material graft containing the same amount (25 μg) of jellyfish collagen mixed with bovine type I collagen (*c*). Two representative pictures are shown from each group of mice. *Arrowheads* indicate the margin of regenerating epithelium recognized by pouring May-Grunwald/Giemsa staining solution. On the right side of the pictures are shown the histograms representing the wound closure rate in the three groups of mice. The histograms represent the means ± SD of 7–8 wounds in each group. The asterisks indicate that the differences are statistically significant (*$P < 0.05$). *N.S.*, not significant.

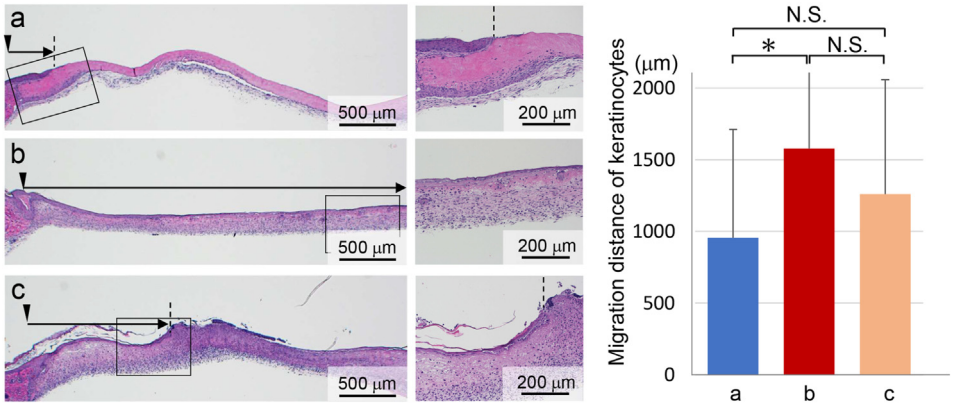


**Fig. 2. Effects of externally applied jellyfish collagen on re-epithelialization after wounding**.Dermal specimens were obtained from mice at day 6 after transplantation of a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*) or 0.5% jellyfish collagen solution (*b*), or transplantation of a composite material graft containing the same amount of jellyfish collagen mixed with bovine type I collagen (*c*). They were subjected to hematoxylin-eosin (H–E) staining. The wound margins, frontlines, and extension span of the regenerating epithelium are indicated by *arrowheads*, *dashed lines*, and *arrows*, respectively. The areas highlighted by a *square* are shown in higher magnification on the right of each picture. On the right side of the histological pictures are shown the histograms representing the migration distance of keratinocytes in the three groups of mice. The histograms represent the means ± SD of 7–8 wounds in each group. The asterisks indicate that the differences are statistically significant (*$P < 0.05$). *N.S.*, not significant.

required in the other two groups of mice (Fig. 4B). Histological examination of the excised specimens indicated that the transplanted jellyfish collagen-containing composite material graft was mostly replaced by endogenous tissue within 14 days and the morphological features of wound scar composed of cell-dense granulation tissue were evident (Fig. 4A, panel c). In marked contrast to these findings, the transplanted bovine collagen graft dripped with jellyfish collagen solution remained in the dermis at day 21 and showed dense collagen fibers (Fig. 4B, panel b), similar to that observed in the bovine collagen graft dripped with control saline (Fig. 4B, panel a).

### 3.4. Externally applied jellyfish collagen to artificial dermal graft accelerated wound closure in diabetic model mice

Based on the stimulatory effects of externally administered jellyfish collagen on physiological wound healing described above, we investigated whether jellyfish collagen solution was

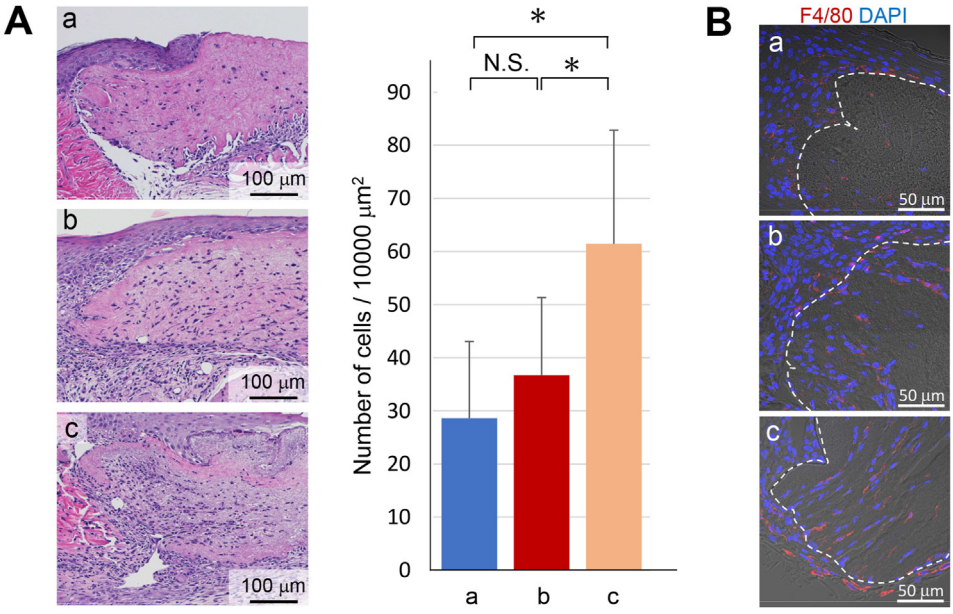


**Fig. 3. Suppression of cell infiltration and excessive inflammatory reaction by external administration of jellyfish collagen solution**. (**A**) Dermal specimens were obtained at day 6 after transplantation of a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*) or 0.5% jellyfish collagen solution (*b*), or transplantation of a composite material graft containing the same amount of jellyfish collagen mixed with bovine type I collagen (*c*). They were subjected to H-E staining. The number of cells infiltrating into the marginal area of the transplanted graft (0–200 μm from the wound edge) was counted and compared among the three groups. On the right side of the histological pictures are shown the histograms representing the means ± SD of 7–8 wounds in each group. The asterisks indicate that the differences are statistically significant (*$P < 0.05$). *N.S.*, not significant. (**B**) Representative pictures of immunofluorescent staining of F4/80 (*red*) are shown from 4 wounds in each group, together with nuclear staining using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, *blue*). *Dashed lines* indicate the border of transplanted dermal grafts, which is located on the right side in each picture. Note that fewer F4/80-positive cells are observed in the graft margin in mice transplanted with bovine collagen graft followed by external administration of saline (*a*) or jellyfish collagen solution (*b*), compared to those with a composite material graft made of jellyfish and bovine type I collagens (*c*).

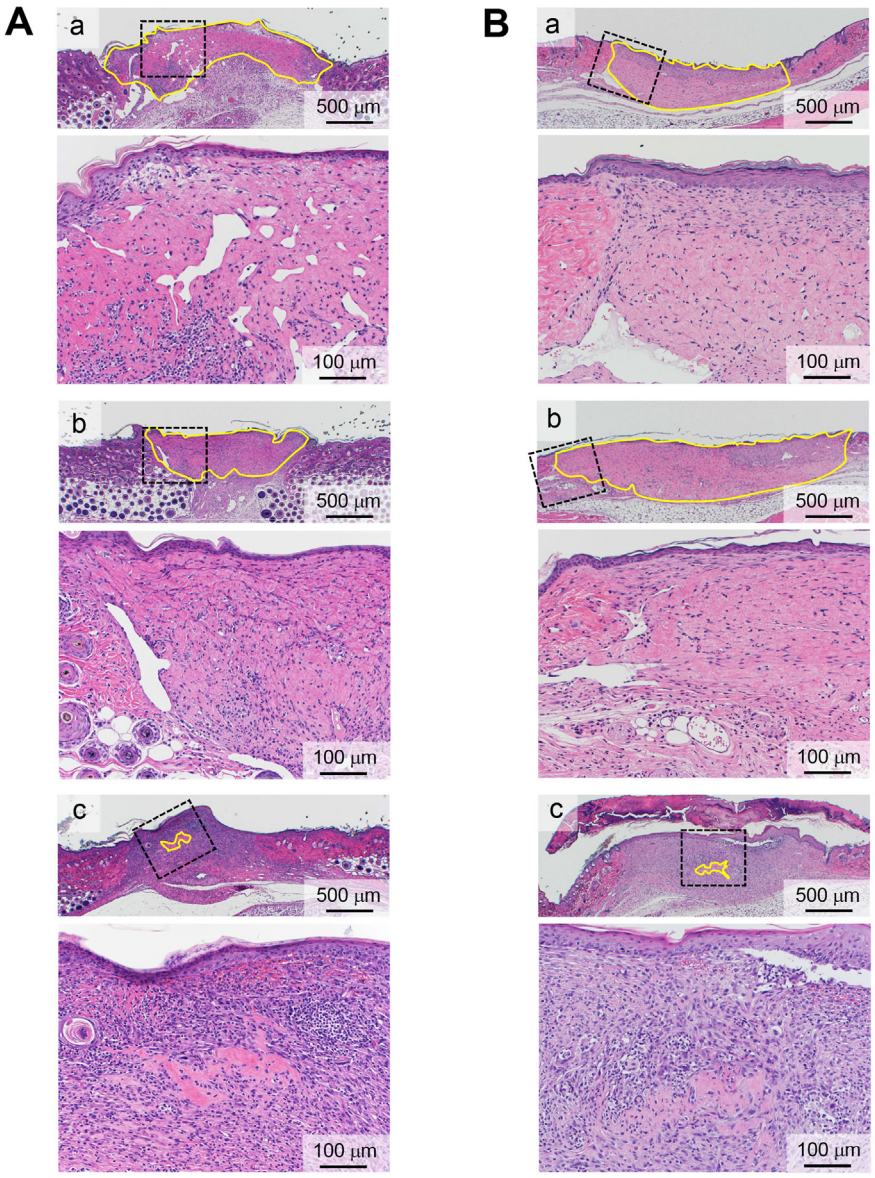


**Fig. 4. Suppression of degradation and absorption of transplanted dermal graft by external administration of jellyfish collagen solution**. Dermal specimens obtained at day 14 (**A**) or day 21 (**B**) after transplantation of a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*) or 0.5% jellyfish collagen solution (*b*), or transplantation of a composite material graft containing the same amount of jellyfish collagen mixed with bovine type I collagen (*c*). They were subjected to H-E staining. The *yellow lines* in each histological picture highlight the remaining transplanted grafts. The areas indicated by a *square* are shown at higher magnification below each picture. Representative images are shown from three to four wounds in each group at day 14 and 21.

also effective in treating impaired skin regeneration under pathological conditions. For this purpose, we used leptin receptor-deficient dB/dB mice, which develop type 2 diabetes mellitus characterized by obesity, hyperglycemia, hyperinsulinemia, and impaired wound healing [14,15]. dB/dB mice underwent full-thickness wounding and subsequent transplantation of artificial dermal grafts in the same way as the wild-type mice described above. Saline and a clinical-grade b-FGF solution were used as controls. As expected, dermal wound healing at day 6 after wounding was remarkably delayed in dB/dB mice (Fig. 5). The mean wound closure rate in dB/dB mice administered with saline (42.6 ± 14.8% in Fig. 5) was significantly lower than that in the wild-type mice administered with saline (57.9 ± 9.6% in Fig. 1) ($P < 0.05$). External administration of jellyfish collagen solution, but not b-FGF, significantly accelerated wound closure compared to saline at both days 6 and 10 (Fig. 5). This stimulatory effect of the jellyfish collagen solution was verified by histological examination showing significant elongation of the regenerating epithelium in dB/dB mice administered with jellyfish collagen externally onto a bovine collagen graft (Fig. 6, panel b) compared to those administered with either saline (Fig. 6, panel a) or b-FGF solution onto the same dermal graft (Fig. 6, panel c).

## 4. Discussion

In the present study, we demonstrated that external administration of moon jellyfish collagen solution onto a bovine collagen graft replicates the stimulatory effect on wound healing, which had been demonstrated previously in a form of composite dermal graft containing a mixture of jellyfish collagen and porcine type I collagen. External administration of jellyfish collagen significantly accelerated the migration of keratinocytes in the epidermis and

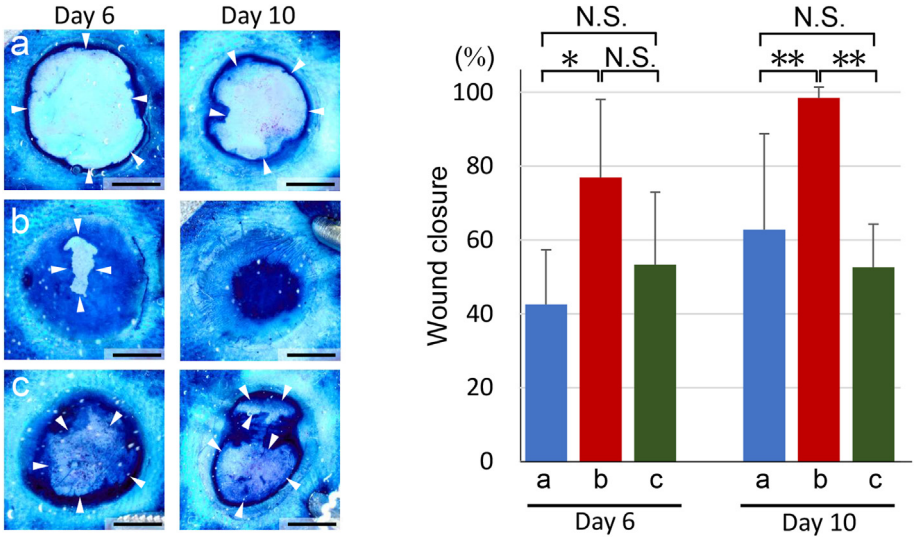


**Fig. 5. Effects of externally applied jellyfish collagen on wound closure in diabetic model mice**.Wound pictures were taken at day 6 and day 10 after making a full-thickness dermal wound on the dorsum of diabetic dB/dB mice. After wounding, they were transplanted with a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*), 0.5% jellyfish collagen solution (*b*), or clinical-grade basic fibroblast growth factor solution (*c*). *Arrowheads* indicate the margin of regenerating epithelium as described in Fig. 1. On the right side of the pictures are shown the histograms representing the wound closure rate in the three groups of mice. The histograms represent the means ± SD of 7–8 wounds in each group of mice. The asterisks indicate that the differences are statistically significant (*$P < 0.05$, **$P < 0.01$). *N.S.*, not significant.

resulted in early wound closure. It also suppressed excessive inflammatory cell infiltration into the granulation tissue and absorption of the transplanted bovine collagen graft, thereby preventing excessive scar formation. Notably, jellyfish collagen applied externally onto the bovine collagen graft was more effective than that given in a form of composite dermal graft in accelerating wound healing. There are several possible reasons that may account for this superior effect of externally applied jellyfish collagen.

First, when applied externally onto the transplanted bovine collagen graft in a small volume (5 μL), effective component(s) of jellyfish collagen remain in the graft surface, which can be delivered to the epithelial wound edge at a relatively high concentration. This is in contrast to the case of the composite dermal graft made of a mixture of jellyfish and bovine collagens, where jellyfish collagen is distributed evenly through the graft at a lower concentration. Second, and along the same line, limited amounts of jellyfish collagen components in the bovine collagen graft may prevent an immune reaction to the unidentified proteins that results in early absorption of the transplanted bovine collagen graft. Finally, while the fibrillar structure of bovine collagen remains intact within the graft in the externally applied jellyfish collagen group, rapid denature and degradation of jellyfish collagen contained in the composite material make the remaining fibral structure loose and denatured, leading to further degradation and absorption of the transplanted graft.

When 5 μL of 0.5% jellyfish collagen solution was dripped externally onto a bovine collagen graft with a 6-mm-diameter, the amount of loaded jellyfish collagen was 25 μg. Although it was only one-third of the amount (75.2 μg) contained in the previously reported composite graft made of 45% jellyfish collagen and 55% porcine type I collagen [10], external administration exerted a comparable effect on the wound healing process. On the other hand, the composite dermal graft used in the present study did not exhibit a significant effect on wound closure despite the presence of the same amount (25 μg) of jellyfish collagen as in the solution (Figs. 1 and 2). This may be because the jellyfish-to-bovine collagen ratio in the current composite graft was 13:87, and the results of our

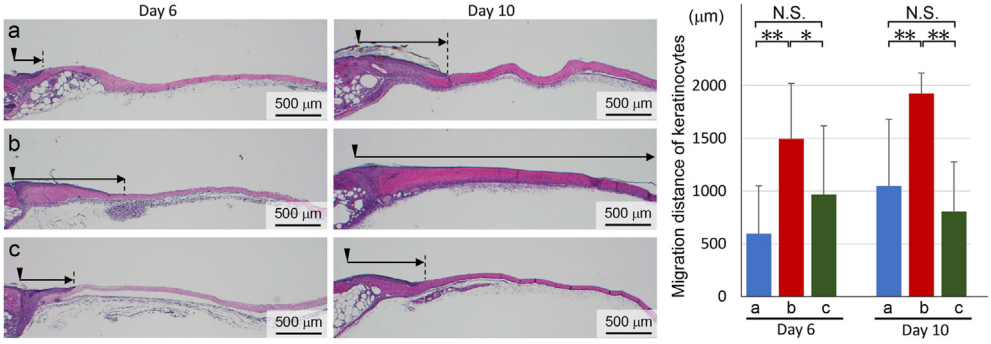


**Fig. 6. Effects of externally applied jellyfish collagen on re-epithelialization after wounding in diabetic model mice**.Dermal specimens were obtained from dB/dB mice at day 6 or day 10 after transplantation of a bovine type I collagen graft followed by external administration of 5 μL of saline (*a*), 0.5% jellyfish collagen solution (*b*), or clinical-grade basic fibroblast growth factor solution (*c*). They were subjected to H-E staining. The wound margins, frontlines and extension span of the regenerating epithelium are indicated by *arrowheads*, *dashed lines*, and *arrows*, respectively. On the right side of the histological pictures are shown the histograms representing the migration distance of keratinocytes in the three groups of mice. The histograms represent the means ± SD of 7–8 wounds in each group of mice. The asterisks indicate that the differences are statistically significant (*$P < 0.05$, **$P < 0.01$). *N.S.*, not significant.

previous study indicated that the stimulatory effect on re-epithelialization was observed by adding 40% or more jellyfish collagen to the composite material [10]. These results suggest that the stimulatory effect of jellyfish collagen on wound healing can be obtained at a lower dose by external administration.

External administration of jellyfish collagen also affected cell infiltration into the artificial dermal graft (Fig. 3A). Proportionally, the number of F4/80-positive activated monocytes and macrophages was lower in the external administration group than in the composite dermal graft group (Fig. 3B). Macrophages, together with other types of inflammatory cells, play important roles in modulating dermal wound healing, and pathological alterations in their functions have been implicated in impaired wound healing [16,17]. For example, insufficient classical macrophage activation in the early wound healing phase and excessive alternative macrophage activation in the later phase were reported in diabetic rats exhibiting impaired wound healing [16]. In addition, it has previously been reported that excessive and prolonged inflammation delays wound healing [18]. Indeed, our previous study indicated that administration of an antagonist against transforming growth factor-β/Smad3 signaling accelerated wound healing, at least in part, by suppressing the infiltration of F4/80-positive cells into the granulation tissue [19]. These findings indicate that proper wound healing requires activation of distinct subtypes of macrophages in a timely fashion. In the present study, external administration of the jellyfish collagen solution onto the bovine collagen graft improved impaired dermal regeneration in a mouse model of type 2 diabetes (Figs. 5 and 6). Further studies clarifying the macrophage subtypes affected by jellyfish collagen are necessary to elucidate the mechanisms by which jellyfish collagen accelerates the wound healing process.

It should be noted that suppression of excessive inflammation also influenced long-term dermal tissue remodeling. The bovine collagen graft remained 3 weeks after transplantation in the external administration group, whereas the composite material graft containing the same amount of bovine type I collagen was biodegraded earlier, possibly by matrix metalloproteinases secreted from infiltrating inflammatory cells including F4/80-positive macrophages (Fig. 4). This suppressive effect of externally administrated jellyfish collagen on the early absorption of the transplanted bovine dermal graft prevented subsequent scar formation.

The results of the present study suggest that the effect of jellyfish collagen on dermal wound healing is attributable to its unknown soluble component(s), rather than an intact collagen triple-helix structure, as jellyfish collagen is effective even after rapid denaturation and degradation at body temperature. Work is in progress in our laboratory to characterize the effective fractions of jellyfish collagen and identify the molecule(s) responsible for the wound healing effect of the jellyfish collagen. Identification of effective small molecule component(s) will be certainly useful to minimize the risk of unexpected immune reactions against the invertebrate collagen. Through these experiments, we aim to eventually contribute to the development of an effective novel skin regeneration therapy for intractable ulcers.

## 5. Conclusion

We have shown in the present study that moon jellyfish collagen can serve as an external medicine that accelerates the wound repair process when applied onto a transplanted conventional dermal graft. The stimulatory effect of jellyfish collagen on wound healing was obtained at a lower dose by external administration compared to transplantation of a composite dermal graft mixed with porcine type I collagen that had been reported previously. Externally applied jellyfish collagen suppressed excessive inflammatory reaction and early absorption of the transplanted bovine collagen graft, thus reducing subsequent scar formation. Unlike b-FGF that primarily enhances granulation tissue formation, the stimulating effect of jellyfish collagen was exerted by its direct biological action on keratinocyte migration, which is not achieved by conventional artificial dermal grafts. Furthermore, external administration of jellyfish collagen was effective in improving the delayed wound closure in diabetic model mice, suggesting its further application to treat intractable skin ulcers.

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## Declaration of competing interest

We disclose a possible conflict of interest in that a part of this study was conducted in collaboration with the Jellyfish Research Inc., Yokohama, Japan, who has taken out a patent (EP2889305B1) for extraction of jellyfish collagen.

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