Original

The effect of the polyglycolic acid/collagen composite nanofiber scaffold with fibroblast growth factor in wound healing

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Regenerative medicine is an attractive option for skin wound healing. Products derived from collagen matrix, a major scaffolding material, are widely used in clinical applications. The fibroblast growth factor (FGF) is used widely in wound healing alone as well as in combination with collagen matrix products. For improved healing efficacy, we previously developed a novel nanofiber composed of polyglycolic acid (PGA) and collagen. This study compared the wound healing ability of three commercially available scaffolds (Terudermis®, Pelnac®, and Integra®) and the PGA/collagen scaffold when used alone and in combination with FGF. The FGF solution was prepared by dissolving Fibrast® powder in normal saline. The scaffold materials were impregnated with 100 μL of FGF solution (100 μg/ml) to prepare FGF-impregnated scaffolds. The scaffolds with or without FGF were evaluated in 9-week-old male db/db mice with bilateral full-thickness wounds (diameter, 6 mm) on the dorsal skin. The FGF-impregnated scaffolds were applied to the defects on the left side, and the saline-impregnated scaffolds were applied to the defects on the right side. The wounds including the surrounding margin of normal skin and the underlying muscle layer were removed from the euthanized animals on postoperative day 5 (n = 6 per group). The wound healing ability of the scaffolds was assessed by vessel density as an indicator of angiogenesis. The angiogenic effect of the FGF-impregnated scaffold was compared to that of the saline-impregnated scaffold for each scaffold type. In addition, the angiogenic effect of the FGF-impregnated versions of the four scaffold types were compared. The vessel density in wounds with the FGF-impregnated PGA/collagen scaffold was significantly higher than that in wounds with saline-impregnated PGA/collagen. Furthermore, the FGF-impregnated PGA/scaffold attained a higher vessel density than the FGF-impregnated Terudermis® and the FGF-impregnated Pelnac® scaffolds. The PGA/collagen scaffold exhibited good affinity for FGF. Additionally, the addition of FGF to the PGA/collagen scaffold significantly increased the vessel density, which was not observed with the other three commercially available scaffolds impregnated with FGF. These results illustrate the promising efficacy of the composite PGA/collagen nanofiber scaffold in advanced wound management as well as regenerative medicine.

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Introduction

Advances in skin regenerative medicine, which afford significant advancement in wound healing, provide attractive options for plastic surgery and wound management. Specifically, tissue-engineered products utilized in regenerative medicine for skin are composed of three prime constituents: cells, growth factors, and biomaterial that is also referred to as the scaffold. Currently, collagen matrix is the major scaffolding material that is officially approved for use in wound treatment. Following application of the collagen matrix to a tissue defect, sprouting capillaries and fibroblasts migrate into the collagen matrix, which acts as a scaffold and promotes angiogenesis and fibroplasia. Terudermis® (Alcare, Japan), Pelnac® (Smith & Nephew, Japan), and Integra® (Century Medical, Japan) are three commercially available scaffolding materials

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that are approved and widely used for clinical applications in Japan.

The ability of the scaffolding material to promote cell proliferation and differentiation is the key for effective wound treatment. To improve the scaffold, we developed a novel nanofiber composed of polyglycolic acid (PGA) and collagen, which, independently, has beneficial properties. Furthermore, the efficacy of the PGA/collagen scaffold in cell migration and neovascularization in an *in vivo* skin defect model. was reported[1].

Conversely, a fibroblast growth factor (FGF) product, Fibrast® (Kaken Pharmaceutical, Japan), is widely employed to promote wound healing. In Japan, FGF products are often used in combination with collagen matrix scaffolds to treat chronic wounds. Therefore, one of the characteristics of an ideal scaffold is compatibility with FGF. Therefore, we determined the wound healing ability of commercially available scaffolds and the PGA/collagen scaffold when used in combination with FGF.

Materials and Methods

Scaffold preparation

The PGA/collagen (PGA) scaffold was prepared as described previously[2]. Briefly, the PGA and collagen solutions were mixed in a 6:4 volume ratio. Following, the PGA/collagen solution was put into a syringe with 22-gause stainless needle. A voltage generator (HSP-30K-2, Nippon- Stabilizer Industry Co. Ltd., Osaka, Japan) was connected to the needle, and a voltage of 18kV was applied to the needle. Electrospinning was conducted for 2 hours. The provided fibers were a diameter of 500 nm and a PGA/collagen weight mixing ratio of 40%[3]. The created nanofiber sponges were sterilized with ethylene oxide gas for in vivo experiments[4]. The other materials (Terudermis®, Pelnac®, and Integra®) were commercially available. All materials were cut into circular shapes that were 6 mm in diameter using disposable biopsy punches (Kai Corporation, Japan). The FGF solution was prepared by dissolving Fibrast® powder (Kaken Pharmaceutical, Japan) in normal saline to obtain an FGF concentration of 100 μg/ml, the recommended optimal dose for clinical use. Next, the cut scaffold materials were impregnated with 100 μL FGF solution to prepare the scaffolds containing FGF.

Mouse model of wound healing

The db/db mice generally take approximately twice as long to heal wounds as non-diabetic mice. So, 9-week-old male db/db mice kept at constant temperature in the institute for laboratory animals and were used in this study. Under anesthesia with isoflurane (1-Chloro-2, 2, 2-trifluoroethyl difluoromethyl ether), the dorsal skin of nine-week-old male db/db mice (Koken, Japan) was depilated. Bilateral full-thickness wounds, 6 mm in diameter, were created on the dorsal skin by a disposable biopsy punch. Silicone stents with an 8-mm inner diameter were glued around the wounds, and the scaffolds were placed on the wounds immediately after impregnation. Specifically, the bFGF-impregnated scaffolds were used for the defects on the left side, and the saline-impregnated scaffolds were used for the defects on the right side (Figure 1a). Next, the wounds were covered with film dressing (OpSite Flexifix; Smith & Nephew) to maintain a moist environment. All steps were performed in a clean environment and according to the guidelines of the Animal Care and Use Committee of Saitama Medical University.

Histological preparation

Animals were sacrificed on postoperative day 5. The wound and the normal skin margin surrounding the wound including the underlying muscle layer were removed from euthanized animals (n = 6 per group; Figure 1b). Each tissue specimen was cut with a surgical blade sagittally along the midline of

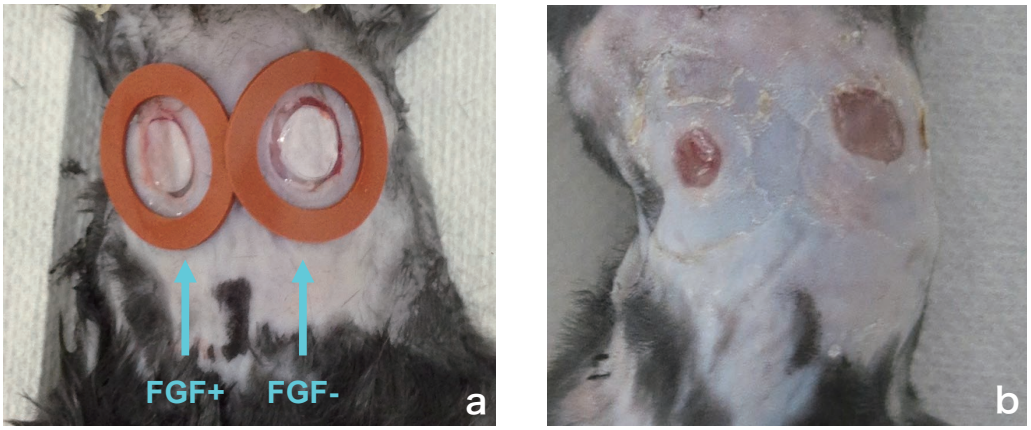

Fig. 1. The wound model mouse

(a) Two symmetrical full-thickness skin wounds (diameter, 6 mm) are created on each side of the dorsal skin. A stent is glued to surround the wound, and the scaffold is placed in the wound. (b) The mouse is sacrificed on postoperative day 5, and the stent is removed.

the wound into two parts. One part was fixed in 10% buffered formalin solution and processed for ordinary hematoxylin and eosin staining (HE). The acetone, methyl benzoate, xylene (AMeX) method visualize blood vessels. Briefly, the specimen was fixed in acetone at −20℃ overnight, was cleared consecutively in methyl benzoate and xylene, and was embedded in ordinary paraffin. Deparaffinized thin sections were stained with antimouse CD31 (BD Pharmingen, San Diego, CA, USA) (CD31) and were visualized by a horseradish peroxidase/diaminobenzidine (DAB) immunostaining system (Dako, Norden A/S, Glostrup, Denmark)[1].

The stained tissue sections were examined at ×200 and ×400 magnification with a stereo intravital microscope (SZH 10, Olympus, Japan). Three fields were randomly selected in each specimen to further assess wound healing.

Assessment of wound healing (Figure 2)

Vessel density was determined to assess the angiogenic ability of the scaffold with WinRoof 2015 image analysis soft-

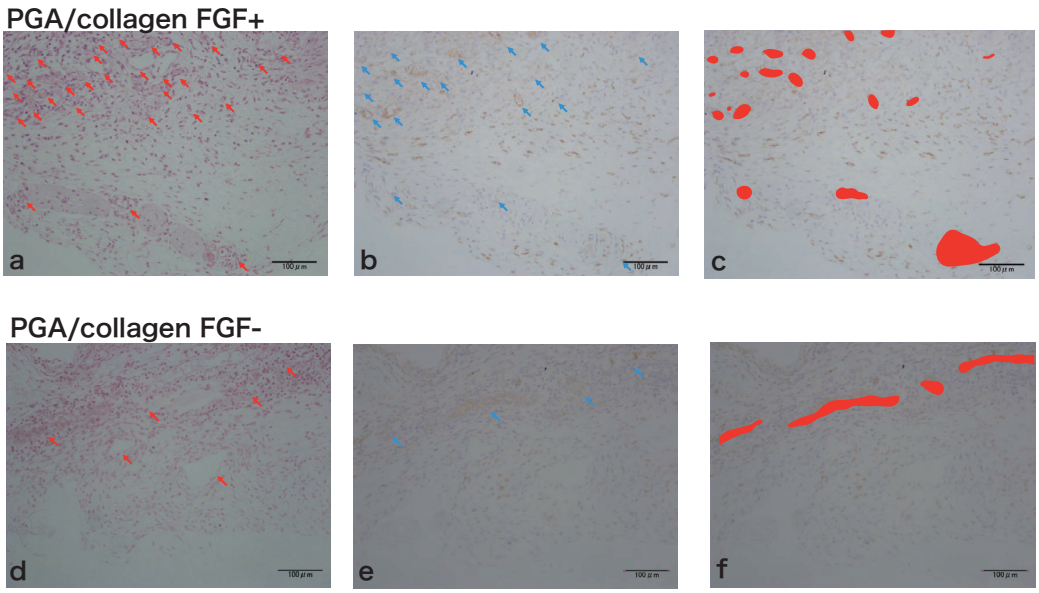

Fig. 2. Histological evaluation of granulation tissue: Hematoxylin/eosin (a, d) and antimouse CD31 (b, c, e, f)
The cavities were confirmed in hematoxylin/eosin (a, d: red arrow) and antimouse CD31 (b, d: blue arrow). The vessels cavities were painted (c, f: red color).

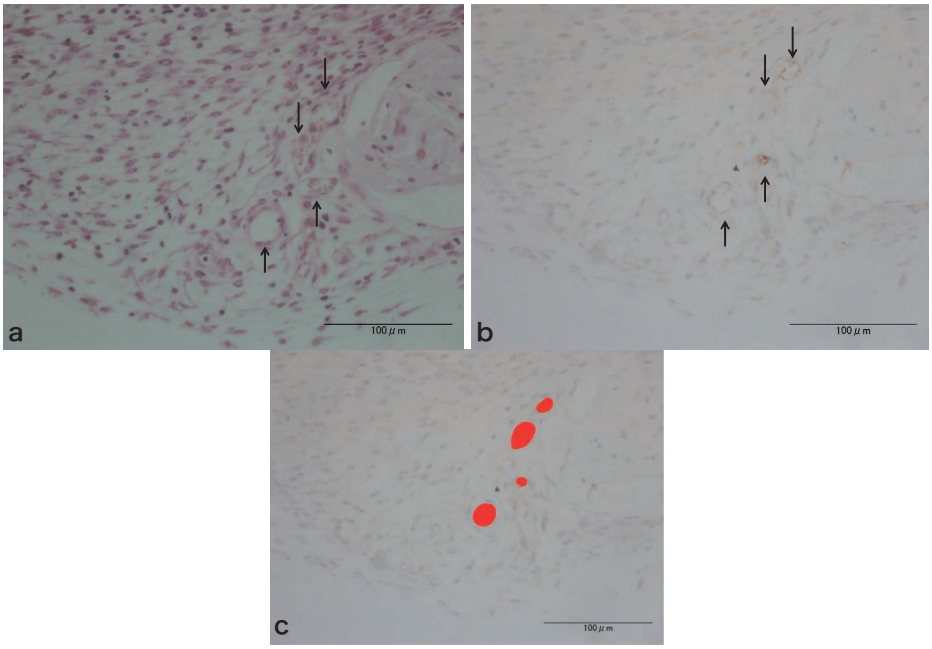

Fig. 3. The confirmation of vessels cavities
The vessel cavities confirmed at ×200 (Figure 2) were re-confirmed at ×400 (a, b, c). The cavities were checked in hematoxylin/eosin and antimouse CD31 (a, b: black arrow). The vessels cavities were painted finally (c: red color).

ware (Mitani Corporation, Japan) (Figure 2c, d). The HE was used as an adjunct identification by CD31 immunostain at ×200 (Figure 2a, b, d, e). Furthermore the vessel identified at ×200 were confirmed by the HE and CD31 at ×400 (Figure 3). CD31-postive vessels in each field were painted over to demarcate the vessels. The vessel density of each field was defined as follows: Vessel density = vessel area/area of selected fields ($\mu m^2/\mu m^2$). The average of vessel densities of the three selected fields were used to express the angiogenic ability of each specimen. To measure the painted area in each field's was vessel area, and the total area of the given field was also measured digitally.

Statistical analysis

Data were expressed as means ± standard deviation. To assess the effect of FGF when used in combination with scaffolds, differences in vessel density of the wounds with FGF-impregnated and saline-impregnated scaffolds were analyzed. To assess the angiogenic ability of scaffolds impregnated with FGF, differences in vessel density among the FGF-impregnated scaffolds were analyzed. Student's paired *t* test was used to assess statistical significance, and *P* values <0.05 were considered to indicate significance. All statistical analyses were performed using R software (Saitama Medical Center, Jichi Medical University, Japan).

Results

The vessel density was higher in wounds with the FGF-impregnated PGA/collagen scaffolds than the saline-impregnated PGA/collagen scaffolds (Figure 2). However, there were no significant differences between the FGF-impregnated and the saline-impregnated versions of the other three scaffolds tested in the current study (Figure 4). The vessel density was higher in the wounds with the FGF-impregnated PGA/collagen scaffolds compared to the wounds with the FGF-impregnated Terudermis®, and the FGF-impregnated Pelnac® scaffolds (Figure 5). The difference was significant between the FGF-impregnated PGA/collagen and the FGF-impregnated Terudermis® as well as between the FGF-impregnated PGA/collagen and the FGF-impregnated Pelnac®.

Discussion

Tissue engineering techniques are promising therapeutic options in plastic surgery and wound management. The ability of scaffold materials to induce cell proliferation and differentiation is the key in tissue engineering. Since approved scaffolds in Japan are currently made from collagen, this study attempted to offer the new material in addition to collagen scaffolds.

Therefore we developed a novel nanofiber composed of PGA and collagen, both of which exert independently beneficial effects[1]. The electrospinning technique produced PGA/collagen nanofibers with a diameter of 500 nm and a weight mixing ratio of 40%[4].

A previous study assessing the *in vivo* efficiency of PGA/collagen nanofibers on granulation and its ability to induce neovascularization by histology revealed that the PGA/collagen nanofibers were superior to the elementary collagen matrix in induction of cellular migration and neovascularia-

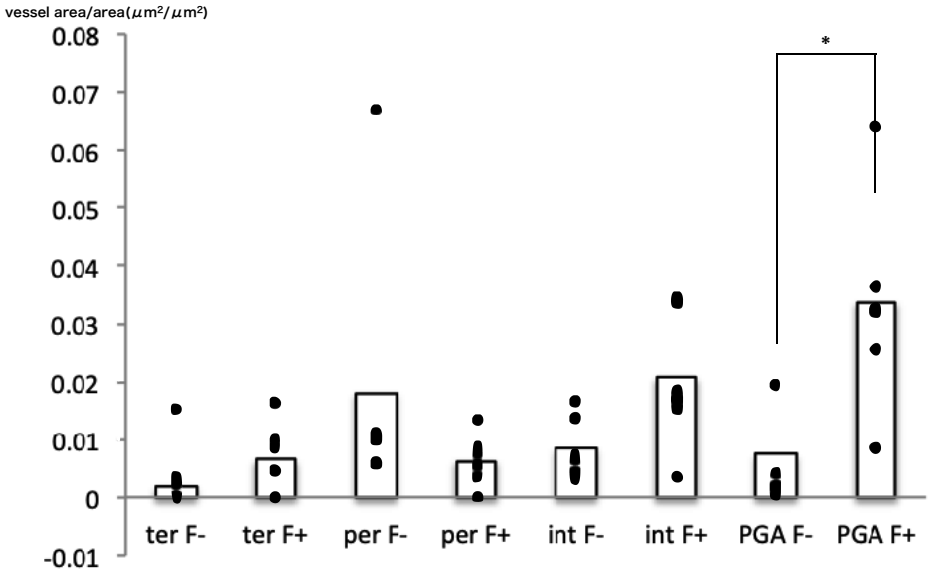

Fig. 4. Mean vessel densities in different scaffold groups, comparing group 1 and group 2
The difference in FGF-and saline-impregnated scaffolds was significant only in the PGA/collagen group.
Abbreviations: ter, Terudermis®; pel, Pelnac®; int, lntegra®; F, fibroblast growth factor
◆: vessel densities values
* $P < 0.05$

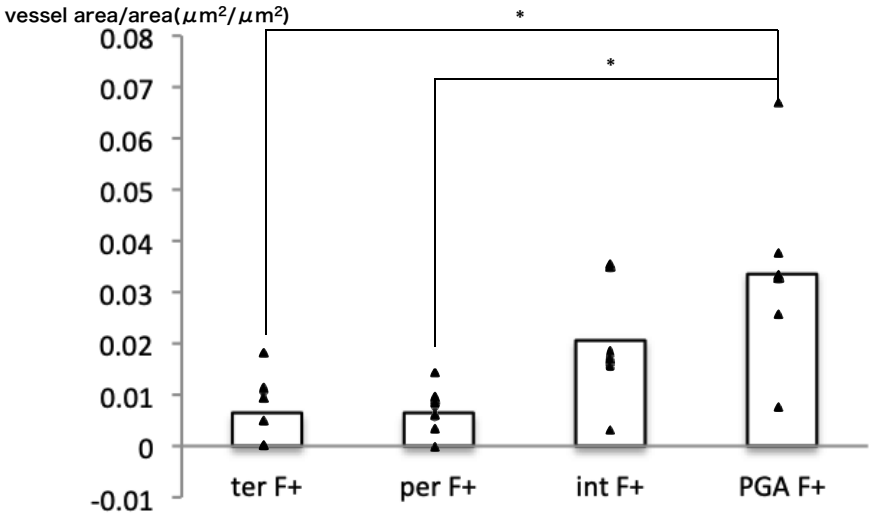

Fig. 5. Comparison of the FGF-impregnated scaffolds for their ability to induce vessel density
The vessel density is significantly higher with the FGF-impregnated PGA/collagen scaffold compared with the FGF-impregnated Terudermis® and Pelnac® groups.
Abbreviations: ter, Terudermis®; pel, Pelnac®; int, Intégra®; F, fibroblast growth factor
◆: vessel densities values
* $P < 0.05$

tion[4]. Furthermore, it attributed to the nanosize effect of the fine structure and the incorporation of PGA, which was associated with enhanced angiogenesis.

In the current study, in addition to the not-yet-approved PGA/collagen scaffold, we also evaluated three approved and commercially available products used as scaffolds in Japan, all of which are composed of a collagen matrix: Terudermis®, Pelnac®, and Integra®. All three scaffolds have been shown to be effective in skin and soft tissue defects[5-8] and to reduce treatment failure and recurrence rates[9].

Concurrently, the FGF product Fibrast® is widely used to promote wound healing. It has been shown to benefit healing of chronic wounds by reducing the ulcer area via the promotion of angiogenesis, re-epithelization, granulation, and scar formation[8,9]. In plastic surgery, FGF products are usually used in combination with collagen matrix scaffolds to treat chronic wounds[10], but the problem unvolves the short half-life of FGF at the wound surface.

Since FGF is rapidly deactivated when it contacts with the raw surface of the wound, the manufacture instruction for Fiblast recommends frequent application (once a day). On the other hand, most products of collagen matrix scaffolds have two layered structures. Collagen components are covered with the silicon membrane. In the operative procedures plastic surgeons generally suture the silicon membrane to the wound edge and apply a tie-over bolster for definite fixation to allow the migration of cells and blood vessels into the scaffolds for several days. During this fixation period we are not able to additionally administer FGF agent. Therefore, FGF impregnated scaffolds desirably demonstrate significant promotion of wound healing at several days after fixation without additional FGF administration. Thus, we estimated the efficacy of PGA/collagen impregnated in FGF agent without additional FGF, and the effect was compared with other commercially available scaffolds.

We found that the impregnation of FGF to the PGA/collagen scaffold led to a significant increase in vessel density in our *in vivo* wound model at 5 post-operative days, whereas the impregnation of FGF to the other three scaffolds did not lead to a significant change in vessel density. Furthermore, the vessel density was higher in the wounds with the FGF-impregnated PGA/collagen scaffold compared to the wounds with the FGF-impregnated Terudermis®, and the FGF-impregnated Pelnac® scaffolds; this difference was significant with FGF-impregnated versions of Terudermis® and Pelnac®. These findings suggest that combination of FGF with the PGA/collagen scaffold enhanced wound healing by inducing angiogenesis more strongly than the FGF-impregnated versions of the commercially available collagen scaffolds. Otherwise, PGA/collagen has no significant difference with Integra®. Integra® includes proteoglycan, and proteoglycan has a function that is similar to FGF.

So far, it has been pointed out that the immediate cellularization of the scaffold accompanied by angiogenesis inside is an important event for regenerative medicine with scaffolds. Therefore, the aim generally pursued is by combining FGF or vascular endothelial growth factor (VEGF) with the scaffold. In previous papers, FGF or VEGF has been combined with

scaffolds to enhance cell migration and blood vessel introduction from host tissues[11-13]. In the precedent study of our group, each of the PGA-collagen sponges, the PGA-collagen sponges with FGF, the PGA sponges, and the PGA non-woven mats were separately implanted in underneath of the fascia lata of rats, and those samples were explanted and observed histologically to evaluate the cell migration into them. The results showed that PGA-Collagen+FGF samples were reasonably most vascularized inside the sponges, but in the case of PGA-Collagen without FGF, half the amount of vascularization was still observed inside the sponge. In the case of PGA sponge without FGF, vascularization was not yet detected inside the sponge[14]. Our current study is compatible with the previous investigation.

For histological observation, we prepared HE staining as well as CD31 immunostaining specimens. In immunohistochemistry, CD31 provides a good marker for endothelial cells and is primarily used to demonstrate the presence of vascular structures in histological tissue sections. CD31 is fairly specific for endothelial differentiation, but reactivity may also be seen in megakaryocytes, platelets, some plasma cells, and macrophages; the latter can have a misleading result when micro-vessels are extracted. In such confusing microscopic images, we comparatively estimated both HE and adjacent CD31 sections.

Microscopic evaluation in our previous study revealed that the very fine nanostructure of the PGA/collagen fibers (diameter, 500 nm) provided a more abundant space for cellular growth compared with the coarser structure of Terudermis® (diameter, approximately 10 μm)[4]. Nanostructured materials have a large surface area relative to their volume. We hypothesize that this nanosize effect of the PGA/collagen fiber provides adhesion sites more abudant for FGF when compared with the commercially available collagen matrices, resulting in superior angiogenic ability[14]. Whether the function is due not only to the surface area but also sustained release will be a future subject for research.

Conclusion

In conclusion, the current study revealed that the PGA/collagen scaffold had a good affinity for FGF, which might be due to the nanosize effect of the fine nanofiber structure of this scaffold, an ideal feature for treatment of chronic wounds. The efficacy of this composite PGA/collagen nanofiber is promising for advanced wound management as well as regenerative medicine.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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ポリグリコール酸コラーゲンナノファイバー足場と塩基性線維芽細胞成長因子の創傷治療における効果の検討

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再生医療の分野は，創傷治癒でも有用な手段となりつつある．形成外科医は，臨床の場で人工真皮という足場を用いて，創傷治療を行なっている．既存の足場のみならず我々は，ポリグリコール酸を使用したナノファイバーの足場（PGA/collagen）を開発し，過去に動物実験でその効果を実証し報告している．

また線維芽細胞成長因子（FGF）は，創傷治癒を促進させるシグナルとして重要な位置を占めており，その製剤が広く本邦では使用されている．臨床の場では，各種足場との組み合わせで，創傷治療の幅を広げている．

今回，3 種の人工真皮および PGA/collagen を FGF と組み合わせて，創傷治癒における血管新生能能力を比較した．

9 週齢の雄の糖尿病マウスを用いた．マウスの背部に創傷を作製し，各種人工真皮および PGA/collagen に FGF を添付した群と添付しない群に分けた．5 日後に周囲組織を含めて創傷部を採取して，組織学的に血管密度を評価した．

PGA/collagen に FGF を添加すると添加しないより有意に血管密度が増加したが，他の 3 種の足場では FGF 添加に起因する有意な血管密度増加がみられなかった．また FGF を添加した全 4 種の足場のなかで PGA/collagen が，2 種の人工真皮（テルダーミス® とペルナック®）との間で統計的有意差があった．

PGA/collagen と FGF の組み合わせは，2 種の人工真皮より血管新生を促し，FGF との相性が良いことが示唆された．これは，既存の足場より PGA による効果およびナノファイバーによる影響が FGF および創傷治癒に有利に働くと考えられた．

