J Mater Sci: Mater Med (2008) 19:3473–3479
DOI 10.1007/s10856-008-3512-4

Development of salmon milt DNA/salmon collagen composite for wound dressing

XuanRi Shen · Nobuhiro Nagai · Masaru Murata · Daisuke Nishimura · Masahito Sugi · Masanobu Munekata

Received: 26 February 2008 / Accepted: 16 June 2008 / Published online: 1 July 2008

Abstract This study aims to develop a novel wound dressing comprising salmon milt DNA (sDNA) and salmon collagen (SC). The sDNA/SC composites were prepared by incubating a mixture of an acidic SC solution, an sDNA solution, and a collagen fibrillogenesis inducing buffer (pH 6.8) containing a crosslinking agent (water-soluble carbodiimide) for gelation, and a subsequent ventilation-drying process to give sDNA/SC films. The conjugation between sDNA and SC were confirmed by sDNA-elution assay and fluorescence microscopy. The sDNA/SC films with various doses of sDNA (sDNA/SC weight ratios of 1:5, 1:10, and 1:20) were used for in vitro cell cultures to evaluate their growth potentials of normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK). It was found that NHDF proliferation was increased by sDNA conjugation, whereas NHEK proliferation was dose-dependently inhibited. In light of the in vitro results, the appropriate dose of sDNA for in vivo study was determined to be the ratio of 1:10. For the implantation in full-thickness skin defects in rat dorsal region, the sDNA/SC films were reinforced by incorporating them on a porous SC sponge, because the sDNA/SC films exhibited early contraction and inadequate morphologic stability when implanted in vivo. The regenerated tissue in the sDNA/SC sponge group showed similar morphology to native dermis, while the SC sponge group without sDNA showed epithelial overgrowth, indicating that additional sDNA could reduce epidermal overgrowth. Furthermore, blood capillary formation was significantly enhanced in the sDNA/SC sponge group when compared to the SC sponge group. In conclusion, the results suggest that the sDNA/SC composite could be a potential wound dressing for clinical applications.

X. Shen (✉) · M. Munekata
Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan
e-mail: sxrcg@yahoo.co.jp

N. Nagai
Creative Research Initiative "Sousei", Hokkaido University, N21-W10, Kita-ku, Sapporo, Hokkaido 001-0021, Japan

M. Murata
Division of Oral and Maxillofacial Surgery, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido 061-0293, Japan

D. Nishimura · M. Sugi
Institute of Nissei Bio Co., Ltd., Megumino, Kita 3-1-13, Eniwa, Hokkaido 061-1374, Japan

1 Introduction

Wound dressings are based on the concept of creating an optimum environment to allow epithelial cells to move unimpeded, for the treatment of wounds. Such optimum conditions include a moist environment around the wound, effective oxygen circulation to aid regenerating cells and tissues, and a low bacterial load. Dressings are classified in a number of ways depending on their functions (antibacterial, occlusive, absorbent, adherence) [1], type of material (collagen, alginate, hydrocolloid) [2], and the physical form (ointment, film, sponge, gel) [3].

Bioactive dressings, such as collagen [4], hyaluronic acid [5], and chitosan [6], are attractive because of their advantages of forming part of the natural tissue matrix. These biomaterials are biodegradable, biocompatible, and play an active part in normal wound healing and new tissue

formation. Among these materials, collagen is more benefit because collagen (1) is a significant constituent of extracellular matrix (ECM) and a major structural protein of any organ [7], (2) functions as a substrate for haemostasis and is chemotactic to cellular elements of healing such as granulocytes, macrophages, and fibroblasts, and (3) promotes wound maturation by providing a scaffold for cellular attachment, migration, and proliferation of fibroblast and endothelial cells [8]. These characteristics, therefore, make collagen attractive choices for wound dressing.

Recently, the use of fish collagen is remarkable in food and cosmetic fields because of its reduced potential for disease transmission, such as bovine spongiform encephalopathy and foot-and-mouth disease, owing to the large evolutionary distance between fish and human beings [9, 10]. Despite the biological advantage, low denaturation temperature has prevented their applications in medical fields. We recently developed a method for improving the thermal stability of collagen gel derived from chum salmon (*Oncorhynchus keta*) [11]. The improvement can be achieved by chemical crosslinking with water-soluble carbodiimide (WSC) during in vitro collagen fibrillogenesis. The resulting salmon collagen (SC) fibrillar gel has a denaturation temperature of 55°C and its biological properties have been demonstrated by several in vitro studies [12, 13].

An idea introduced recently to improve biomaterial performance is the use of salmon milt DNA (sDNA). The sDNA extracted from industrial discarded salmon milts has been widely applied in various areas. For example, sDNA has been used for environmental purposes, biological and genetic applications, and medical therapeutic aims [14–17]. The phosphate groups of sDNA provide the poly-anionic character which allows bonding with the cationic polymers by electrostatic stabilization. The complex of sDNA with positively charged materials have been studied and useful results have been obtained. However, accompanying problems that ionic bonded sDNA is solubility in the biogenic environment before it plays sufficient functions have been noted. Several techniques to fabricate water-insoluble sDNA complexes are reported [18, 19], but little is known about the composite comprising sDNA and SC.

The aim of this study was the development of a novel wound dressing comprising sDNA and SC. The use of such marine sources, which are discarded as industrial wastes, is advantageous to the reduced risk for pathogen transmission and effective use of natural resources. Here, sDNA/SC composites were prepared by incubating a mixture of an acidic SC solution, a neutral sDNA solution, and a fibrillogenesis inducing buffer (pH 6.8) containing WSC for gelation, and a subsequent drying process. We investigated the appropriate dose of sDNA for in vivo study by evaluating the growth of normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) in vitro on the sDNA/SC composites with various doses of sDNA. The implantation study in full-thickness skin defects in rat dorsal region was performed to evaluate the biocompatibility and skin regeneration potential of the sDNA/SC composites.

2 Materials and methods

2.1 Preparation of sDNA/SC composites

sDNA (sodium salt, molecular weight is ca. 100 kDa) was purchased from Biochem (Japan) and SC (molecular weight is ca. 330 kDa) was kindly supplied by Ihara & Company (Japan). The SC solutions (5 mg/ml) in diluted HCl (pH 3.0) were mixed with 0.5 M NaCl solutions containing sDNA (pH 7.0) to give sDNA/SC weight ratios of 1:5, 1:10, and 1:20. Then the mixtures were mixed with sodium phosphate (NaP) buffers (pH 6.8) containing WSC (Dojindo, Japan) at 4°C. The final concentrations of SC, NaCl, NaP, and WSC in the mixtures were 1.67 mg/ml, 333 mM, 15 mM, and 60 mM, respectively. The mixtures were immediately transferred into a 24-well tissue culture plate (Asahi Techno Glass, Japan) at 1 ml per well and incubated at 4°C for 24 h for gelation and crosslinking. Resulting gels were washed 6 times with deionized water to remove excessive WSC and NaCl, and were ventilation-dried in the clean bench overnight to make sDNA/SC films for the following assays (sDNA elution assay, fluorescence microscopy, and in vitro cell cultures).

For the implantation study, the gelation of sDNA/SC mixture was performed on a porous SC sponge (10 mm × 10 mm; thickness: 1 mm) to give an sDNA/SC sponge. The porous SC sponge was prepared by the method reported previously [20]. Briefly, the sDNA/SC mixture containing 1.67 mg/ml SC, 333 mM NaCl, 15 mM NaP, 60 mM WSC, and 167 μg/ml sDNA (sDNA/SC weight ratio was 1:10) was poured on the porous SC sponge at 1 ml per sponge and placed at 4°C for gelation and crosslinking. Resulting sponge/gel structure was washed 6 times with deionized water to remove excessive WSC and NaCl, then lyophilized (FDU-830; EYELA Tokyo Rikakikai, Japan). The untreated SC sponge was used as control.

2.2 sDNA elution assay

To confirm the sDNA conjugation with SC, the elution rates of sDNA from the sDNA/SC films were investigated. The films were rinsed 4 times with phosphate-buffered saline (PBS). The sDNA content of the supernatant after a

fourth rinse was evaluated by measuring the absorbance at 260 nm with spectrophotometer (UV mini 1240, Shimadzu, Japan). The elution rates of sDNA from the sDNA/SC films were calculated as follows:

$$\text{Elutionrate}(\%) = 100 \times \text{elution volume of sDNA}(\text{mg}) / \text{initial volume of sDNA (mg)}$$

Volume of sDNA was calculated from standard sDNA calibration.

2.3 Fluorescence microscopy

Dimeric cyanine nucleic acid dyes (YOYO-1, Molecular probes, USA) were used for sDNA staining in the sDNA/SC films. The staining was performed by manufacturer's instructions. Fluorescence was observed with the wavelengths of 491 nm and 509 nm using fluorescence microscopy (Axio Imager A1, Carl zeiss, Germany) to excite the dimeric cyanine.

2.4 In vitro cell culture

The NHDF and NHEK were purchased from Takara Bio (Japan), and were used at passage 4 and 6, respectively. The NHDF were suspended in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Japan) containing 10% fetal bovine serum (FBS; Gibco BRL, USA) at a concentration of 5×10^4 cells/ml, then seeded on the sDNA/SC films placed in 24-well tissue culture plates (Asahi Techno Glass). Cell numbers were determined using Cell Titer 96®AQ_{ueous}Non-Radioactive Cell Proliferation Kit (MTS; Promega, Japan) according to the manufacturer's instruction. Prior to addition of 0.5 mg/ml MTS working solution, the films were rinsed three times with PBS. After incubation for 1 h, absorbance was measured at 492 nm using a microplate reader (Thermo electron, Japan). In the case of NHEK experiments, KGM-2 (Takara Bio, Japan) was used for culture medium.

2.5 SEM observation

The sDNA/SC sponge (sDNA/SC weight ratio was 1:10) and the SC sponge were dehydrated with a sequential immersion in 50, 75, 85, 95, and 99% ethanol. The immersion in 99% ethanol was repeated three times. The ethanol in the dehydrated sponge was thoroughly exchanged with *t*-butyl alcohol and subjected to a critical point drying. The dried sponges were coated with *Au* using an ion coater (E-1010; Hitachi, Japan) and subjected to observation under high-resolution scanning electron microscope (SEM; JSM-6500F; JEOL, Japan). The SEM apparatus was operated at 5.0 kV.

2.6 In vivo study

Wistar rats were used for the implantation study. The rats were anesthetized with isoflurane (concentration, 1.5% and flux, 500 ml/min; Nissan Chemical, Japan). Two full-thickness defects (round shapes; diameter, 8 mm) were created on the dorsum of each rat. Each rat had a wound (no treatment), the sDNA/SC sponge, and the SC sponge. At the end of the predetermined time, the rats were sacrificed by an overdose of ether for histological observation. The study protocol was in compliance with the Health Sciences University of Hokkaido's guidelines as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by National Institutes of Health (NIH Publication No. 86-23, 1985).

2.7 Histological observation

The specimens were excised along with the adjacent tissue and fixed in 10% formalin solution and embedded in paraffin. The specimens were cut into 5-μm-thick sections for von willebrand factor (vWF) staining and subjected to histological evaluation. The sections were incubated with specified primary antibodies (rabbit polyclonal anti-human vWF/HRP; 22 μg/ml, Dako, Japan) for 1 h at room temperature, and visualized by addition of immunoperoxidase substrate diaminobenzidine using DakoCytomation Liquid DAB Substrate Chromogen System (Dako). The sections were observed by a phase-contrast microscope (CKX41N; Olympus, Japan).

The quantitative measurement of the number of positive-stained area in vWF stained sections was performed in the areas of 4,000 × 1,800 μm at original magnification × 200. The results are expressed as the mean of the five specimens.

3 Results

3.1 Preparation of sDNA/SC composites

The elution rates of the sDNA from the sDNA/SC films with the sDNA/SC ratios of 1:5, 1:10, and 1:20 were 6.8, 5.0, and 3.2%, respectively. The fluorescence microscopy observation demonstrates that sDNA was chemically conjugated with SC matrix (Fig. 1).

3.2 In vitro cell cultures

Figure 2 shows the growth curves of the NHDF and NHEK cultured on the sDNA/SC films. The NHDF proliferation was significantly increased in sDNA-conjugated samples

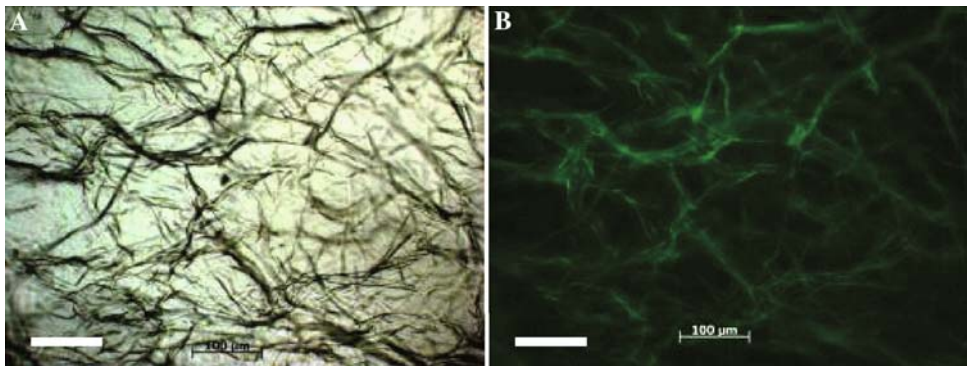

Fig. 1 Light microscopy (**a**) and fluorescence microscopy (**b**) of sDNA/SC film with sDNA/SC weight ratio of 1:10. Dimeric cyanine nucleic acid dyes were used for staining sDNA. Bars, 100 μm

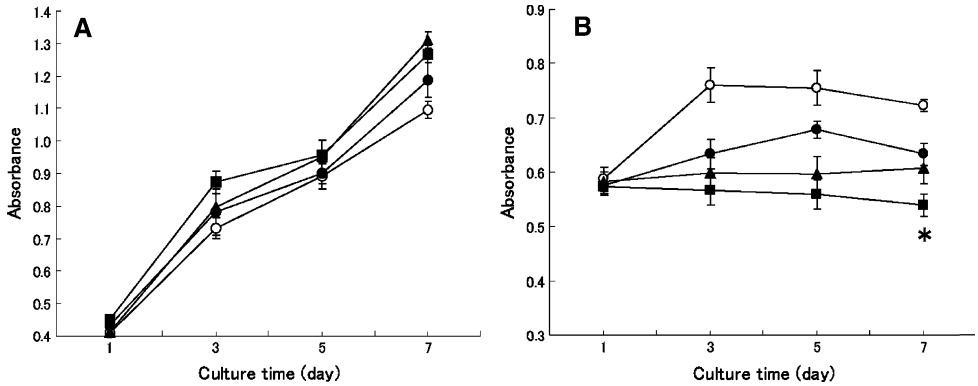

Fig. 2 Growth curves of NHDF (**a**) and NHEK (**b**) cultured on sDNA/SC films with sDNA/SC weight ratios of 1:5 (closed squares), 1:10 (closed triangles), and 1:20 (closed circles). SC films without sDNA were used as control (open circles). Cell number was expressed as absorbance obtained by MTS assay. Data are expressed as means ± standard deviations (n = 5). *$P < 0.05$ (1:10 vs. 1:5)

(Fig. 2a). The sample with the sDNA/SC ratio of 1:10 showed the highest rate of the NHDF proliferation. There was no difference in attachment rates of the NHEK among the samples, while the NHEK proliferation was dose-dependently inhibited in the sDNA-conjugated samples (Fig. 2b). In particular, the sample with the sDNA/SC ratio of 1:5 shows significant decrease in NHEK growth at 7 d compared to other samples (Fig. 2b). These assays were repeated three times and similar results were obtained.

3.3 SEM observation

Figure 3 shows the SEM images of the sDNA/SC sponge and the SC sponge. Pore surfaces of the sDNA/SC sponge were rough in whole area (Fig. 3a), while those of the SC sponge were smooth (Fig. 3b), indicating successful incorporation of sDNA on the pore surfaces. The pore structure was maintained after the sDNA incorporation. Both sponges had microporous structure with pore sizes of 50–150 μm.

3.4 Histological observation

Figure 4 shows the histological sections of the vWF-stained samples implanted in full-thickness skin defects in rat dorsal region at 10 d after implantation. The sDNA/SC sponge group demonstrates favorable skin regeneration with abundant granulation tissue, epidermis, and blood

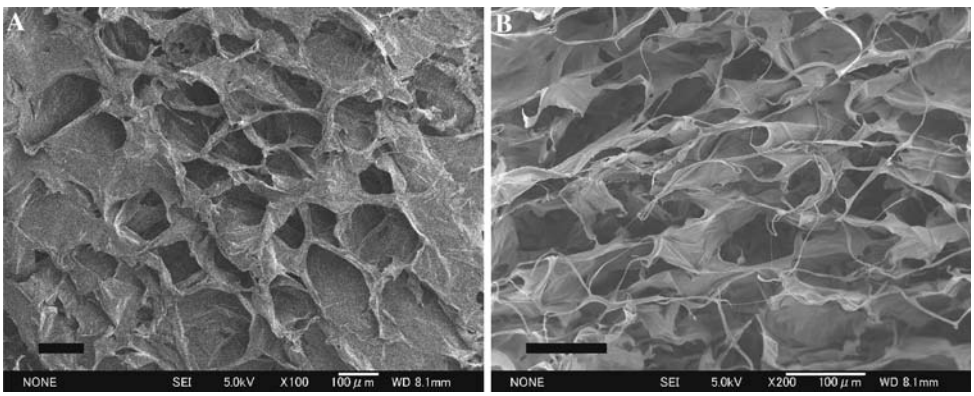

Fig. 3 SEM images of sDNA/SC sponge (1:10) (**a**) and SC sponge (**b**). Bars, 100 μm

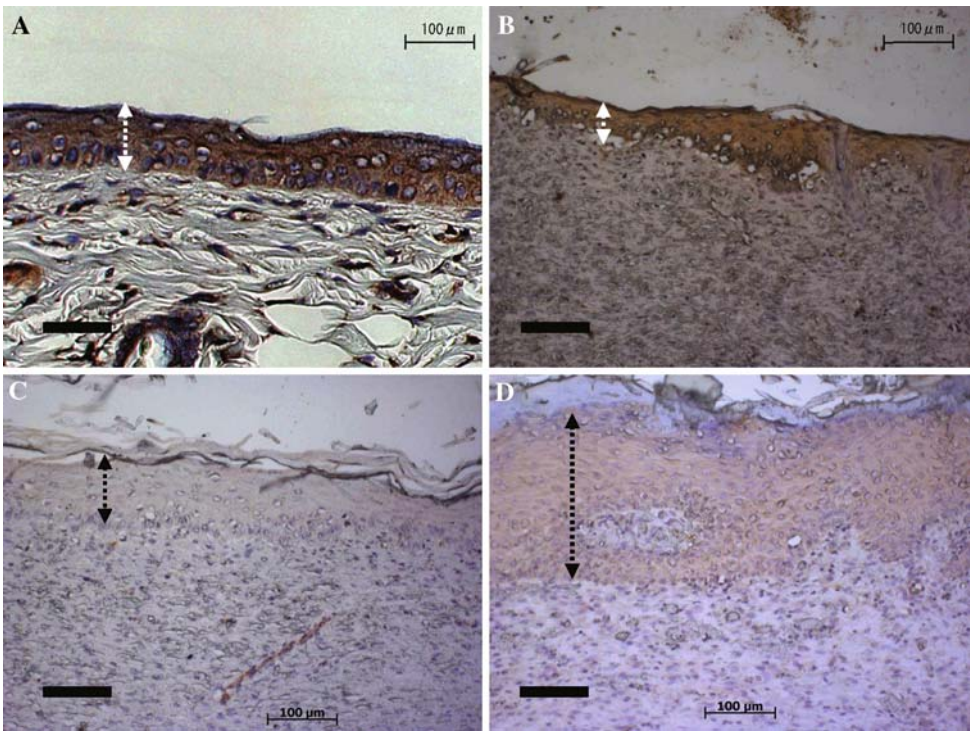

Fig. 4 Histological sections of normal skin (**a**), non-treated wounds (**b**), wounds treated with sDNA/SC sponge (1:10) (**c**), and SC sponge (**d**). The sections were stained with vWF antibodies. Two-headed arrows indicate the epidermal regions. Bars, 100 μm

capillaries (Fig. 4c). The sDNA/SC sponge was nearly biodegraded with little inflammatory response. The thickness of neo-epidermis was almost the same as the native epidermis (Fig. 4a); the thickness was about 100 μm. On the other hand, the SC sponge group demonstrates thicker neo-epidermis formation compared to the sDNA/SC sponge group (Fig. 4d); the thickness was about 220 μm, although the granulation tissue formation with blood capillaries was observed. No treatment group demonstrates the incomplete healing with deficient epidermal regeneration and incomplete closure of wound surface (Fig. 4b).

Figure 5 shows the region at the depth of 250 μm from skin surface at 5 d and 7 d after implantation. The vWF-positive cells or capillaries were present in both samples,

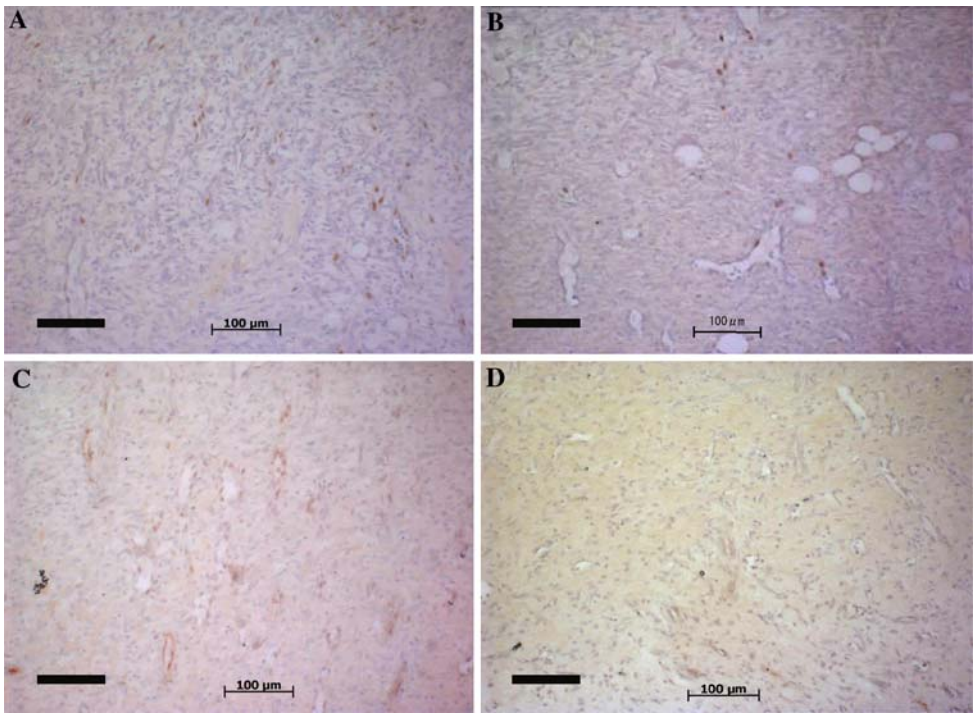

Fig. 5 Histological sections of the region at the depth of 250 μm from skin surface in the wounds treated with sDNA/SC sponge (1:10) (**a**, 5 d; **c**, 7 d) and SC sponge (**b**, 5 d; **d**, 7 d). The sections were stained with vWF antibodies. Bars, 100 μm

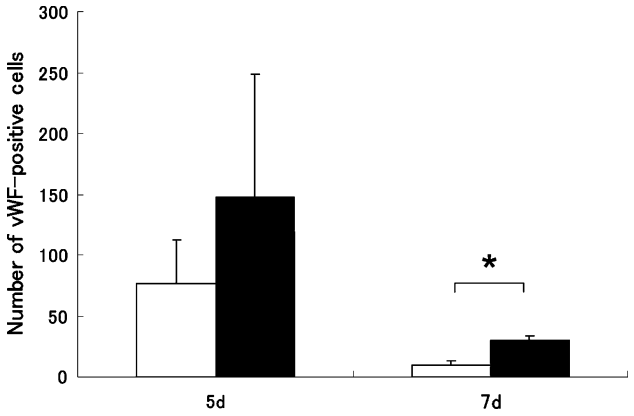

Fig. 6 Quantitative results of the number of the cells or capillaries positively stained with vWF antibodies in sDNA/SC sponge group (closed columns) and SC sponge group (open columns). Data are expressed as means ± standard deviations (n = 5). $^*P < 0.05$

indicating angiogenesis. The stain area of the vWF-positive cells was macroscopically large in the sDNA/SC sponge group (Fig. 5a, c) compared to that in the SC sponge group (Fig. 5b, d). Meanwhile, the positive cells were hardly observed at 10 d after implantation in both groups (data not shown).

Figure 6 shows the quantitative results of the number of vWF-positive cells or capillaries in the region in Fig. 5. The number of the immunopositive one in the sDNA/SC sponge group was higher than that in the SC sponge group up to 7 d after implantation. The difference in the number between both groups at 7 d was significant. The number decreased with time, then reached to almost zero at 10 d in both groups (data not shown).

4 Discussion

This study aimed to develop a novel wound dressing comprising sDNA and SC. First, we fabricated the sDNA/SC films to determine the appropriate dose of sDNA for in vivo study. The conjugation of sDNA with SC was achieved by the WSC crosslinking during in vitro collagen fibrillogenesis. It is well known that cross-linking with WSC involves activation of carboxylic acid groups to give *O*-acylisourea groups, which form cross-links with free amine groups [21]. Single strand sDNA has plenty of amine groups in its polymeric chains; hence, the activated carboxylic acid groups in collagen reacted with the amine groups in sDNA, providing cross-links with the formation of amide bonds. In the present study, simultaneous cross-linking with collagen fibrillogenesis was used because sDNA was expected to be introduced uniformly in the collagen fibril network. Collagen fibrillogenesis is basically a self-assembly process inspired by hydrophobic interaction at neutral pH in the presence of a physiological concentration of saline [22]. Our previous study demonstrates that the introduction of crosslinking during collagen fibrillogenesis provides uniform crosslinks between collagen molecules and a drastic increase in thermal stability [11]. Although we applied the method to prepare the sDNA/SC films, it was found that the addition of sDNA caused interference to the collagen fibrillogenesis, resulting in a fibril-unformed gel with less thermal stability. Actually, precipitation was occurred by the addition of sDNA into an SC solution due to electrostatic interaction. The precipitation, however, was solved by changing the final concentration of NaCl from 35 to 333 mM. The increase in ionic strength might reduce the electrostatic interaction between anionic sDNA and cationic collagen due to electron screening by additive electrolytes. As a result, the sDNA/SC films with various doses of sDNA were successfully fabricated.

Next, the sDNA/SC films were used for the in vitro cultures of NHDF and NHEK. It was found that NHDF proliferation was increased by sDNA conjugation, whereas NHEK proliferation was dose-dependently inhibited. These results indicate that the application of the sDNA/SC films to wound dressings requires particular attention to an appropriate dose of sDNA. We assume that hydrolysate of sDNA, deoxyadenosine, enhanced or inhibited cell proliferation with activation of adenosine receptors. Thellung et al. reported that adenosine enhanced the proliferation of skin dermal fibroblasts by adenosine receptor activation [23]. On the other hand, Cook et al. reported that adenosine was antiproliferative for normal human epidermal keratinocytes [24]. These reports are in good agreement with our data. Consequently, we chose the ratio of 1:10 for in vivo study, because the ratio of 1:5 showed decreased growth of the NHEK and the ratio of 1:10 showed enhanced growth of NHDF.

For the implantation study in full-thickness skin defects in rat dorsal region, the sDNA/SC films were reinforced by incorporating them on a porous SC sponge, because the sDNA/SC films exhibited early contraction and inadequate morphologic stability when implanted in vivo. The porous SC sponge prepared by the method we reported previously shows no contraction during 14 days culture period. Hence, subsequent freeze-drying of the resulting sponge/gel structure succeeded in the preparation of the sDNA/SC sponge with extra strength and stability. Microscopic observation reveals the homogenous incorporation of the sDNA on pore surfaces of the SC sponge. There was no contraction of the sDNA/SC sponge throughout the implantation period.

The implantation study shows that the regenerated tissue in the sDNA/SC sponge group was a similar morphology to native dermis, while the SC sponge group without sDNA

showed epithelial overgrowth, indicating that sDNA could reduce epithelial overgrowth. The in vivo results were consistent with the in vitro results demonstrating that sDNA dose-dependently inhibited the growth of the NHEK. Therefore, sDNA might be effective in skin regeneration while reducing epidermal overgrowth. Furthermore, the number of blood capillaries significantly increased in the sDNA/SC sponge group when compared to the SC sponge group, indicating that exogenous sDNA enhanced neovascularization at early stage. Montesinos et al. reported that adenosine mediated angiogenesis and promoted wound healing [25]. We speculate that the deoxyadenosine derived from biodegraded sDNA mediated the neovascularization by activating the adenosine receptors, similar to the previous reports. Extensive study to evaluate the mechanism underlying the enhanced angiogenesis is ongoing and the results will be reported in near future.

5 Conclusion

We first developed the sDNA/SC composite for wound dressing. Both in vitro and in vivo results show that the modification of the SC sponge with sDNA could reduce epidermal overgrowth and enhance neovascularization, leading to appropriate skin regeneration. The results suggest that the sDNA/SC composite could be a potential dressing for clinical applications.

Acknowledgement Shen thanks the Japan Society for the Promotion of Science (JSPS) for a post-doctoral fellowship for foreign researchers.

References

1. S.K. Purna, M. Babu, Burns **26**, 54 (2000). doi:10.1016/S0305-4179(99)00103-5
2. D. Queen, H. Orsted, H. Sanada, G. Sussman, Int. Wound J. **1**, 59 (2004). doi:10.1111/j.1742-4801.2004.0009.x
3. A.F. Falabella, Dermatol. Ther. **19**, 317 (2006). doi:10.1111/j.1529-8019.2006.00090.x
4. J.A. Ramshaw, J.A. Werkmeister, V. Glattauer, Biotechnol. Genet. Eng. Rev. **13**, 335 (1996)
5. C.J. Doillon, F.H. Silver, Biomaterials **7**, 3 (1986). doi:10.1016/0142-9612(86)90080-3
6. M. Ishihara, K. Nakanishi, K. Ono, M. Sato, M. Kikuchi, Y. Saito et al., Biomaterials **23**, 833 (2002). doi:10.1016/S0142-9612(01)00189-2
7. C.H. Lee, A. Singla, Y. Lee, Int. J. Pharm. **221**, 1 (2001). doi:10.1016/S0378-5173(01)00691-3
8. M. Mian, F. Beghe, E. Mian, Int. J. Tissue React. **14**(Suppl), 1 (1992)
9. E. Song, S. Yeon Kim, T. Chun, H.J. Byun, Y.M. Lee, Biomaterials **27**, 2951 (2006). doi:10.1016/j.biomaterials.2006.01.015
10. M.M. Giraud-Guille, L. Besseau, C. Chopin, P. Durand, D. Herbage, Biomaterials **21**, 899 (2000). doi:10.1016/S0142-9612(99)00244-6
11. S. Yunoki, N. Nagai, T. Suzuki, M. Munekata, J. Biosci. Bioeng. **98**, 40 (2004)
12. N. Nagai, K. Mori, Y. Satoh, N. Takahashi, S. Yunoki, K. Tajima et al., J. Biomed. Mater. Res. A **82**, 395 (2007). doi:10.1002/jbm.a.31110
13. N. Nagai, S. Yunoki, Y. Satoh, K. Tajima, M. Munekata, J. Biosci. Bioeng. **98**, 493 (2004)
14. M. Yamada, K. Kato, K. Shindo, M. Nomizu, M. Haruki, N. Sakairi et al., Biomaterials **22**, 3121 (2001). doi:10.1016/S0142-9612(01)00061-8
15. J.J.J.P. Van Den Beucken, M.R.J. Vos, P.C. Thune, T. Hayakawa, T. Fukushima, Y. Okahata et al., Biomaterials **27**, 691 (2006). doi:10.1016/j.biomaterials.2005.06.015
16. W. Liu, S. Sun, Z. Cao, X. Zhang, K. Yao, W.W. Lu et al., Biomaterials **26**, 2705 (2005). doi:10.1016/j.biomaterials.2004.07.038
17. E. Holen, O.A. Bjørge, R. Jonsson, Nutrition **22**, 90 (2006). doi:10.1016/j.nut.2006.01.001
18. T. Fukushima, T. Hayakawa, Y. Inoue, K. Miyazaki, Y. Okahata, Biomaterials **25**, 5491 (2004). doi:10.1016/j.biomaterials.2004.01.006
19. M. Yamada, K. Kato, M. Nomizu, N. Sakairi, K. Ohkawa, H. Yamamoto et al., Chemistry (Easton) **8**, 1407 (2002)
20. N. Nagai, S. Yunoki, T. Suzuki, M. Sakata, K. Tajima, M. Munekata, J. Biosci. Bioeng. **97**, 389 (2004)
21. L.H. Olde Damink, P.J. Dijkstra, M.J. Van Luyn, P.B. Van Wachem, P. Nieuwenhuis, J. Feijen, Biomaterials **17**, 765 (1996). doi:10.1016/0142-9612(96)81413-X
22. K.E. Kaldler, D.F. Holmes, J.A. Trotter, J.A. Chapman, Biochem. J. **316**(Pt 1), 1 (1996)
23. S. Thellung, T. Florio, A. Maragliano, G. Cattarini, G. Schettini, Life Sci. **64**, 1661 (1999). doi:10.1016/S0024-3205(99)00104-6
24. P.W. Cook, N.M. Ashton, M.R. Pittelkow, J. Invest. Dermatol. **104**, 976 (1995). doi:10.1111/1523-1747.ep12606228
25. M.C. Montesinos, A. Desai, J.F. Chen, H. Yee, M.A. Schwarzschild, J.S. Fink et al., Am. J. Pathol. **160**, 2009 (2002)