MATERIALS AND METHODS

Animals

Seven-week-old KSN/Slc nude mice, obtained from the Chubu Kagaku Shizai Corporation (Nagoya, Japan), were used. The animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Nagoya University School of Medicine.

Cell Culture of Human Deciduous Teeth Dental Pulp Cells (hDPC)

The experimental protocol is summarized (Fig. 1). hDPC were obtained from clinically healthy extracted deciduous teeth from 7 to 8 year-old children. The ethics committee of Nagoya University approved our experimental protocols. hDPC were isolated and cultured as previously described.[9,10] Briefly, the pulp was gently removed and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 hour at 37 °C. Cells were cultured in conditioned medium consisting of low-glucose Dulbecco's modified Eagle medium with growth supplements (50 mL of fetal bovine serum, 10 mL of 200-mmol/L L-glutamine, and 0.5 mL of penicillin-streptomycin mixture containing 25 U of penicillin and 25 Kg of streptomycin [Lonza, Inc, Walkersville, MD]) at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide. The medium was changed every 3 days. When the cells were confluent, they were passaged. Cells up to 5 passages were used in this experiment.

Basic Fibroblast Growth Factor (b-FGF)

Recombinant human basic fibroblast growth factor (b-FGF) (Kaken Pharmaceutical Co, Ltd, Tokyo, Japan), was dissolved in phosphate buffered saline (PBS) before use. The concentration of b-FGF (100μg/ml) was according to the manufacturer's instructions.

Wound Healing Model

The excisional wound splint model was utilized as described previously.[18] Mice were individually anesthetized, and two 8 mm full thickness skin defects were created on the dorsal surface each side of the midline. A doughnut-shaped silastic splint was placed so that the wound was centered within the splint. A fast-bonding adhesive (Krazy Glue, Columbus, OH) was used to fix the splint to the skin, followed by interrupted 4-0 silk sutures to stabilize its position.

Cell Transplantation

The cultured hDPC were detached from culture dishes by enzymatic treatment with 0.05% trypsin/EDTA. hDPC labeled with PKH26 (Sigma-Aldrich, St Louis, MO), were then prepared. The animals were randomly divided into four groups: 100μl of PBS was applied to the wound bed (control group), 100μl of 100μg/ml b-FGF solution was applied (b-FGF group), 5×10^6 cells of hDPC suspended with 100μl of PBS was applied (hDPC group), and 5×10^6 cells of hDPC suspended with 100μl of 100μg/ml b-FGF solution was applied (hDPC/b-FGF group). Tegaderm (3M, London, ON, Canada) was placed over the wounds. The animals were housed individually.

Wound Healing Analysis

Digital photographs were taken at day 0, 3, 5, 7, 10, and 14 to evaluate wound area, which was measured using an image analyzing software.[18] Wound area was calculated as a percent area of the original wound as follows: Area of actual wound /Area of original wound × 100.

Histological and Histomorphometric Analysis

Mice were sacrificed at day 7 and 14 after cell transplantation. Skin samples including the wound and 4 mm of the surrounding skin were collected using a scalpel and scissors, fixed in 4 % paraformaldehyde and embedded in OCT compound (Tissue-Tek; Miles Inc, Elkhart, IN). Immunofluorescent staining was used to confirm the presence of human Type I collagen generated by the injected cells (Rockland Immunochemicals Inc., Gilbertsville, PA). Immunofluorescent staining followed standard methodology. The slides were mounted in the mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA). And the next slide was stained with hematoxylin and eosin. In addition, histomorphometric analysis used azan staining, following standard methodology. Collagenous fibre area was measured using an image analyzing software.[18] Collagenous fibre area was calculated as a percent area of dyed light blue. The percentage of collagenous fibre area was calculated as follows: Area of dyed light blue/ Area of all tissue × 100.

Statistical Analysis

Statistical differences among the defect area and collagenous fibre area in each group were evaluated by the Tukey-Kramer test after 1-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to be statistically significant.

RESULTS

hDPC/b-FGF Enhance Wound Healing by Macroscopic Findings

Each skin sample was harvested at day 7 and 14. At day 7, all samples were filled with the effusion, and the surface was still raw under macroscopic observation (Fig. 2). On the other hand, at day 14, the wound of hDPC/b-FGF group was almost completely closed in contrast to other groups and the epithelium appeared thicker (Fig. 2). Digital image analysis showed that the percentage of wound area was 85.38 ± 2.46 %, 35.82 ± 3.32 % (control group), 74.16 ± 2.78 %, 19.30 ± 3.40 % (b-FGF group), 69.13 ± 3.96 %, 17.83 ± 4.06 % (hDPC group), 48.72 ± 3.22 %, 6.50 ± 2.61 % (hDPC/b-FGF group) at day 7 and 14, respectively. Both b-FGF group and hDPC group demonstrated accelerated wound healing compared to control group (Fig. 3). The enhancement appeared at day 5 after implantation and became more evident after day 7. Differences in the mean wound area were statistically significant at all time points after day 7 ($P<0.05$; Fig. 3). There was no statistically significant difference in wound area observed between hDPC and b-FGF treated groups at all time points ($P<0.05$; Fig. 3). hDPC/b-FGF treated group appeared to accelerate wound healing compared to control group (Fig. 3). There was statistically significant difference in wound area observed between hDPC/b-FGF and control groups at all time points ($P<0.05$; Fig. 3). hDPC/b-FGF treated group accelerated wound healing compared to b-FGF and hDPC groups (Fig. 3). The enhancement appeared at day 3 after implantation and became more evident after day 5. Differences in the mean wound area were statistically significant at all time points after day 5 ($P<0.05$; Fig. 3).

Histological Observations

Day 14 after cell transplantation, basophilic nuclei were scattered throughout the tissue, in which great numbers of collagen fibril bundles could be found in hDPC/b-FGF group by histological observation (Fig. 4). PKH 26 is a lipophilic dye that stains the membrane of viable cells and is distributed among cells when mitosis occurs. It is reported that the fluorescence of PKH 26 is not transferred to other cells, but rather to daughter cells with no cellular toxicity. Most of the injected cells labeled with PKH 26 were located as a single mass in the subcutaneous tissue in hDPC and hDPC/b-FGF groups (Fig. 4).

Detection of Human Type I Collagen Produced by Transplanted hDPC and hDPC/b-FGF Groups

Immunohistological evaluation using anti-human type I collagen antibody was performed to confirm the presence of type I collagen derived from injected hDPC group in mice skin. At day 14 PKH 26-positive cells were surrounded by human type I collagen in hDPC and hDPC/b-FGF groups (Fig. 4). These results showed that the hDPC group produced human type I collagen and bundles of new collagen fibrils were derived from the injected cells.

Histomorphometric Analysis

Wound sections were collected on day 7 and 14 after cell transplantation for azan staining. Images were taken with a conventional microscope with bright field light. At day 7, there appeared to be fewer inflammatory cells in hDPC/b-FGF group compared with the

other groups. All groups were missing epidermis and control group was covered with a large amount of clots (Fig. 5). At day 14, many collagen fibrils were observed in the hDPC/b-FGF group compared with the other groups. Collagen fibrils were observed in the hDPC and b-FGF groups compared with control group (Fig. 5). In addition, epidermis in hDPC/b-FGF group appeared to be thicker. Re-epithelialization was advanced and the thickening of the epidermis was observed in hDPC/b-FGF group. The percentage of collagen fibrils area at day 7 and day 14 were 22.4 ± 2.57 %, 28.4 ± 1.74 % (control group), 33.4 ± 2.8 %, 39 ± 3.03 % (b-FGF group), 35.0 ± 4.15 %, 40.0 ± 3.03 % (hDPC group), 47.6 ± 3.44 %, 52.2 ± 2.99 % (hDPC/b-FGF group), respectively (Fig. 6). Significantly increased collagen fibrils area in wounded tissue was observed b-FGF, hDPC and hDPC/b-FGF groups as compared with control group at day 7 an 14 ($P<0.05$; Fig. 6). Moreover hDPC/b-FGF group showed significantly higher collagen fibrils area compared to b-FGF and hDPC groups ($P<0.05$; Fig. 6). There was no statistically significant difference in between b-FGF and hDPC group ($P<0.05$; Fig. 6).

DISCUSSION

In this study, we demonstrate for the first time that hDPC accelerated wound healing, similar to b-FGF, and that they enhanced wound healing more efficiently in the presence of b-FGF. Our immunohistological staining showed that hDPC produced human type I collagen, as previously reported, and hDPC/b-FGF group displayed greater production compared to hDPC group. These results meant that b-FGF accelerated human type I collagen production made by hDPC, the first report of its kind. And our azan staining results demonstrated that collagen fibril production, which meant human collagen made by hDPC and mice collagen, was consistent to human type I collagen. These results demonstrated hDPC enhanced wound healing via increasing collagen production.

There are some kinds of wound healing models. The most simple skin defect model is skin punch-out, but the rodent skin defect is easy to contract and shrink. To eliminate this bias, we utilized an excisional wound splinting model, resulting in uniform wound closure due to minimization of variations such as skin contraction and wound dressings through granulation and re-epithelialization, as reported previously.[19] Therefore, our results are reasonable as a first step to evaluate the wound healing effect of hDPC. But, rodent and human skin wound healing is considerably different. Researches reported human skin xenografted onto SCID mouse as a chimeric model, which maintains much of its original human skin function.[20] We will utilize this chimeric model to advance to the next step in our future research.

Of all growth factors, b-FGF is one of the most fascinating in regenerative medicine. Experimental studies have demonstrated that b-FGF administration to the skin wound

accelerates angiogenesis, granulation, and epithelialization, resulting in accelerated wound healing.[11] Clinical studies with the use of recombinant b-FGF have shown that it is highly effective and safe for skin ulcers and decubitus ulcer.[21] And b-FGF is also well known as one of the most common reagents to maintain the self-renewal and efficient proliferative capabilities of stem cells.[22] Therefore, we evaluated hDPC for wound healing with or without b-FGF, and our results showed that b-FGF enhanced human type I collagen production by hDPC. Our co-workers reported that b-FGF stimulated hDPC proliferation.[23]The fact that hDPC/b-FGF group enhanced collagen production resulted from increasing collagen production per cell and/or hDPC proliferation. Further mechanism need to be studied for hDPC with b-FGF in wound healing process.

Stem cells play an important role in regenerative medicine. We can isolate them from born marrow, fat tissue, meniscus and so on. Dental pulp is a fascinating stem cell source, not only in craniofacial area but also in the whole human body. Compared to other tissue, dental pulp has great advantages; it is "medical waste" everyone has some dental pulp; there is no need for an invasive, painful procedure; and hDPC has a stem cell population, so it has the capacity to be induced to osteoblasts, chondrocyte, adipocyte etc., and express several growth factors such as TGF-β2 and 3 and CTGF NGF, BMP1, IL-1β. It may be suitable for a wide range of human diseases.[10,23] This study demonstrated that hDPC accelerated wound healing, so it can be an effective, unique stem cell resource for potential of new cell therapies for intractable ulcer such as radiation ulcer, leg ulcer, decubitus ulcer.

In conclusion, our results showed that hDPC accelerated wound healing, similar to

b-FGF, and they enhanced wound healing more efficiently in the presence of b-FGF. We believe that a better understanding through these investigations will help us understand skin regeneration and the wound healing process, and lead us towards developing novel cell therapies for skin defects in the future.

ACKNOWLEDGMENTS

We thank M. Okada, A. Yamawaki-Ogata, A. Kimura for technical assistance, Dr H. Hibi and Dr. A. Yamamoto for discussion. We thank Kaken Pharmaceutical Co. Ltd. Tokyo, Japan for the basic fibroblast growth factor.

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Figure Legends

FIGURE 1. Scheme of experimental protocol.

FIGURE 2. Macroscopic observations of the wounds at day 7 and 14 after procedure. Left panel (A, C, E, G) shows day 7, right panel (B, D, F, H) shows day 14. A, B is control group, C, D is b-FGF group, E, F is hDPC group, G, H is hDPC/b-FGF group, respectively. Bars= 3mm

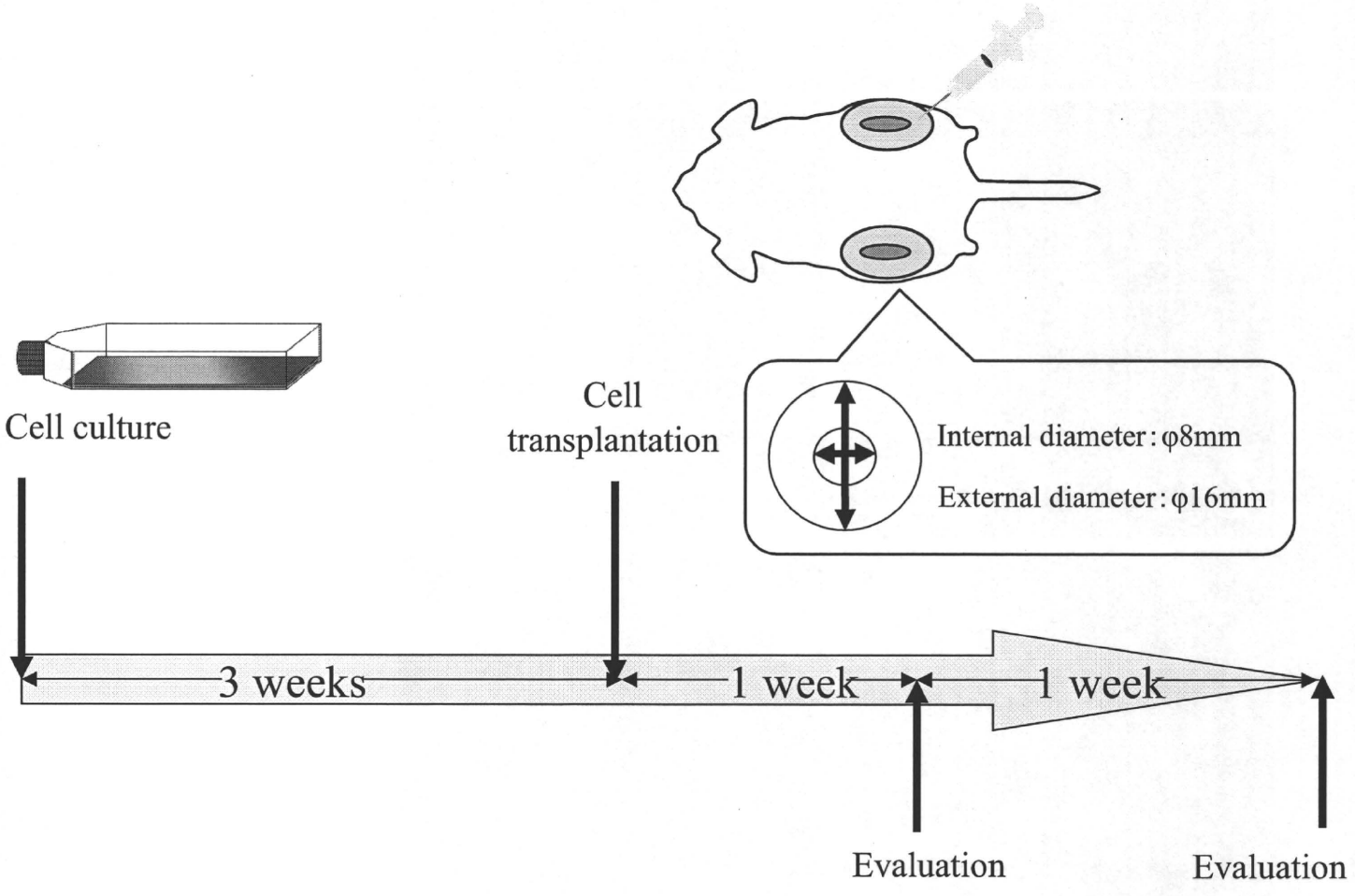
FIGURE 3. Wound area was measured by using Scion Image. The percentage of wound area was calculated as follows: Area of actual wound / Area of original wound x 100. Analysis of variance (ANOVA). hDPC/b-FGF group versus control group, $*P<0.05$

FIGURE 4. Histologic evaluation at day 14 after procedure. Left and middle panels shows hematoxylin and eosin staining, and right panel shows human type I collagen staining. control group is A, B, C, b-FGF group is D, E, F, hDPC group is G, H, I and hDPC/b-FGF group is J, K, L, respectively. Bar=3mm (A, D, G, J); 25μm (B, C, E, F. H, I, K, L)

FIGURE 5. Azan staining of the wounds at day 14 after procedure. Left panel (A, C, E, G) shows day 7 and right panel (B, D, F, H) shows day 14. A, B is control group, C, D is b-FGF group, E, F is hDPC group, G, H is hDPC/b-FGF group, respectively. Bars= 200μm

FIGURE 6. The percentage of collagenous fibre area (%) in control group, b-FGF group, hDPC group and hDPC/b-FGF group, at day 7 and 14 after procedure. Bar=SD. *$P<0.05$

FIGURE 1.


Cell culture
Cell
transplantation
Internal diameter: φ8mm
External diameter: φ16mm
3 weeks
1 week
1 week
Evaluation
Evaluation

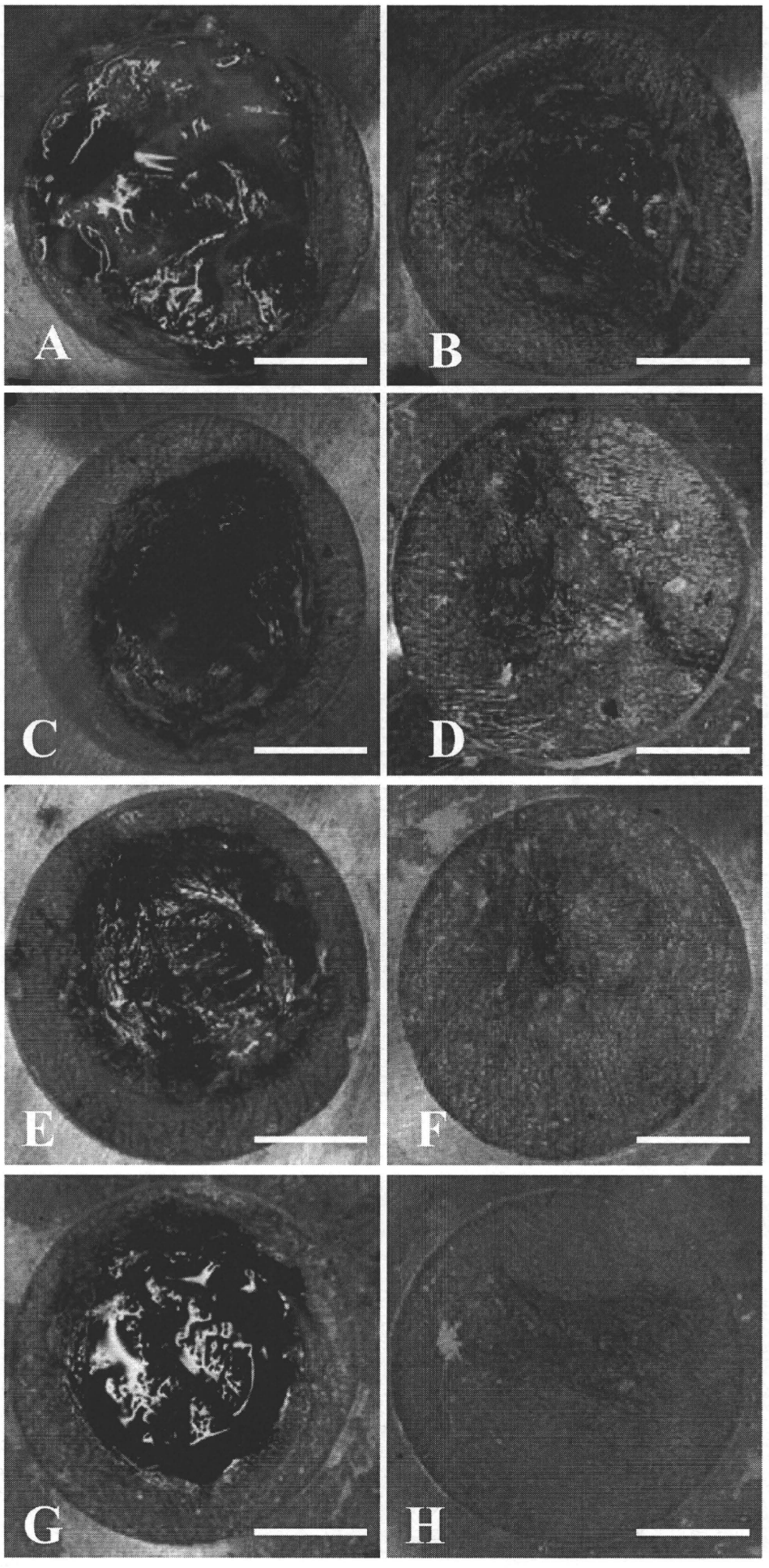


FIGURE 2.

FIGURE 3.

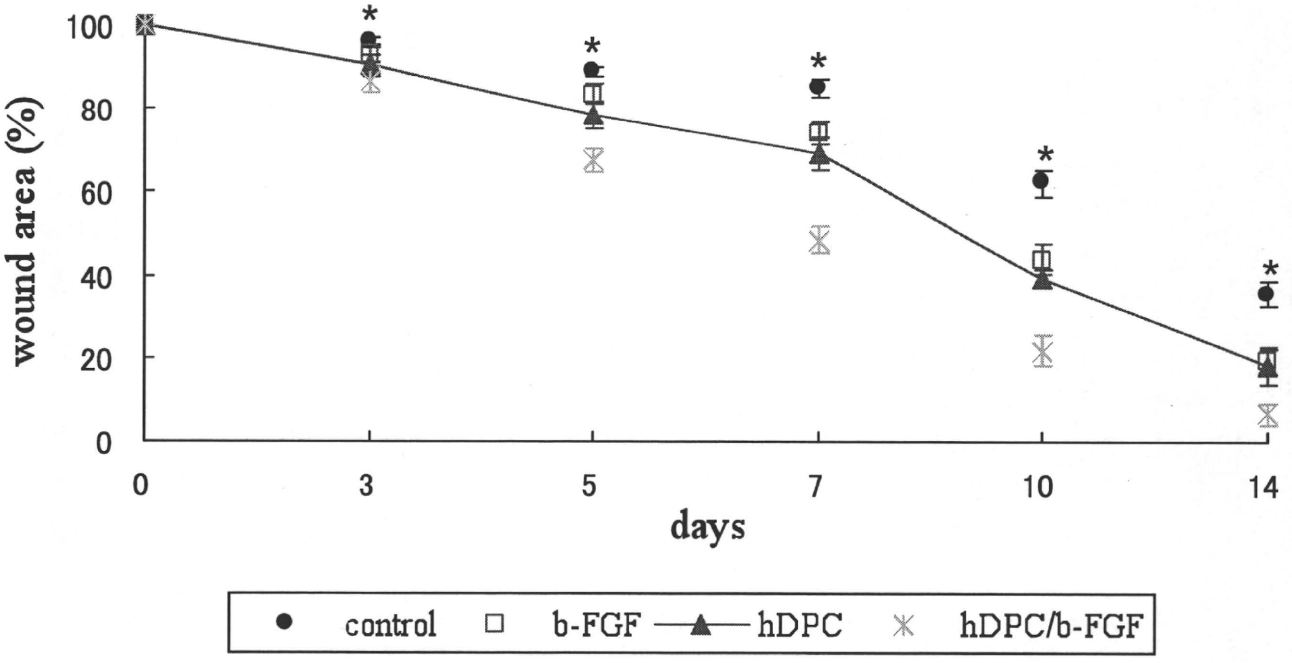

wound area (%)
100
80
60
40
20
0
0
3
5
7
10
14
days
*
control
b-FGF
hDPC
hDPC/b-FGF

FIGURE 4.