Evaluation of a novel collagen-gelatin scaffold for achieving the sustained release of basic fibroblast growth factor in a diabetic mouse model.

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CITATION:

Kanda, Norikazu ...[et al]. Evaluation of a novel collagen-gelatin scaffold for achieving the sustained release of basic fibroblast growth factor in a diabetic mouse model.. Journal of tissue engineering and regenerative medicine 2012, 8(1): 29-40

ISSUE DATE:

2012-05-24

URL:

http://hdl.handle.net/2433/196766

Title Page

Full title: **Evaluation of a novel collagen/gelatin scaffold for achieving the sustained release of basic fibroblast growth factor in a diabetic mouse model**

Short title: "Sustained release of bFGF from CGS accelerated tissue regeneration in diabetic mice."

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This work was supported by a grant from the Japan Science and Technology Agency.

ABSTRACT

The objective of this study was to evaluate the ability of a scaffold, collagen/gelatin sponge (CGS), to release basic fibroblast growth factor (bFGF) in a sustained manner using a pressure-induced decubitus ulcer model involving genetically diabetic mice. We confirmed that the CGS impregnated with a bFGF concentration of up to 50μg/cm^2 were able to sustained the release of bFGF throughout their biodegradation. We prepared decubitus ulcers on diabetic mice. After debriding the ulcers, we implanted CGS (diameter: 8mm) impregnated with normal saline solution (NSS) or bFGF solution (7, 14, 28, or 50μg/cm^2). At one and two weeks after implantation, the mice were sacrificed, and tissue specimens were obtained. The wound area, neoepithelium length, and numbers and total area of newly formed capillaries were evaluated. The CGS impregnated with NSS became infected and degraded, whereas the CGS impregnated with 7 or 14μg/cm^2 of bFGF displayed accelerated dermis-like tissue formation, and the CGS impregnated with 14μg/cm^2 of bFGF produced significant improvements in the remaining wound area, neoepithelium length, and numbers and total area of newly formed capillaries compared with the NSS group. No significant difference was observed between the NSS and 50μg/cm^2 bFGF groups. CGS impregnated with 7μg/cm^2 to 14μg/cm^2 bFGF accelerated wound healing, and an excess amount of bFGF did not increase the wound-healing efficacy of the CGS. Our CGS is a scaffold that can release positively charged growth factors such as bFGF in a sustained manner and shows promise as a scaffold for skin regeneration.

Keywords: bFGF, artificial skin, collagen/gelatin sponge, scaffold, sustained release, wound healing, diabetic mice

1. INTRODUCTION

We were involved in the development of a bilayered acellular artificial dermis (AD, Pelnac®, Gunze Co. Ltd, Kyoto, Japan) as a biodegradable scaffold containing an upper layer composed of a silicone sheet and a lower layer made of collagen sponge by modifying the artificial dermis proposed by Yannas and Burke (Yannas et al., 1980; Suzuki et al., 1990a). After the AD has been grafted onto a full-thickness skin defect, the collagen sponge is biodegraded and gradually replaced with regenerated dermis-like tissue within 2 to 3 weeks (Suzuki et al., 1990b). Basic FGF was released during the biodegradation of the CGS.

Artificial dermises have been used in clinical practice for the treatment of full-thickness skin defects caused by severe burns and tumor excision for more than 10 years. However, some problems remain to be solved. Before capillaries have infiltrated the collagen sponge, the artificial dermis is not resistant to infection (Matsuda et al.,1992). Therefore, it is difficult to apply artificial dermises to chronic ulcers such as decubitus, diabetic, and leg ulcers, because of the high probability of infection (Matsuda et al., 1988).

Basic fibroblast growth factor (bFGF), which was identified in 1974 (Gospodarowicz et al., 1974), promotes the proliferation of fibroblasts and capillary formation and accelerates tissue regeneration (Uchi et al., 2009). In Japan, human recombinant bFGF (FIBRAST SPRAY® Kaken Pharmaceutical, Tokyo, Japan) has been used clinically for the treatment of chronic skin ulcers since 2001, and its clinical effectiveness has been demonstrated (Kawai et al., 2000). Recently, combination therapy involving bFGF and artificial dermis has been reported to accelerate dermis-like tissue formation (Muneuchi et al., 2005;

Ito et al., 2005; Akita et al., 2008). In spite of its effectiveness, this combination therapy has not become a standard treatment because bFGF must be applied every day as it rapidly diffuses away from the site of administration and is also inactivated quickly after its administration (Kawai et al., 2000).

To overcome these problems, we have developed a novel scaffold, collagen/gelatin sponge (CGS), containing a 10wt% concentration of acidic gelatin that is capable of releasing positively charged growth factors such as bFGF for more than 10 days in vivo via the formation of ion complexes between bFGF and gelatin (Takemoto et al., 2008). Human bFGF, which has an isoelectric point (IEP) of 9.6 (Kanda et al., 2011; Artem et al., 2011; Takemoto et al., 2008; Kawai et al., 2005; Kawai et al.,2000; Tabata Y et al., 1999 ; Muniruzzaman et al., 1998), is ionically complexed with acidic gelatin, which has an IEP of 5.0 (Muniruzzaman et al., 1998). CGS acts in the same manner as a scaffold such as AD, and the bFGF impregnated into the CGS is released during its biodegradation (Takemoto et al., 2008). In our previous study involving normal mouse skin defects, CGS impregnated with 7μg/cm^2 bFGF accelerated dermis-like tissue formation 2 or 3 fold compared with AD (Kanda et al., 2011). In another study in which we created full-thickness palatal mucosa defects in beagles, CGS impregnated with 7μg/cm^2 bFGF accelerated the regeneration of the palatal mucosa, induced good levels of neovascularization, and produced less wound contracture (Artem et al., 2011). We expect that CGS impregnated with bFGF will prove to be an effective treatment for full thickness skin defects including chronic ulcers such as diabetic foot ulcers and decubitus ulcers. In this study, we examined the optimal bFGF dosage with which to impregnate CGS and the release profile of bFGF from CGS after

impregnation. Then, we investigated the effectiveness of CGS impregnated with bFGF and the optimal bFGF dosage in an impaired wound healing model involving genetically diabetic mice with pressure-induced decubitus ulcers.

2. MATERIALS AND METHODS

2.1. Animals and operations

The animals 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.

2.2. Preparation of CGS

We used gelatin isolated from pig dermis with an isoelectric point (IEP) of 5.0 and a molecular weight of 99,000 (Nippi, Inc., Tokyo, Japan) and atelocollagen isolated from pig tendons with an IEP of 8.5 and a molecular weight of 300,000 (Nitta Gelatin, Inc., Osaka, Japan). CGS was produced according to production procedure has described in Takemoto's paper (Takemoto et al., 2008). CGS with a gelatin concentration of 10wt% of the total solute was prepared by mixing 3wt% gelatin solution with 0.3wt% collagen solution. We then spread a thin layer of silicone paste onto a polyester mesh. Before the silicone paste had dried, the top of the CGS was attached to the silicone paste covered polyester mesh. As the silicone paste dried, it formed a sheet that adhered to the CGS.

2.3. In vitro bFGF release study and degradation rate of CGS

We prepared CGS of 10mm×20mm in size and 3.0mm in thickness. We weighed all of the CGS (n=10) and then placed them into 15ml test tubes (Thermo Fisher Scientific Inc. Osaka, Japan). We prepared distilled water (DW, Otsuka Pharmaceutical, Tokyo, Japan) and distilled water solution containing bFGF (FIBRAST SPRAY® Kaken Pharmaceutical, Tokyo, Japan) at concentrations of 0.07, 0.14, 0.28, and 0.5μg/μl and applied 200 μl of each bFGF solution to CGS. Thus, we prepared four bFGF groups, in which CGS was impregnated with 7, 14, 28, or 50μg/cm^2 bFGF and incubated overnight at 4℃.

We prepared Tris-HCl buffer solution (pH7.4) containing 4 units/ml collagenase (Collagenase Type A, Sigma-Aldrich Corporation Japan, Tokyo, Japan) and poured 5ml of collagenase solution into the test tubes to dissolve the CGS at 37°C. At 1, 2, 4, and 6 hours after the degradation, we collected 1 ml of the solution from the test tubes and used it to estimate the bFGF concentrations in the four bFGF groups. After collecting the solutions, collagenase was completely removed from the test tubes, and the CGS inside the test tubes were immediately washed with distilled water to stop the enzyme reaction. The CGS were removed from the test tubes and freeze-dried for 12 hours to remove any water using a freeze dryer (BRZ350WA, ADVANTEC Toyo Kaisha, Ltd., Tokyo, Japan). The CGS were then weighed in order to calculate the degradation rate of the CGS impregnated with bFGF at each time point.

The analysis was performed using ELISA (Enzyme-Linked Immunosorbent Assay) kits (Human FGF basic immunoassay kit: R&D Systems, Inc, Minneapolis, USA). To estimate

the concentrations of the bFGF solutions, solutions and standards were assayed in duplicate according to the manufacturer's instructions. The test wavelength of each well was set at 490 nm using a microplate reader (MTP-450 CORONA ELECTRIC Co., Ltd., Ibaragi, Japan) and compared to a reference wavelength of 650 nm. The bFGF concentrations were determined by plotting their values on a standard curve. The amount of bFGF released from the CGS was calculated at each time point.

2.4. Pressure-induced ulcer model in diabetic mice

We prepared 40 genetically diabetic mice (Nine-week-old BKS.Cg-+ Leprdb/+ Leprdb/Jcl, CLEA Japan Inc, Osaka, Japan). All mice had their backs and abdomens shaved and depilated under anesthesia with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan) and then were positioned on experimental tables. In our previous study, we developed a pressure induced ulcer model using diabetic mice and a pneumatic compressor （Kawai et al., 2005). In this study, 4 hours prolonged pressure (2h×2 pressure sessions; 2h interval between pressure sessions; 500 g/cm^2) was loaded onto the area above the femoral trochanters of the mice using a pneumatically driven compressor for two consecutive days (EARTH MAN AC-20 OL, TAKAGI Co., Ltd. Japan, Niigata, Japan). The air pressure was regulated with a precision regulator providing a constant pressure level. Five days after the completion of the pressure loading, the area of necrosis was clearly demarcated (Fig. 3A).

2.5. Impregnation of bFGF into CGS and the implantation of the CGS

We used CGS of 8mm in diameter and 3mm in thickness. As for the dosage of bFGF, the recommended therapeutic dose of bFGF for chronic ulcers is 1μg/cm^2 per day (Uchi et al., 2009). In a diabetic mouse study, the daily application of bFGF produced a similar bell-shaped dose-response pattern with a peak at 1μg/cm^2 per day (Okumura et al., 1996). This shows that the effective dosage of bFGF is not very different between humans and diabetic mice. According to the daily bFGF dosage recommendations and a CGS release period of about 10 days, we hypothesized that the optimal bFGF dosage for impregnating the CGS ranged from 7μg/cm^2 to 14μg/cm^2 for 7 or 14 days. In this experiment, we prepared CGS containing NSS or one of four different doses of bFGF (7, 14, 28, or 50μg/cm^2) as in the in vitro study and then incubated them overnight at 4°C.

The mice were anesthetized via the intraperitoneal injection of 25mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL, USA) and the inhalation of diethyl ether (Wako Pure Chemical Industries, LTD., Osaka, Japan). Five days after the completion of the pressure loading, the necrotic tissues were resected, and skin defects of 8mm in diameter were created using a 8mm-diameter skin punch biopsy tool (Kai industries, Gifu, Japan) and scissors (Fig. 3B). CGS impregnated with NSS or bFGF solution were implanted into the defects and sutured into the marginal skin wounds with 5-0 nylon sutures (Johnson & Johnson K.K., Tokyo, Japan) (Fig. 3C). All wounds were covered with gauze and fixed in place with adhesive tape (ALCARE®, ALCARE Co., LTD. Tokyo, Japan).

2.6. Assessment of the wound area and histological assessment of neoepithelization

One and two weeks after implantation, the mice were sacrificed via the inhalation of

carbon dioxide. After the removal of the silicone sheets, the wounds were photographed, and the wound area was measured using the imaging analyzer ImageJ software (version 1.38, National Institutes of Health, USA). The wound area is expressed as a percentage of the original wound area.

The implanted CGS and dermis-like tissue were harvested using scalpels and scissors and sectioned axially. Specimens were then fixed with 20% formalin fluid, paraffin-embedded, and sliced into 4μm thick sections, before the sections were stained with hematoxylin and eosin. Using a light microscope and NIS Elements (Nikon Instruments Company, Tokyo, Japan), the neoepithelium length of each specimen was measured from the innermost hair root of the marginal skin to the end of the neoepithelium on each side of each cross-section at a magnification of x 100.

2.7. Immunohistological staining and evaluation of the area and number of newly formed capillaries

Using 4μm thick paraffin-embedded sections, immunohistological staining with von Willebrand factor was performed to detect newly formed capillaries in the CGS. After the sections had been dewaxed and rehydrated, they were incubated in PBS with 0.1% trypsin (Vector Laboratories Inc., Burlingame, CA) for 15 minutes at 37°C for antigen retrieval. Anti-Von Willebrand factor rabbit polyclonal antibody (DAKO Japan, Tokyo, Japan) was used as the primary antibody (1: 500 dilutions), and EnVision+Rabbit/HRP (DAKO Japan, Tokyo, Japan) was used as the secondary antibody. These sections were exposed to DAB (3-3`-diaminobenzidine-4HCl) (DAKO Japan, Tokyo, Japan) for 2 minutes at room

temperature. Counterstaining was performed with hematoxylin.

Digital light micrographs of the sections of the CGS and dermis-like tissue beyond the muscle layers were taken at a magnification of x 100. In each section, two square areas of 500μm in width and height were chosen from the dermis-like tissues beneath the marginal skin. The area and number of newly formed capillaries in two squares in each section were measured twice. Using a microscope, we measured the epithelium length directly using NIS Elements. An assessment of neoepithelization and newly formed capillaries was also performed using NIS Elements.

2.8. Statistical analysis

All data were analyzed using Fisher's protected least significant difference test (Fisher's PLSD) and expressed as the mean+standard error. A value of $p<0.05$ was accepted as significant.

3. RESULTS

3.1. The degradation of CGS and basic FGF release in vitro

The degradation rates of the CGS are shown as percentages compared to their original weight or their collagenase treated weight (Fig.1). The CGS were digested by collagenase throughout the degradation period. There were significant differences in the degradation rate at 1, 2, and 4 hours after the start of the degradation between the CGS impregnated with 50μg/cm^2 of bFGF and those containing 7 or 14μg/cm^2 of bFGF, but no significant

differences were seen at 6 hours after the start of the degradation. The time course of bFGF release from the CGS is shown in Fig.2. No initial burst of bFGF release from CGS was observed; rather, the bFGF was continuously released throughout the degradation of the CGS (Fig.2).

3.2. Wound area

The gross appearance of the wounds at one and two weeks after implantation is shown in Fig. 4 and Fig.5. One week after implantation, the CGS in the NSS group were infected, whereas dermis-like tissue had begun to form in the wounds treated with the bFGF impregnated CGS (Fig. 4). Two weeks after the implantation, the CGS in the NSS group displayed implantation failure. In contrast, the wound areas covered with CGS containing 7 or 14μg/cm^2 of bFGF had markedly reduced and were infection-free, and the wounds treated with 14μg/cm^2 of bFGF were almost completely epithelized (Fig. 5). In the wounds treated with CGS containing 28 or 50μg/cm^2 bFGF, dermis-like tissue had formed but epithelization had not proceeded as quickly as that seen in the wounds treated with CGS containing 7 or 14μg/cm^2 bFGF.

The time course of the remaining wound area is shown in Fig. 6. One week after implantation, the wound area in the 14μg/cm^2 bFGF group was significantly smaller than that in the control group. Two weeks after implantation, the wound areas in the 7 and 14μg/cm^2 bFGF groups were significantly smaller than those in the control group and 50μg/cm^2 bFGF group.

3.3. Histological assessment of neoepithelium length

Light microphotographs of the histological sections are shown in Fig. 7 and Fig.8. The low magnification image (x 40) shows the neoepithelium, CGS, and marginal skin. The high magnification image (x 100) shows neoepithelium formation. The neoepithelium formation was especially marked in the 7 and 14μg/cm^2 bFGF groups.

The time course of neoepithelium length is shown in Fig. 9. One week after implantation, the neoepithelial lengths in the 7 and 14μg/cm^2 bFGF groups were significantly longer than those in the control group. Two weeks after implantation, the neoepithelium length in the 14μg/cm^2 bFGF group was significantly longer than those in the control and 50μg/cm^2 bFGF groups.

3.4. Evaluation of newly formed capillaries in the wounds

Light microphotographs of newly formed capillaries stained with von Willebrand Factor are shown in Fig.10. The number of capillaries in the bFGF impregnated group was significantly larger than that in the NSS group, although no significant difference was observed among the bFGF impregnated groups（Fig. 11）. The capillary areas in the bFGF impregnated groups treated with 7, 14, or 28μg/cm^2 bFGF were significantly larger than those in the control (almost 20 times larger) and 50μg/cm^{2}bFGF groups (almost 8 times larger). No significant difference was observed between the control and 50μg/cm^2 bFGF groups (Fig. 12).

4. DISCUSSION

Chronic skin ulcers can result from diabetic neuropathy, pressure sores, venous insufficiency, peripheral vascular disease, infectious disease, or acute surgical wounds in cases in which the healing process is disturbed. Chronic skin ulcers have a significant impact on public health through increased disability, morbidity, and mortality, all of which increase the cost of healthcare (Ho et al., 2005). In patients suffering from these conditions, diabetic foot ulcers are a leading cause of hospitalization and amputation. Many advanced technologies, such as wound dressings, topical ointments, enzymatic debridement compounds, and hyperbaric oxygen therapy, have been developed to improve the treatment of chronic skin ulcers (Heyneman et al., 2008; Dunn et al., 2008; McCallon et al., 2008; Collier et al., 2009; Baroni et al., 1987; Oriani et al., 1990; Kessler L et al., 2003, Ong M. 2008). Recently, due to advances in tissue engineering and cell culture techniques, cultured skin substitutes have been used in the treatment of diabetic ulcers, pressure ulcers, and venous leg ulcers (Karr et al., 2008;Ohara et al., 2010; Cervelli et al., 2010). Negative pressure wound therapy such as Vacuum Assisted Closure (VAC) Therapy (KCI, Texas, U.S.A.) involves the delivery of intermittent or continuous subatmospheric pressure, thereby providing an occlusive environment in which wound healing can proceed under moist, clean, and sterile conditions (Labanaris et al., 2009; Nather et al., 2010). In addition, various growth factors such as basic FGF (Fu et al., 2002; Kurokawa et al., 2003), PDGF-BB（platelet-derived growth factor (Bhansali et al., 2009), EGF (epidermal growth factor）(Kim et al., 2010), and PRP (platelet-rich plasma) (Cervelli et al., 2009; Kathleen et al., 2010) have been used for the clinical treatment of chronic wounds.

A major clinical problem with the use of AD is their low resistance to infection when applied to chronic ulcers. We have attempted to experimentally impregnate AD with antibiotics or sulfadiazine silver to prevent infection; however, the clinical application of these techniques has not been actualized because there is a danger of the emergence of antibiotic-resistant bacteria or silver toxicity (Matsuda et al., 1991; Kawai et al., 2001). Recently, the combination of AD and the daily application of bFGF was reported to accelerate granulation tissue formation during the treatment of uninfected chronic ulcers (Muneuchi et al., 2005; Ito et al., 2005; Akita et al., 2008). We previously reported that AD containing bFGF-impregnated gelatin microspheres (MS) sustained the release of biologically active bFGF, accelerated angiogenesis, and promoted dermis-like tissue formation (Tabata et al., 1999; Kawai et al., 2000; Kawai et al., 2005). This therapy did not require the daily application of bFGF; however, the injection of MS into AD is complicated and time consuming. Our CGS is a scaffold that can sustain the release of positively charged growth factors such as bFGF, PDGF-BB, and TGF-β (transforming growth factor) (Takemoto et al., 2008). As for the optimal dosage of bFGF to impregnate into CGS, it is reported that bFGF forms a polyion complex with acidic gelatin at a bFGF/ gelatin molar ratio of 1/1 (Muniruzzaman et al., 1998). Therefore, our CGS was able to sustain a bFGF concentration of more than 60μg/cm^2 depending on the gelatin content of the CGS. In our previous study, we achieved the sustained release of bFGF from CGS impregnated with 20 μg/cm^2 bFGF. In this study, we confirmed that CGS is able to sustain bFGF at concentrations ranging from 7μg/cm^2 to 50μg/cm^2 during its biodegradation (Fig.2).

It has been reported that the dose-effect relationship of bFGF is bell-shaped (Okumura et

al., 1996; Motomura et al., 2008; Uchi et al., 2009). In a clinical study of diabetic foot ulcers, Uchi reported that the bFGF showed a bell-shaped dose-response pattern with a peak at 1μg/cm^2 bFGF per day (Uchi et al., 2009). In our previous studies, we compared the wound healing processes induced by various bFGF concentrations. In our previous study in which we created skin defects in C57BL mice, CGS impregnated with 7μg/cm^2 of bFGF accelerated dermis-like tissue formation the most, and CGS impregnated with 14μg/cm^2 of bFGF had the second strongest effect. However, the application of CGS impregnated with 50μg/cm^2 bFGF did not accelerate wound healing (Kanda et al., 2011 Jul 5. [Epub ahead of print].). In another study in which palatal mucosal defects were created in beagles, CGS impregnated with 7μg/cm^2 of bFGF also accelerated the tissue regeneration the most, and 14μg/cm^2 of bFGF had the second strongest effect (Artem et al., 2011 Jul 23. [Epub ahead of print].). According to these results, we considered that a bFGF concentration of between 7μg/cm^2 and 14μg/cm^2 would be optimal for accelerating wound healing.

In this study, as expected, CGS impregnated with 7 or 14μg/cm^2 of bFGF accelerated dermis-like tissue formation, and CGS containing 14μg/cm^2 of bFGF produced a significant reduction in the remaining wound area compared with CGS impregnated with 7μg/cm^2 bFGF. The remaining wound area, neoepithelium length, and area of newly formed capillaries in the wounds treated with CGS containing 50μg/cm^2 bFGF were significantly inferior to those of the wounds treated with CGS containing 7 or 14μg/cm^2 bFGF. It has been reported that treatment with an excess amount of bFGF prolonged wound closure in diabetic mice and prevented keratinocyte proliferation in vitro (Okumura et al., 1996; Motomura et al., 2008). CGS impregnated with 50μg/cm^2 of bFGF released a

persistently high dose of bFGF throughout their biodegradation, which might have inhibited the formation of the neoepithelium and capillaries. Our results regarding the degradation rate of CGS suggest that 50μg/cm^2 bFGF inhibits the activity of collagenase. This inhibition might prolong the biodegradation of CGS and hence bFGF release in vivo and so have an adverse effect on wound healing.

Our CGS containing bFGF showed resistance to infection. Some combination therapies have been reported to solve the problem of the lower resistance of AD to infection. Combination therapy involving AD (Integra ®, Integra LifeSciences Corp., Plainsboro, NJ, USA) and the VAC Therapy System has been reported to be effective at increasing granulation tissue formation (Molnar et al., 2004; Pollard et al., 2008). This therapy does not require daily treatment, but does need a specialized pump to maintain a constant negative pressure, and the patient's movements are restricted by the device. As another combination therapy, AD seeded with autologous or allogeneic cultured fibroblasts has been reported to be effective at accelerating granulation tissue formation. The fibroblasts contained in the AD release various growth factors and extracellular matrix molecules and accelerate wound healing (Ohara et al., 2010). Kuroyanagi reported that spongy collagen containing allogeneic fibroblasts was an effective therapy for patients with intractable skin ulcers including burns, venous ulcers, and autoimmune disease (Kuroyanagi et al., 2001). Although tissue-engineered substitutes are attractive, some problems remain, such as the possibility of disease transmission, unfavorable immune and local inflammatory reactions and their high cost, the latter of which is very important from a clinical perspective. Tissue-engineered skin substitutes, such as Dermagraft® (Advanced Tissue Sciences, Inc,

USA.), Transcyte® (Advanced Tissue Sciences, USA.), and Aprigraf® (Organogenesis, Inc., Canton, MA, USA and Novartis Pharmaceuticals Corp, USA.) are expensive, and access to these treatments is limited in many parts of the world (Eran et al., 2006). CGS is cheap compared with AD, and bFGF is cheaper than treatment with the VAC Therapy System or living cells. The procedure for our novel combination therapy involving CGS and bFGF is simple; i.e., bFGF is sprayed onto the CGS just before their application.

Basic FGF has not been authorized for use in most countries. The acidic gelatin contained in CGS can sustain the release of not only bFGF but also other positively charged growth factors such as PDGF-BB and TGF-β (transforming growth factor-β) found in PRP (Hong et al., 2000; Kanematsu et al., 2004). PRP can be prepared from a patient's blood and PL (platelet lysate) produced from donated platelets (Mirabet et al., 2008). When bFGF can not be used, combination therapy involving CGS and autologous or allogeneic PRP is an alternative.

Recently, tissue engineering has been recognized as a newly emerging biomedical technology for regenerating and repairing body defects using various combinations of cells, scaffolds, and growth factors (Tsuji-Saso et al., 2007). Collagen sponge scaffolds are one of the most common scaffolds, and it has been reported that collagen scaffolds treated with growth factors are effective at regenerating various kinds of tissues such as the dermis, epidermis, fat, bone, and cartilage (Langer et al., 2007; Kimura et al., 2010; Nishizawa et al., 2010). Our CGS, which is capable of the sustained release of positively charged growth factors, is useful as a scaffold for tissue engineering.

CONCLUSIONS

CGS impregnated with bFGF at concentrations ranging from 7μg/cm^2 to 14μg/cm^2 accelerated wound healing in decubitus ulcer models involving diabetic mice, and an excess amount of bFGF did not increase their wound-healing efficacy. Our CGS is a novel scaffold that can sustain the release of positively charged growth factors such as bFGF. Combination therapy involving CGS and bFGF or PRP is a promising strategy for the treatment of chronic skin ulcers.

ACKNOWLEDGMENT

This work was supported by a grant from the Japan Science and Technology Agency.

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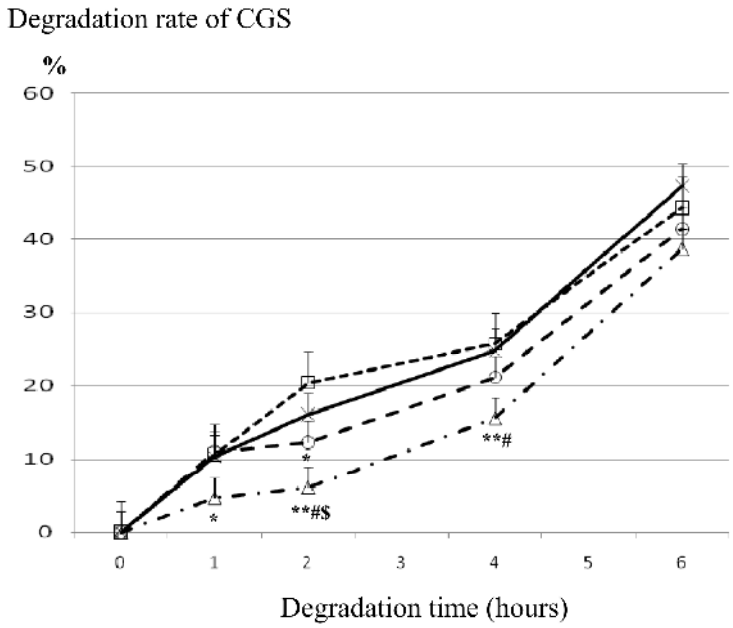

Fig.1. Time course of the degradation of CGS by collagenase. CGS were impregnated with 7μg/cm2 (□), 14μg/cm2 (×) 28μg/cm2 (○), or 50 μg/cm2 of bFGF (△). *p<0.05, **p<0.01 versus 7μg/cm2 of bFGF; #p<0.01 versus 14μg/cm2 of bFGF. $p<0.05 versus 28μg/cm2 of bFGF.
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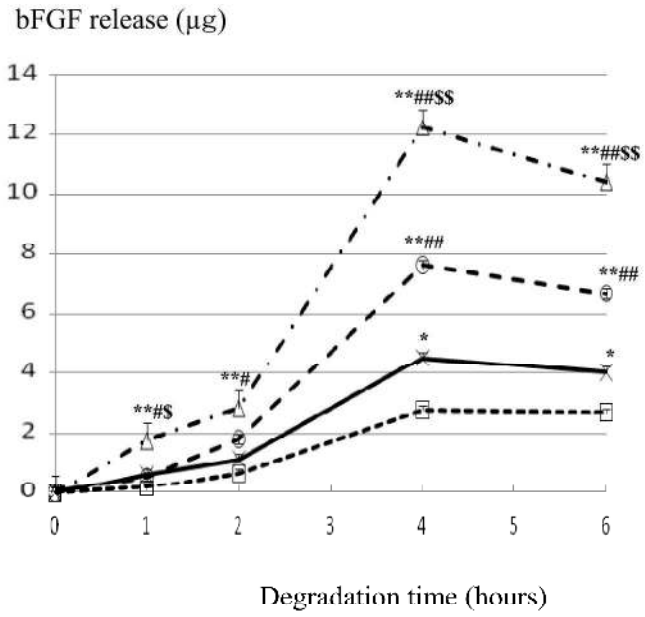

Fig. 2. Time course of bFGF release from CGS. CGS were impregnated with 7μg/cm2 (□), 14μg/cm2 (×), 28μg/cm2 (○), or 50 μg/cm2 of bFGF (△). *p<0.05, **p<0.01 versus 7μg/cm2 of bFGF; #p<0.05, # #p<0.01 versus 14μg/cm2 of bFGF. $p<0.05, $$p<0.01 versus 28μg/cm2 of bFGF.
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Fig. 3. （A） The gross appearance of the decubitus ulcers at 5 days after the completion of the pressure loading.
(B) The necrotic tissue was resected.
(C) The CGS were implanted into the defect and sutured in place.

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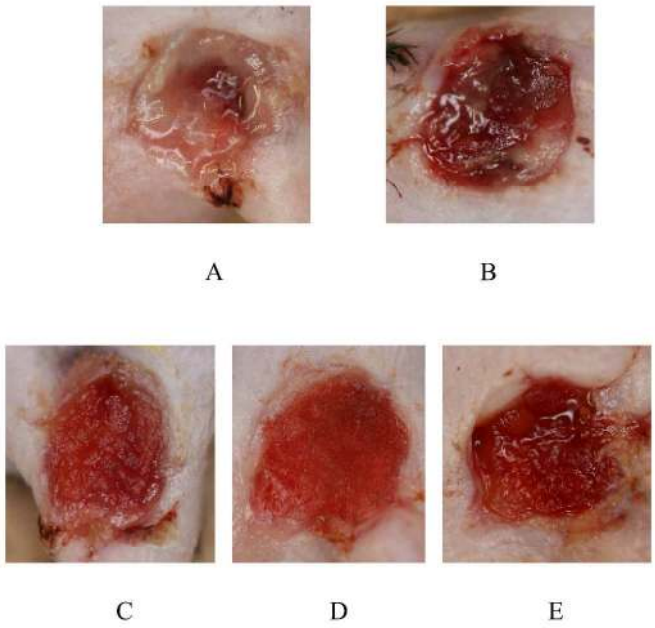

Fig. 4. Gross appearance of the wounds at one week after CGS implantation. The wounds were treated with CGS impregnated with NSS (A) or 7μg/cm2 (B), 14μg/cm2 (C), 28μg/cm2 (D), or 50μg/cm2 of bFGF (E). One week after implantation, the CGS impregnated with NSS were infected, whereas dermis-like tissue had begun to form in the wounds treated with the bFGF impregnated CGS.
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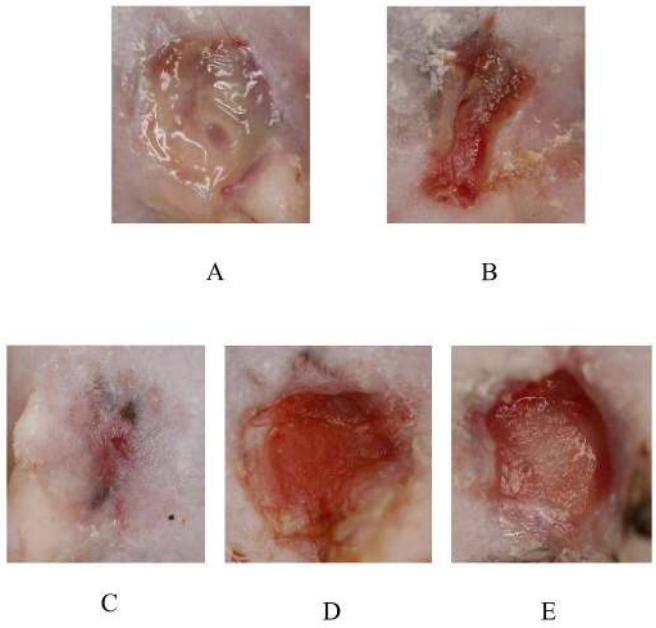

Fig. 5. Gross appearance of the wounds at two weeks after CGS implantation. The wounds were treated with CGS impregnated with NSS (A) or 7μg/cm2 (B), 14μg/cm2 (C), 28μg/cm2 (D), or 50μg/cm2 of bFGF (E). Two weeks after implantation, the CGS impregnated with NSS had become infected and degraded. In contrast, the wound areas covered with CGS containing 7 or 14μg/cm2 of bFGF had markedly reduced without infection. The wounds treated with 14μg/cm2 of bFGF had become almost completely epithelized. 297x420mm (300 x 300 DPI)

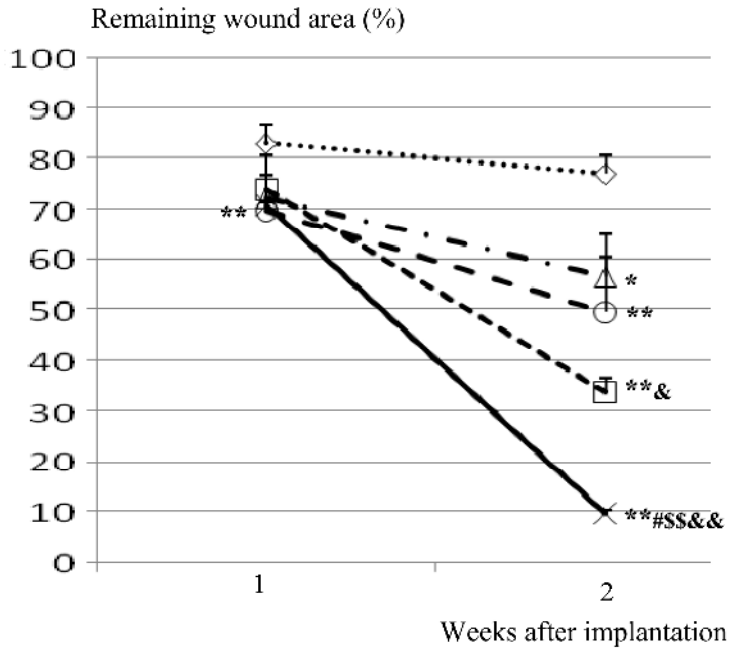

Fig. 6. Time course of the remaining wound area. Wounds treated with CGS impregnated with NSS (□) or 7μg/cm2 (□), 14μg/cm2 (×), 28μg/cm2 (○), or 50μg/cm2 of bFGF (△). *p<0.05, **p<0.01 versus NSS; #p<0.05, ##p<0.01 versus 7μg/cm2 of bFGF. $p<0.05, $$p<0.01 versus 28μg/cm2 of bFGF. &p<0.05, &&p<0.01 versus 50μg/cm2 of bFGF.
One week after implantation, the wound area in the group treated with CGS containing 14μg/cm2 bFGF was significantly smaller than that in the control group. Two weeks after implantation, the wound areas in the groups treated with CGS impregnated with 7 or 14μg/cm2 bFGF were significantly smaller than those in the control and CGS with 50μg/cm2 of bFGF groups.

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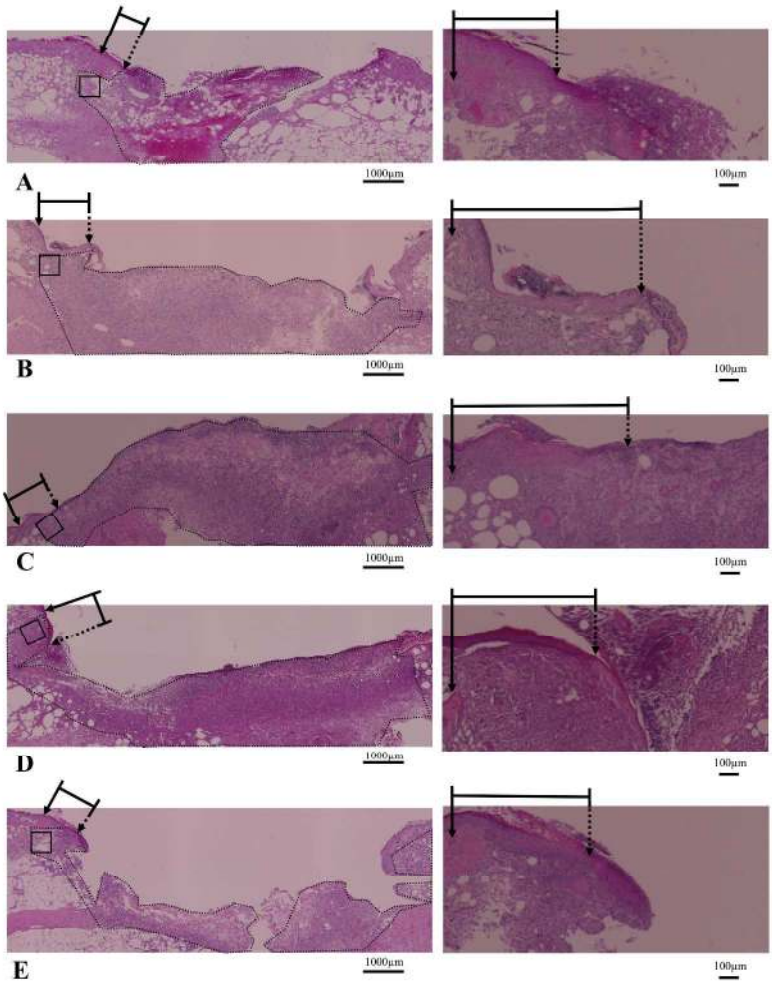

Fig. 7. Hematoxylin and eosin stained sections of wounds at one week after implantation. The wounds were treated with CGS impregnated with NSS (A) or 7μg/cm2 (B), 14μg/cm2 (C), 28μg/cm2 (D), or 50μg/cm2 of bFGF (E). The low magnification image (original magnification ×40, left side) shows a whole image of the implanted CGS. The area of remaining CGS is indicated by a broken line. The square bounded by solid lines shows the area used for the immunohistological staining of newly formed capillaries. The high magnification image (original magnification ×100, right side) shows the newly formed epithelium on the left side. The black arrow with the solid line indicates the hair root. The black arrow with the broken line indicates the end of the neoepithelium. The neoepithelium is shown in the upper section as a black line that is closed at both ends.
297x420mm (300 x 300 DPI)

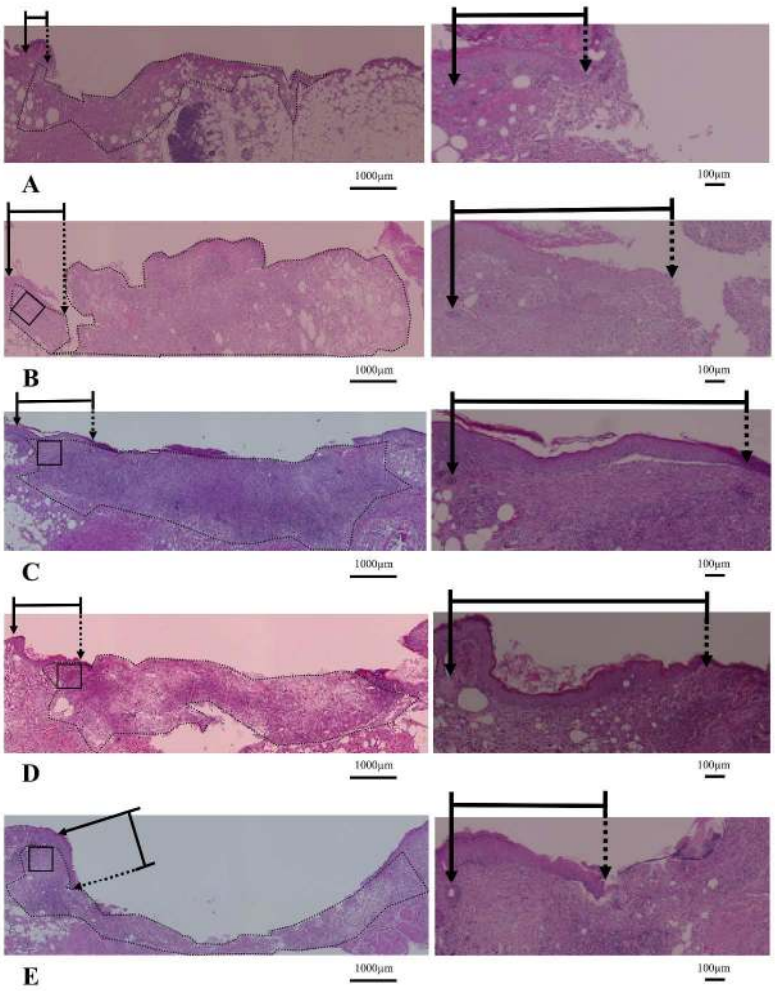

Fig.8. Hematoxylin and eosin stained wound sections at two weeks after implantation. The wounds were treated with CGS impregnated with NSS (A) or 7μg/cm2 (B), 14μg/cm2 (C), 28μg/cm2 (D), or 50μg/cm2 of bFGF (E). The low magnification image (original magnification ×40, left side) shows a whole image of the implanted CGS. The two black solid squares (500μm in width and height) show the area of the CGS in which the area and number of newly formed capillaries were investigated. The area of remaining CGS is surrounded by a broken line. The square bounded by solid lines shows the area used for the immunohistological staining of newly formed capillaries. The high magnification image (original magnification ×100, right side) shows the newly formed epithelium on the left side. The black arrow with the solid line indicates the hair root. The black arrow with the broken line indicates the edge of the epithelium. The neoepithelium is shown in the upper section as a black line that is closed at both ends.
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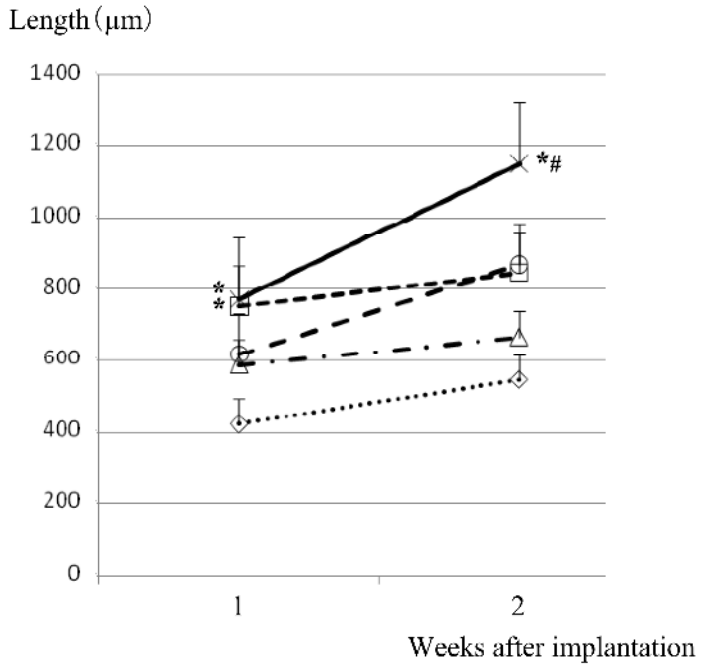

Fig. 9. Time course of neoepithelium length. Wounds treated with CGS impregnated with NSS (◇) or 7μg/cm2 (□), 14μg/cm2 (×) 28μg/cm2 (○), or 50 μg/cm2 of bFGF (△). *p<0.01 versus NSS, #p<0.01 versus 50μg/cm2 of bFGF. One week after implantation, the neoepithelial lengths in the wounds treated with 7μg/cm2 or 14μg/cm2 of bFGF were significantly longer than that of the control group. Two weeks after implantation, the neoepithelium length of the wounds treated with 14μg/cm2 of bFGF was significantly longer than those of the control group and the group treated with 50μg/cm2 of bFGF.
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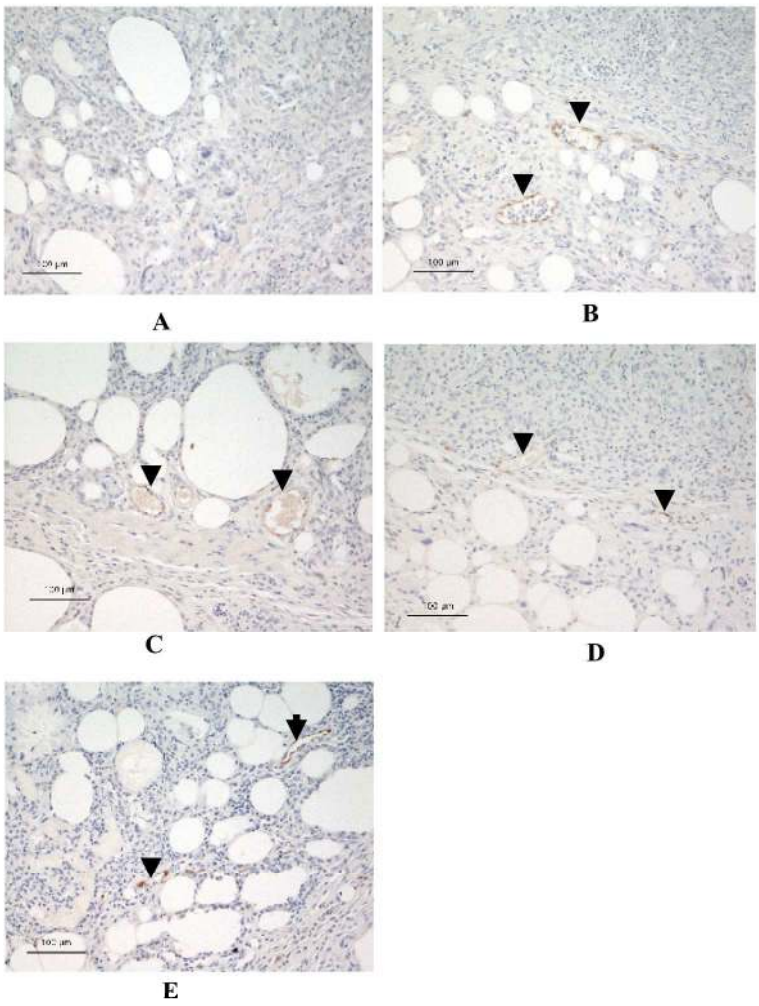

Fig. 10. Immunohistological staining of CGS two weeks after implantation. The newly formed capillaries were immunostained with Von Willebrand Factor (original magnification ×200). CGS with NSS (A), 7μg/cm2 of bFGF (B), 14μg/cm2 of bFGF (C), 28μg/cm2 of bFGF (D), and 50μg/cm2 of bFGF (E). The black arrowheads indicate capillaries.
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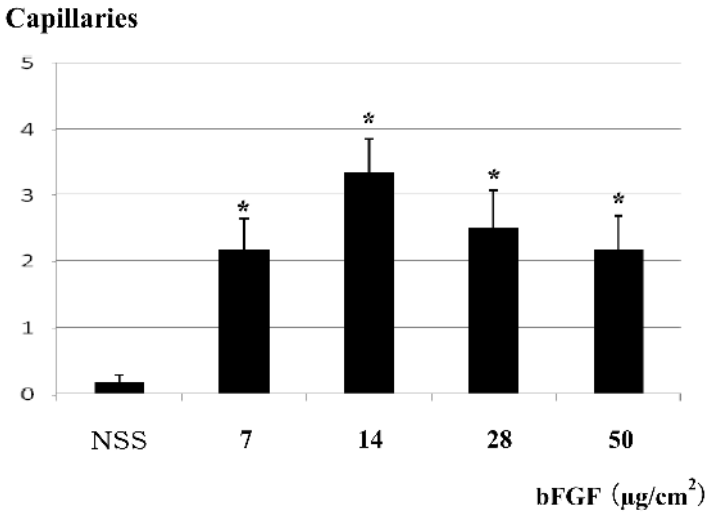

Fig.11. The area of newly formed capillaries immunostained with Von Willebrand Factor （two weeks after implantation).
CGS treated with NSS or 7μg/cm2, 14μg/cm2, 28μg/cm2, or 50μg/cm2 of bFGF. *p<0.01 versus NSS ; #p<0.01 versus 50μg/cm2 bFGF.
The area of newly formed capillaries in the group treated with CGS impregnated with 7μg/cm2 bFGF was significantly larger than those in the control group and the group treated with CGS impregnated with 50μg/cm2 of bFGF. The areas of newly formed capillaries in the groups treated with CGS impregnated with 14μg/cm2 or 28μg/cm2 bFGF were significantly larger than that in the control group.

297x420mm (300 x 300 DPI)